Review

RNA quality assessment: a view from plant qPCR studies

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Much work to be done in plant qPCR analyses : RNA quality

Abstract

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is probably the most commonly published molecular technique used in transcriptome analyses today. The simplicity of the technology and protocols that generate results even by inexperienced users without the need to understand the underlying technical mechanisms, has made RT-qPCR the method of choice for RNA quantification. Rather than the 'gold standard technology' used to define it, the performance of qRT-PCR often suffers from pitfalls during the general workflow. The inconsistency of conventional methods to evaluate RNA quality is summarized here and its influence on both qPCR performance and stability of reference genes are discussed in the frame of this area has been distinguished by a prevalent lack of concern.

Key words
MIQE, qPCR, RNA integrity, 3':5' ratio

1. Introduction

Since the relatively recent introduction of qPCR in the plant science community, interest in the technique has increased exponentially (Gachon, 2004). However, one of the major drawbacks of qPCR are the numerous critical steps required through the entire workflow that may influence the accuracy and reliability of results (Derveaux *et al.*, 2010; Huggett and Bustin, 2011; Huggett *et al.*, 2005). The apparent simplicity of the qPCR technology has made it vulnerable to a lack of clarity and transparency in the literature, leading to few publications reporting in detail how results have been obtained. Therefore, in spite of its superiority over other methods available for evaluating gene expression, RT-qPCR remains underused, due in part to conflicting results and difficulty to replicate experiments. While the technology itself is not intrinsically inaccurate, the absence of strict guidelines has led to researchers performing experiments and analysing data based on information gathered from disparate sources, which has led to data of variable quality (Taylor *et al.*, 2010). Two of

main obstacles impeding a more extensive adoption of RT-qPCR assays are concerns over quality assessment and data normalization, both of which affect reproducibility. Efforts to adopt methods for the systematic validation of reference genes applying a robust normalization strategy are growing and are currently being led by the molecular researchers from the medical field (Radonić *et al.*, 2004; Dheda *et al.*, 2004; Dheda *et al.*, 2005; Vandesompele *et al.*, 2009). In recent years, the plant scientific community has gradually recognized the need for robust validation, and not surprisingly, awareness of the importance of such validation has increased over the recent years (Gutierrez *et al.*, 2008a; Gutierrez *et al.*, 2008b; Udvardi *et al.*, 2008; Guenin *et al.*, 2009).

The other main issue is related to the quality of the template. This is arguably the most important determinant of the reproducibility and biological relevance of subsequent RT-qPCR results (Huggett *et al.*, 2005; Pfaffl, 2010; Pérez-Novo *et al.*, 2005; Fleige and Pfaffl, 2006; Fleige *et al.*, 2006; Bustin and Nolan, 2004; Imbeaud *et al.*, 2005; Bustin, 2002). To this end, numerous articles have elaborated on the theme of producing high-quality and reliable data from RT-qPCR have always highlighted the importance of RNA sample quality (Taylor *et al.*, 2010; Becker *et al.*, 2010). The instability of RNA and its sensitivity to degradation (introduced during storage under suboptimal conditions, the variety of additional steps set out in the isolation protocols or inter-laboratory sample shipments) is well known by the RNA research community. Assessing transcript quantification of RNA preparations should confront the essential problem of whether obvious detected differences in gene expression are related to the hypothetical assumptions of the particular study or whether they are explainable to some extent by a certain bias in the composition of the sample set concerning RNA quality, with the final goal that results should reflect true biological differences (data of

biological significance) and not differences due to poor RNA quality (data of statistical significance). Acknowledgement of this potential conflict should lead to a widespread debate mainly about which operational procedure is the standard for RNA quality assessment and which requirements must be met from the technical point of view. However, current proposals for adequate RNA integrity measurements are not taken into account and quality control in gene expression studies is an often ignored consideration; this is an issue shared with the biomedical sciences but in the plant field research is especially significant.

