Journal of Experimental Botany, Vol. 60, No. 2, pp. 487–493, 2009
doi:10.1093/jxb/ern305

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references

Stéphanie Guénin[1], Mélanie Mauriat[2], Jérôme Pelloux[3], Olivier Van Wuytswinkel[3], Catherine Bellini[2,4] and Laurent Gutierrez[1,2,*]

[1] Molecular Biology Centre, Centre de Ressources Régionales en Biologie Moléculaire, Université de Picardie Jules Verne, Faculté des Sciences, 33 Rue St Leu, F-80039 Amiens, France
[2] Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, 901 83 Umeå, Sweden
[3] EA3900 Biologie des plantes et contrôle des insectes ravageurs, Université de Picardie Jules Verne, Faculté des Sciences, 33 Rue St Leu, F-80039 Amiens, France
[4] Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, F-78026 Versailles Cedex, France

Received 8 July 2008; Revised 31 October 2008; Accepted 10 November 2008

Abstract

Quantitative RT-PCR (reverse transcription polymerase chain reaction, also known as qRT-PCR or real-time RT-PCR) has been used in large proportions of transcriptome analyses published to date. The accuracy of the results obtained by this method strongly depends on accurate transcript normalization using stably expressed genes, known as references. Statistical algorithms have been developed recently to help validate reference genes but, surprisingly, this robust approach is under-utilized in plants. Instead, putative 'housekeeping' genes tend to be used as references without any proper validation. The concept of normalization in transcript quantification is introduced here and the factors affecting its reliability in qRT-PCR are discussed in an attempt to convince molecular biologists, and non-specialists, that systematic validation of reference genes is essential for producing accurate, reliable data in qRT-PCR analyses, and thus should be an integral component of them.

Key words: Gene expression, normalization, quantitative RT-PCR, reference gene, transcript quantification.

Introduction: normalization in biology

In addition to its rates of biosynthesis, metabolism, and degradation, the quantity of a given compound in a biological sample obviously depends on the size of the sample. Therefore, quantified amounts should be normalized for every sample to account for variations in the amounts of starting material, before comparing samples. The normalization procedure usually consists simply of dividing measured quantities by the amounts of material in the respective samples. However, normalization should, ideally, also take into consideration possible between-sample variations in the efficiency of the quantification procedures. Internal standards, added to the starting material and quantified in parallel to the target analytes, are commonly used for this purpose.

However, the normalization procedure is quite different in analyses of gene expression, for several reasons. Firstly, the compounds to be quantified are specific mRNA molecules with the same chemical constituents as other mRNA molecules, differing only in their sequences. Secondly, due to overall differences in transcriptional activity between tissues, normalization cannot be based simply on the amounts of starting material. Instead, quantification of mRNA species should be normalized according to the total amounts of mRNA present in the samples. Since all mRNA

* To whom correspondence should be addressed: E-mail: laurent.gutierrez@u-picardie.fr

molecules are subject to the same variations in both the amounts of starting material and the efficiency of the quantification process, the quantification of a target gene in a sample can be validly normalized using the ratio [target mRNA]/[total mRNA]. Hence, the key requirement for reliable normalization in such analyses is a robust, accurate assessment of the total amount of mRNA in each sample.

Normalization in Northern, microarray, and RT-PCR analyses

Three methods are extensively used for transcript quantification: Northern blotting, microarray analysis, and RT-PCR (reverse transcription polymerase chain reaction) analysis. Northern blotting consists of hybridizing a labelled probe, which is specific to a target mRNA, on a membrane to which a total RNA population has been transferred after electrophoretic separation in a gel. In order to normalize a Northern blot, the quantity of mRNA loaded is commonly estimated by staining the gel with ethidium bromide (EtBr) before the transfer, or by hybridizing the membrane with a labelled probe that specifically binds to an RNA molecule that is supposedly present at a level proportional to the total amount of mRNA. These controls are not very accurate and do not provide reliable indications of the consistency of the mRNA loadings in each lane. Usually, the controls used are ribosomal RNAs, which have been shown to be inappropriate because the ratio between mRNA and risosomal RNA may vary widely depending on the cell population that is being analysed (Spanakis, 1993; Johnson *et al.*, 1995; Warner, 1999; Hansen *et al.*, 2001; Solanas *et al.*, 2001; Tyler *et al.*, 2004). However, Northern blotting is used to assess large variations in expression levels and, thus, errors due to approximate normalization appear to be acceptable since they will not dramatically affect the derived trends in the expression of the target genes. Microarray analysis has enabled gene expression analysis to be expanded from investigations of one gene at a time to wide-scale analyses, in which expression levels of many genes can be monitored simultaneously. In this type of analysis, which is also based on hybridization, a wide range of probes and targets are used in single experiments, allowing the data acquired in each experiment to be normalized by applying various statistical procedures to the global hybridization signals rather than relying on specific genes that are supposedly representative of the total amount of mRNA (Quackenbush, 2002).