Fig. 1 shows the distribution of methods that have been used to perform RNA quality assessment from 520 studies based on RNA transcription analysis published in three leading high impact journals in the past five years: *Journal of Experimental of Botany*, *BMC Plant Biology* and *Plant Biotechnology Journal*. The aforementioned time period also includes the seminal Minimum Information for publication of Quantitative real-time Experiments (MIQE) in April 2009 (Bustin *et al.*, 2009). Of the papers reporting RNA quality control, 1 in 2.2 addressed it using two alternative methods. Additionally, 14% of studies performed quality check based on 15 to 20 year-old methods, although it is certainly not the best option (Fig. 1A). However, by far the most difficult data to explain is the more than 74% of gene expression papers published that have not even mentioned performing RNA quality assessment (Fig 1B). The low percentage of papers including RNA quality reports in the last five years raises the question of whether the importance of the critical sample quality control section of the MIQE guidelines has fully penetrated the plant research community. It is evident from these data that this area requires urgent attention and considerable changes to the way qPCR assays are

both performed and reported.

This review aims to address the effect of RNA quality on gene expression profiling using qPCR data, and to place the progress in quality assessments into the context of high technology requiring rigid quality controls.

2. Total RNA quality assessment

The term RNA quality is defined as the combination of RNA purity and RNA integrity (Becker *et al.*, 2010). Purity and integrity are unrelated and therefore both should be assessed independently, but the most common techniques for controlling the quality of RNA have focused on purity. The use of techniques emphasizing purity was acceptable for conventional or end-point PCR assay, but translating such assays directly into the qPCR format is just not possible. The advent of qPCR has meant the potential to quantify target nucleic acids accurately. When the sample is to be used for measurements of transcript quantity, other relevant measurement is a determination by whether the mRNA molecules are intact or fragmented, i.e. RNA integrity (Nolan and Bustin, 2008).

2.1 Denaturing agarose gels

Conventional methods based on RNA electrophoretic separation aim at the establishment of an environment for complete denaturation in order to fully disrupt the hydrogen bond formation that hampers the estimation of RNA molecular weights. The most widespread method makes use of MOPS/formaldehyde gel electrophoresis stained

with ethidium bromide, though faster and safer alternatives have been developed (Aranda *et al.*, 2012; Masek *et al.*, 2005). The method relies on the assumption that ribosomal RNA (rRNA) quality reflects those of the underlying mRNA population. rRNA quality is assessed visually using the intensities of ribosomal bands with a ratio of ~2 considered as a good indicator of high level integrity (Sambrook *et al.*, 1989), but as the appearance of rRNA bands is affected by the electrophoretic conditions, the amount of loaded RNA and the saturation of ethidium bromide fluorescence, this method can be less than reliable. The main disadvantage is the significant amount of precious RNA required (typically on the order of 300-800 ng of RNA), as this is a low-throughput method, it is also dependent on the researcher's perception. Furthermore, the initial premise is considered questionable because it is not clear how rRNA degradation actually reflects the quality of the underlying mRNA population (Dotti and Bonin, 2011). Though RNA quality assessment methods have advanced well beyond denaturing agarose gels, confidence in RNA electrophoresis analysis has continued, due in part to reliance on traditional reasoning over data produced through more reliable methods. The fact of the matter is that the lack of sensitivity and specificity of agarose gels is especially clear in its inability to detect small RNA degradation (Fleige and Pfaffl, 2006; Imbeaud *et al.*, 2005). It should be further stated that this method relies on the use of formaldehyde and ethidium bromide, both toxic chemicals requiring special handling and waste disposal procedures (Sambrook *et al.*, 1989). It is for these reasons that the denaturing of agarose gels as the singular method for evaluating the degree of RNA degradation cannot be recommended.

2.2 *Spectrophotometric measurement*

Another common technique for controlling the quality of RNA is optical density (OD) measurement. RNA purity can be verified on the basis of the 260/280 ratio. An $OD_{260/280}$ >1.8 and maximization of $OD_{260/230}$ and $OD_{260/320}$ are usually considered acceptable indicators of good RNA quality (Sambrook *et al.*, 1989; Manchester, 1996). However, the OD260 can be compromised by the presence of genomic DNA, while the OD 280 will estimate the presence of protein but provide no hint on possible residual organic contaminants (Manchester, 1995). In the literature, the reliability of spectrophotometrics for quality measurement is discussed, and since it is limited in the range of substances detected, it does not reveal the state of degradation or the integrity of the sample (Imbeaud *et al.*, 2005). Therefore, the spectrophotometric method by itself may give misleading results. Moreover, conventional A260/A280 measurement does not detect the presence of inhibitor components that are clearly detrimental to qPCR amplification in an assay-specific manner; these inhibiting agents may be co-purified components from the biological sample or reagents used during nucleic acid extraction, and is particularly relevant to formalin-fixed, paraffin-embedded (FFPE) samples. Although full discussion on this concern lies beyond the scope of the present review, there are some sound articles available on this topic (Nolan and Bustin, 2008; Nolan *et al.*, 2006a).

2.3 *Microfluidic capillary electrophoresis*

For all reasons aforementioned it was highly desirable to develop an user-independet strategy based on a reliable system with an appropriate metrics for evaluation of RNA integrity. A major improvement in RNA analysis occurred with the advent of microfluidics-based electrophoresis systems (lab-on-a-chip technology developed by

Caliper Life Sciences Inc.). In 1999, Agilent Technologies, Inc. introduced an automated device using microfluidics technology that provided electrophoretic separation of DNA, RNA and protein samples, the Agilent 2100 Bioanalyzer (Mueller *et al.*, 2000). The first software system for the instrument calculated the ratio of the two ribosomal bands, following the traditionally used approach for RNA quality assessment; this proved to be far superior to the gel based approach, as it was free of the subjective visual interpretation. However these ribosomal ratios showed a practical value only as long as there were no prominent degradation products (Schoor *et al.*, 2003; Auer *et al.*, 2003). Moreover, when ratios were calculated from identical samples but through independent runs, a large degree of variability was observed (Imbeaud *et al.*, 2005). Still newer technologies continue to report that ribosomal ratios show a weak correlation with RNA integrity (Pfaffl *et al.*, 2008) as well as no significant correlation between the 28S/18S ratio and qPCR performance (Fleige *et al.*, 2006).

Thus, because of the limited reproducibility of rRNA ratios to asses RNA integrity, Agilent Technologies introduced an user-independent classifier algorithm, the so-called RIN (RNA Integrity Number) for standardization of RNA quality control (Schroeder *et al.*, 2006). A total of 1300 electropherograms of RNA samples from various tissues of three mammalian species (human, mouse and rat), showing varying levels of degradation and an adaptive learning approach were used in order to assign a weight factor to the relevant features that describe the RNA integrity from the electrophoretic trace: the total RNA ratio (ratio of area of ribosomal bands to total area of the electropherogram), the height of the 28S peak, the fast area ratio (ratio of the area in the fast region to the total area of the electropherogram) and the height of the lower marker. The RIN number was computed for each RNA profile resulting in the classification of

RNA samples in 10 numerically predefined categories of integrity. Thus, the output RIN of the Agilent Bionalyzer is a decimal or integer number in the range of 1–10: a RIN of 1 is returned for a completely degraded RNA samples whereas a RIN of 10 is achieved for intact RNA sample. Similarly, Bio-Rad (Bio-Rad Laboratories, Inc.) introduced the RQI (RNA Quality Indicator) algorithm in the Experion system as a method to standardize and quantitate RNA integrity (Denisov *et al.*, 2008). Here, only three regions of the electropherogram are taken into account when mapping a sample for RQI calculation: the 28S region, the 18S region, and the pre-18S regions. Comparability and validity of results in terms of RNA quality delivered by both lab-on-chip-systems has been investigated, and overall, both algorithms have been shown to be functionally equivalent in reliably determining RNA integrity (Denisov *et al.*, 2008; Riedmaier *et al.*, 2011).