The approach is very different from that applied in RT-PCR analysis, in which the efficiency of the enzymatic reactions involved (including the reverse transcription that produces the cDNA and the polymerase chain reaction) affects the strength of the detected signals and thus should be considered in the normalization. In such analyses, the expression level of each target gene is normalized to the expression of a stably expressed gene, called a 'reference', which is presumed to be representative of the cDNA concentration in each sample, and subject to the same errors during cDNA preparation as the target gene(s) (Bustin *et al.*, 2005; Huggett *et al.*, 2005).

Normalization in semi-quantitative versus qRT-PCR

RT-PCR analysis is widely used because it can detect very low quantities of a target transcript with very high specificity due to the high temperature used when annealing the specific primers to the target sequence during the PCR, in comparison with the lower stringency applied for probe hybridization in Northern blotting and microarray analysis. The high sensitivity is conferred by the exponential nature of the PCR reactions, which enable specific sequences to be detected in samples even if only a few copies are present. Accordingly, however, it is extremely important to use highly reliable reference genes to normalize the results of RT-PCR analyses.

Two kinds of RT-PCR are commonly applied in published studies: semi-quantitative RT-PCR and quantitative RT-PCR (qRT-PCR or real-time RT-PCR). In semi-quantitative RT-PCR, a target cDNA species is amplified using the same number of cycles for all investigated samples. After electrophoretic separation in a gel and staining with EtBr (or some other nucleic acid dye), the expression rate of the target gene is assessed by measuring the intensity of the band corresponding to the generated amplicon. The band's intensity reflects the number of copies of the target cDNA (i.e. of the target mRNA) at the beginning of the PCR, and thus the level of expression of the target gene in the sample (Fig. 1A). To ensure that the analysis yields reliable results, the concentration of total cDNA must be the same in all of the samples analysed. Each cDNA sample is, therefore, initially diluted until the intensity of the band corresponding to a reference gene (which is supposedly representative of the total amount of cDNA) obtained from each sample is the same after a defined number of PCR cycles (Fig. 1B). In such semi-quantitative RT-PCR, the intensity of a given band is only correlated to the level of expression of the corresponding gene during the exponential phase of the PCR, which spans a window of just a few cycles that differs both between genes and between samples according to the amounts of transcripts originally present. As illustrated in Fig. 1C, the window is limited and so it is impossible to define a number of cycles that will be an appropriate endpoint for all samples. This technique is, therefore, mainly applied to differences detected during the linear phase. This major limitation, together with the low accuracy of quantifications of bands' intensities, the lack of consideration of PCR efficiency, and the requirement for several dilution adjustments during the normalization process, make semi-quantitative RT-PCR (as implied by the term) barely quantitative. Consequently, although this method is still currently used to assess very weak changes in gene expression, it should really only be used in cases where there are substantial variations.

By contrast, the development of qRT-PCR has provided a powerful tool for quantifying gene expression, combining improvements in both sensitivity and specificity with efficient techniques for signal detection (Nolan *et al.*, 2006). Nevertheless, to ensure reproducible and accurate measurements of

transcript abundance, this sensitive technique should be performed by following golden rules which have been detailed recently in Udvardi *et al.* (2008).