Altough initially, both calculation algorithms were established and tested using various mammalian tissues, at present the method has shown to be useful for RNA samples from a variety of organs and organisms, including bacterial RNA (Jahn *et al.*, 2008; Pinto *et al.*, 2012). Evidently, 'lab-on-a-chip' technology has also been widely adopted by the plant community. For example, Agilent maintains (although no updates) a freely accesible data base showing species where RIN has been used (http://www.chem.agilent.com/RIN/). However, while plants have the ubiquitous major ribosomal subunits, they also have other rRNA species that can be present, including plastid-specific rRNAs found in photosynthetic tissues that will be present at high levels in leaves but at lower levels or missing in other tissues. Therefore, as a precautionary measure, the electropherogram should be visually inspected to confirm that ribosomal peaks have been properly identified by the software. In this way, the

RNA quality assessment with Bioanalyzer or Experion has been performed for a number of different topics: gene expression analysis during plant-pathogen interactions (Klink *et al.*, 2007), plant-symbiotic interactions (Branscheid *et al.*, 2010; Grunwald *et al.*, 2009), comparison between relative quantification approaches (Regier and Frey, 2010) or identification of stable reference genes (Klie and Debener, 2011; Lilly *et al.*, 2011).

The significant advantage of microfluidic systems is the large decrease in the amount of RNA needed to evaluate integrity down to the submicrogram scale; an aspect which will likely lead to its increased use in the future. Although the cost of automated electrophoresis stations renders the method unfeasible for laboratories with resource constraints, the services provided by external genomics companies are making the approach more and more accessible.

2.4 *The 3':5' ratio mRNA integrity assay*

In the absence of an alternative, the use of a 3':5' assay has been proposed for assessing mRNA integrity (Nolan *et al.*, 2006b). The data obtained are independent of ribosomal RNA integrity, provide a reasonable measure of the degradation of the transcripts and are modelled using the standard approach adopted by microarray users and conventional techniques applied to end-point PCR assays (Auer *et al.*, 2003). The main advantage of using a 3':5'ratio to assess the RNA integrity is that this method specifically focuses on the integrity of a messenger RNA molecule instead of addressing the ribosomal RNA transcripts. As such, the 3':5' assay appears to constitute the most useful parameter to qualify RNA samples (Vermeulen *et al.*, 2011). Moreover,

RNA integrity is inferred using the same technology used for RT-qPCR, giving the 3':5' assay greater relevance compared to other methods. The assay is also particularly applicable for analysis of precious samples when little RNA is available (Nolan and Bustin, 2008). The method aims at measuring of the integrity of a reference gene mRNA that is considered to be representative of the integrity of all mRNAs in a given RNA sample by amplifying different amplicons. It is based on the evidence that cDNA yield from sequence near the 5' end of partially degraded mRNAs is significantly less than from sequence near the polyA tail (Swift *et al.*, 2000): i.e. poor RNA quality adversely affects synthesis of first strand cDNA resulting in under-representation of the 5' moiety of the transcript.

The usefulness of the 3':5' ratio relies on the oligo(dT) priming method for cDNA synthesis, consequently the progress of the reverse transcriptase is wholly-reliant on the intactness of the mRNA. Under ideal conditions the reverse transcription will generate full-length cDNA including the 5'-end of the RNA, but the process will be interrupted wherever the mRNA is fragmented. Unlike other methods, the use of oligo(dT) and random primers is unsuited for this assay, as random sequence primers will copy RNA at multiple origins along the template and thereby produce more than one cDNA target per original mRNA target, including cDNA produced from rRNA: performed under these conditions, the final PCR yield may be somewhat higher while the template becomes partially degraded (Fig. 2).

The ideal 3':5' ratio of 1 corresponds with the highest quality material, while other ratios depend on the differing number of nucleotides between the 5'- and 3' amplicons that can discriminate between different integrity level of the RNA samples. Each amplicon may represent a 3' or 5' assay as that designation is merely of location: the 3'

target assay is designed to amplify of an amplicon near the 3' end, and the 5' target assay targets the 5' end of the mRNA sequence. Anchoring the 5' assay ~1500 nt from the 3' end of the sequence and by spacing the two assays at a distance ~1100 nt, a 4.43-fold differences cut-off value may characterize the material as unreliable for downstream quantification studies (Die *et al.*, 2011).

3. Influence of RNA quality on gene expression profiling using RT-qPCR

The need for high quality RNA standards has been an unparalleled challenge in the microarray field, as gene expression profiles change considerably upon RNA degradation. Probably the cost of such technology led to recognized soon the influence of the issue on the overall success of experiments. The inclusion of samples with degraded RNA showed an important influence on the statistical analysis and hence the interpretation of expression levels. It was therefore concluded that the reliability of the microarray technology is affected by the quality of the extracted RNA, and that degraded samples could not be reasonably considered for analysis (Schoor *et al.*, 2003; Copois *et al.*, 2007; Strand *et al.*, 2007). In RT-qPCR, however, nice-shaped sigmoidal amplification curves can usually be obtained, even from degraded templates. The truth is that despite the vast number of publications pointing out that starting with low quality RNA may strongly compromise the results of downstream applications, quality control prior to qPCR measurement is still an often overlooked consideration (Fig. 1).

3.1 *Influence of RNA quality on RT-qPCR performance*

Today, it is acknowledged that RT-qPCR performance is affected by RNA integrity.

Imbeaud and colleagues (2005) have shown that artifactual detection of false positive and negative differential expression may be observed due to RNA integrity differences: their results indicated that up to 7-fold differences may be expected in the relative expression levels measured in samples that differ only by their quality. On the other hand, Fleige and colleagues (2006) proved a significant negative relationship between RNA quality and Cq for all samples they tested (by using RIN as quality metric). Similarly, Koppelkamm *et al.* (2011) have recently reported Cq shifts between highest and lowest RIN values showing statistically significant correlation coefficients.

In February 2011, Vermeulen *et al.* (2011) reported the more complete framework to measure the impact of RNA quality on the gene expression results to date. They studied the impact of RNA quality on the significance of differential expression of marker genes between two risk groups of cancer patients using six RNA quality parameters. Their results clearly showed an influence of RNA quality on single gene differential expression for a substantial number of genes. While all quality measures were correlated, assessments based on the mRNA rather than rRNA were the best indicators of reliable amplification.

Joining these technical studies, there are a number of other publications showing the biological relevance of high quality RNA for obtaining reliable data from qPCR experiments. For example, Lipska and colleagues (2006) focusing on schizophrenia analysis showed that differences in RNA quality led to crucial effects much more pronounced than the underlying disease related effects and that is inappropriate to compare samples with different degradation extent. At that time, Kerman *et al.* (2006) found significant differences in the quantification of gene expression in microdissected tissues for LCM showing the impact of RNA quality on the outcome of RT-qPCR

studies. More recently, Taylor *et al.* (2011) categorized breast cancer RNA samples by integrity based on RQI and used the minichromosome maintenance protein MCM7 as a model target gene to determine the importance of appropriate sample quality on the results. The relative expression of *mcm7* was assessed between normal samples of low-quality and tumor samples showing no significant differences, and contrasting with the opposite results when comparing normal samples of high-quality and tumor samples.

3.2 *Influence of RNA quality on reference gene expression stability*

One of the more relevant concerns that have caused debate is related to the elucidation of whether a data normalisation step can eliminate the influence of impaired RNA integrity. The gold standard for normalization of qPCR expression data is normalization against multiple, validated reference genes (Derveaux *et al.*, 2010). The reference-gene-based normalisation corrects for variable starting amounts of RNA and for differences in reverse transcription efficiency, since the references are exposed to the same preparation steps as the gene of interest (Huggett *et al.*, 2005; Radonić *et al.*, 2004; Udvardi *et al.*, 2008; Bustin, 2005). Nevertheless, it is a general misconception that the influence of degraded RNA will be corrected by the normalization step using the reference genes expression from the same degraded RNA template. Perez-Novo and colleagues (2005) have shown that this faulty thinking may lead to misinterpretation of target-gene expression level information when there is no prior knowledge of the RNA degradation status. Quantifying 10 commonly used reference genes in both intact and degraded RNA, they showed that the stability of those references was different within the same tissue types according to the degradation status of the samples. By determining the average gene-specific variation of all reference genes, the authors

found that the levels of those genes were always higher in degraded versus intact samples, and therefore proposed performing RNA quality control and discarding degraded samples. Although normalisation may improve mRNA quantification, even significant difference (up to 4-fold, meaning around 75% mRNA is degraded) in gene expression may be expected in samples differing only in their quality (Fleige *et al.*, 2006); a fact which is especially relevant in accurately quantifying small differences in expression. More recent studies have confirmed the influence of RNA quality on reference gene expression stability. The results obtained from those studies indicate that the process of normalization does not completely resolve the effect of compromised RNA quality on the final results (Vermeulen *et al.*, 2011).