In qRT-PCR, for both target and reference genes, a constant volume of crude cDNA sample, which does not require any preliminary adjustment of cDNA concentrations between samples, is directly used for the PCR. The data generated, called crossing thresholds (CTs) or crossing points (CPs), correspond to the numbers of cycles required to reach a defined fluorescence intensity (Fig. 2). This intensity, measured in real-time, is related to the amount of DNA produced during the PCR and originates from either a non-sequence-specific dye (eg SYBR Green) that fluoresces when bound to a double-stranded DNA molecule, or from a fluorescing probe that anneals to a specific DNA sequence. In either case, the intensity of the fluorescence is always related to the number of amplicons generated during the PCR.

As in every type of PCR, the numbers of cycles required to enter the visible, exponential phase of the PCR depends on the initial amount of the target sequence at the beginning of the PCR. In semi-quantitative RT-PCR, the variations in band intensities at the endpoint provide indications of the shifts between PCR curves. In qRT-PCR, the shifts can be directly visualized in the real-time PCR curves and expressed as the differences between the CTs, called ΔCT (Fig. 2A). After assessing the PCR efficiency (E), which is specific for each primer pair and is seldom (if ever) exactly 2, the difference in the expression level of a target (T) gene between two samples, 1 and 2, is calculated from $E_T^{\Delta CT(1-2)}$. The normalization consists of using the ΔCT and the efficiency measured for the reference (R) gene to calculate the $E_T^{\Delta CT(1-2)}/E_R^{\Delta CT(1-2)}$ ratio, which gives the normalized efficient-corrected relative quantification of the target gene expression in sample 2 compared to sample 1; sample 1 being, in this case, the calibrator (Fig. 2B). Clearly, using a reference gene that is

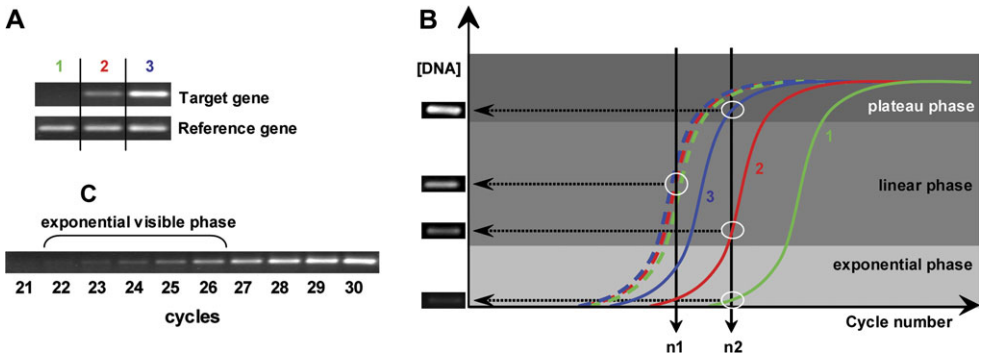

Fig. 1. Normalization in semi-quantitative RT-PCR. (A) Example of expression quantification of a target gene in three cDNA samples (1, 2, and 3) by semi-quantitative RT-PCR. (B) Curves corresponding to the PCR performed in (A). Amplification is shown by coloured lines for the target gene and by dotted lines for the reference gene. Each cDNA sample (1 in green, 2 in red, and 3 in blue) is diluted until the intensity of the band corresponding to the reference gene obtained from each sample is the same after a defined number (n1) of PCR cycles. After this normalization step, the target gene expression can be analysed at endpoint, after n2 cycles of PCR. (C) In order to define the exponential phase, PCR are performed using a range of cycle numbers (here from 21 to 30).