Obviously, the larger benefit of quality prediction prior to qPCR assays is the determination of a cut-off point from which one can move forward with additional experiments: knowing the degree to which results may be compromised may prevent substantial cost in the form of wasted reagents and technical time. Data obtained with the most degraded samples cannot be reasonably considered for downstream application, but this raises questions regarding moderately degraded samples and there is an on-going debate as to what extent level of RNA degradation is too low to be included in analyses.

4. Gene expression profiles from partially degraded RNA

Intact RNA obviously constitutes the best representation of the natural state of the transcriptome, however there are situations in which gene expression analysis may be desirable even on partially degraded RNA. In plant research, as in other disciplines,

there is an increasing interest in extracting nucleic acids from formalin-fixed, paraffin-embedded (FFPE) samples. Nevertheless, a major challenge of FFPE material is related to the extensive degradation of RNA due to the fixation procedure. Given the fact that in human clinical studies each patient sample is extremely valuable, it is not surprising that most of the attempts to address the utility of partially degraded RNA from non-ideal samples came from the biomedical research field. To our knowledge, Schoor and colleagues (2003) were the first to study the quality of RNA preparations in the context of gene expression analysis by microarrays. These investigators were interested in the impact of varying amounts of RNA degradation on the expression profile of the samples, inducing RNA degradation in human tumor and healthy tissue samples by endogenous ribonucleases. The study established that expression differences from partially degraded RNA samples with still visible ribosomal bands were similar to those obtained from high-quality samples. Some moderate degradation therefore does not preclude microarray analyses and it might still lead to meaningful results if used carefully. In the context of qPCR, the points that deserve attention are as follows:

(1) RT-qPCR technique could be particularly suitable for quantitating mRNA levels in tissue samples containing partially degraded RNA due to the short length of amplicons (Fleige *et al.*, 2006; Bustin, 2002; Antonov *et al.*, 2005; Li *et al.*, 2008; Li and Reilly, 2008). Since qPCR generates amplicons as small as 60 bp (Bustin, 2002), the likelihood of fragmentation between priming regions is significantly reduced and thus yields more consistent results.

(2) It is important to ensure that data analyses are performed using samples of comparable RNA quality (Fleige and Pfaffl, 2006; Auer *et al.*, 2003). It is inappropriate to compare degraded and intact samples: this compels a systematic RNA integrity

control prior to any qPCR analysis (Pérez-Novo *et al.*, 2005; Bustin and Nolan, 2004; Imbeaud *et al.*, 2005).

(3) Special attention should be given to the extent of gene expression differences. Although large differences in gene expression may be reliably detected and quantified, even with partially degraded input RNA, small expression differences from low quality RNA samples are prone to misinterpretation (Pérez-Novo *et al.*, 2005; Kerman *et al.*, 2006). For example, Koppelkamm et al. (2011) working with post-mortem human tissues, determined the sensitivity of the approach by means of a threshold of 7-fold change for a particular assay, below which the changes could not be distinguished from differences caused by impaired integrity. In this sense, all those cases wherein partial RNA degradation is expected or detected, the systematic validation of degradation profiles for all transcripts of interest in order to reveal detection limits of assays would greatly improve the plant qPCR analysis.

(4) From an experimental point of view one of the more practical approaches is the 3'-based amplification method. The oligo(dT) priming method for cDNA synthesis will yield cDNAs that extend from the 3' end to the 5' end of mRNA, or to the cleavage site in case RNA degradation: this might be used for the more reliable detection of gene expression by targeting against 3' regions of the corresponding genes, a fact that has been the bottom line in the priming strategy for reverse transcription in microarray experiments. Not surprisingly, some gene expression profiling studies have shown tolerance to degraded RNA samples (Lee *et al.*, 2005): the Affymetrix GeneChip design, which is 3' biased, shows oligonucleotide probes that are usually designed to be within the last 600 nucleotides of the mRNA end (Li and Reilly, 2008). It is of particular interest that Opitz *et al.* (2010) have not observed a global effect of RNA quality on

gene expression. The authors stated that RNA degradation seemed to have a significant influence only on a small number of genes. Interestingly, the relative positions of probes from these genes in the corresponding cDNA sequences were shifted to the 5' region, while probes of the rest of normally-represented genes were closer to the 3'-end. This may allow for a so-called noise-reduction strategy by limiting microarray analysis to probe sets closest to the 3' end of the transcripts (Turchin, 2006). Analogously, using qRT-PCR, statistically significant differences have not been observed in expression levels between intact and degraded RNA samples of two target templates designed within ~ 800 nucleotides of the 3'-end of the transcript, suggesting a high confidence-region associated with the 3' end that may be utilized through careful primer design in quantifying mRNA levels (Die *et al.*, 2011). The approach's relevance is limited by the choice of oligo(dT) priming methods for cDNA synthesis, as well as the availability of information in public databases regarding gene structures.

5. RNA quality in MIQE guidelines

There is an increasing consensus within the scientific community that the need to strengthen published information with relevant qPCR experimental detail is urgent (Huggett and Bustin, 2011). While guidelines that define the minimum information required for interpretation of microarray data have been available since 2001 (Brazma *et al.*, 2001), similar specifications for qPCR experiments have been more recently developed. In 2009, a set of qPCR best-practice guidelines was published by an international consortium of leading qPCR scientists, establishing the MIQE guidelines (Bustin *et al.*, 2009). Among the essential items to be reported, the quality assessment of RNA templates holds a prominent position. These guidelines are not a set of dogma

principles; instead, they provide a technical common-sense approach for enhancing the reproducibility and transparency of qPCR data. Although adherence to the MIQE guidelines is not explicitly required by most leading journals (including those in plant field), there has been a general positive response to MIQE from a broad sector including researchers and authors (Bustin *et al.*, 2011), with more than 460 citations in peer-reviewed literature since 2011. From the papers surveyed while writing this review, it is evident that some areas of the pre-analysis qPCR steps require reformulation. Without providing information on RNA quality it is difficult to evaluate, as readers or reviewers, the relevance of any other reported data; this lack of transparency makes it difficult to attempt to reproduce experiments in different laboratories. A set of MIQE key parameters was recently reported in 2010 underlining the critical need to record RNA quality measurement once again. Although no perfect assessment method is postulated, microfluidics-based systems or 3':5' ratio assays are defined as basic indicators of RNA integrity (Bustin *et al.*, 2010).

6. Conclusion

Analyses on a transcriptome-wide level using RNA-seq, or next-generation sequencing will undoubtedly transform transcriptomic biological research similarly to how the development of microarrays or RT-qPCR technique changed the possibilities of mRNA quantification more than one decade ago. However, qPCR is clearly the more cost and time-effective method currently available for a broad range of applications. The future of the technique most likely includes the standardization of practices and transparency in reporting data. An increasing number of studies deal with recommendations for refining RT-qPCR standards. There are abundant examples where it is difficult to

collect sufficient samples that meet the minimum quality threshold from the biomedical research. In plants, despite some exceptions, one should make use of the opportunity offered by the easier procurement of samples. Samples of the highest quality RNA possible, as well as the method(s) required to assess the critical issue of RNA quality, must be mandatory and freely accessible to revisers and colleges. This will help to maintain the level of quality and high standard of both works and publications.

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Figure Legends

Figure 1 RNA quality assessment in gene expression studies. (a) Distribution of methods used to perform RNA quality check from studies published in three leading plant journals in the past five years. Data recorded until May 2012. (b) Percentage of published papers reporting to have performed RNA quality assessment from 2008 to May 2012. MIQE guidelines publication in April 2009 is shown.

Figure 2 Normalized relative expression levels of different amplicons throughout the ubiquitin cDNA sequence (DFCI Medicago Gene Index, TC112803) from *Medicago truncatula* RNA root samples. Two different priming strategies were performed using the ImProm-II Reverse Transcription System (Promega). Bars represent mean expression level ± SEM from two independent partially RNA degraded samples (RQI<5). The X-axis represents amplicon distance in nucleotides from the 3' end. Normalized values are rescaled to the 543 assay.