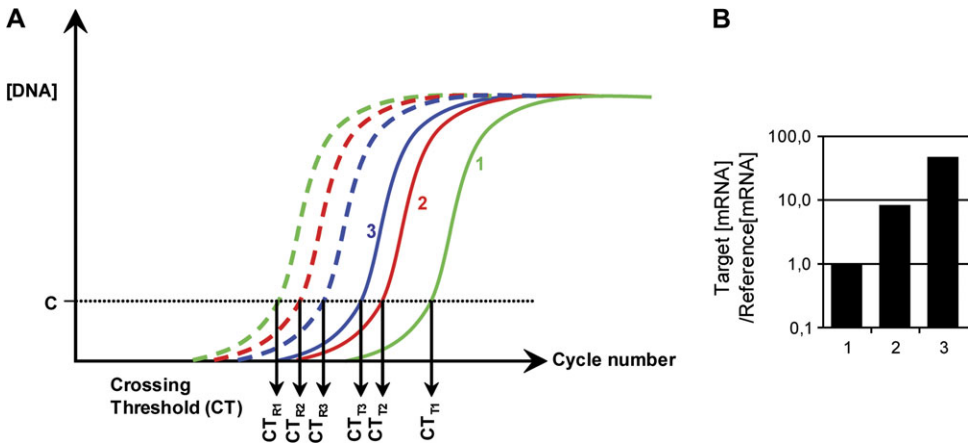

Fig. 2. Normalization in qRT-PCR. (A) In qRT-PCR, the fluorescence intensity is measured in real-time, allowing a CT to be defined for each cDNA sample. PCR for the target gene is shown by coloured lines and PCR for the reference gene by dotted lines. Each colour corresponds to a cDNA sample. C is the threshold, a constant fluorescence intensity, used to determine each CT. (B) Data generated by qRT-PCR are used to assess quantitatively the expression of a target gene.

consistently expressed, i.e. that is accurately representative of the total mRNA amount in each sample, is essential since any variation in its expression would directly affect the final result of the normalized-expression ratio of the target gene (Dheda *et al.*, 2005; Hendriks-Balk *et al.*, 2007). It is therefore essential to use valid reference genes in order to ensure the accuracy of qRT-PCR analysis.

Reference validation: the need for systematic assessment

Validating a reference gene is challenging since it requires the resolution of a circular problem: assessing the stability of the expression of a gene without using any other references. Indications regarding the stability of the expression levels of specific genes in model organisms may be acquired from the data compiled in huge public microarray databases. However, this approach raises several questions regarding, for instance, the reliability of using indicators of the stability of genes' expression levels derived by averaging values obtained from large numbers of samples collected under widely varying experimental conditions, and the inconsistency of using data obtained in microarray analyses to obtain values for normalizing the results of qRT-PCR analyses, which are known to be more accurate than microarray analyses (Holland, 2002; Czechowski *et al.*, 2004; Dallas *et al.*, 2005).

In attempts to solve this conundrum, several statistical algorithms for processing qRT-PCR data have been developed to identify the best reference genes to use under given experimental conditions (Pfaffl *et al.*, 2002, 2004; Vandesompele *et al.*, 2002; Andersen *et al.*, 2004). The geNorm software (Vandesompele *et al.*, 2002) is one of the most commonly used algorithms, which has been cited hundreds of times and downloaded thousands of times [http://medgen.ugent.be/~jvdesomp/genorm/]. In order to assess the reliability of candidate genes as references, their expression is measured by qRT-PCR under the same experimental conditions as those used for the target genes (i.e. using the same set of cDNAs). The crude (non-normalized) expression data are then exported, for instance, to the geNorm software which determines, for each gene, the pairwise variation with all other genes in terms of the standard deviation of their logarithmically transformed expression ratios. *M*, a measure of the gene expression stability, is the average pairwise variation of a particular gene compared to that of all other genes. Genes with the lowest *M* values have the most stable expression. The reliability of such a strategy for reference validation can be easily checked, for instance, by comparing the expression patterns for a given target gene obtained by normalization using several of the best candidates. If the differences between the patterns obtained using these candidates are minor, then the choice of candidate will not greatly affect the target gene's expression profiles, thus providing reassurance regarding the reliability of the normalization. The robustness and convenience of this approach explain why evaluations using geNorm software are now commonly included in qRT-PCR analyses of gene expression in animals, yeast, and bacteria [http://medgenugentbe/~jvdesomp/genorm/citationsphp]. This approach has been successfully, although unfortunately rarely, used in the plant field, allowing the identification of the best reference genes to use under given experimental conditions in rice (Caldana *et al.*, 2007), in *Medicago truncatula* (Kakar *et al.*, 2008), and in grapevine (Reid *et al.*, 2006).

Figure 3 shows the results of a geNorm assessment of gene expression stability in *Arabidopsis* using both data from microarray public databases, compiled from analyses covering a huge range of types of samples and experimental conditions—i.e. the Affymetrix ATH1 data from the developmental series—(Fig. 3A), and data generated by qRT-PCR analysis of much more limited types of sample (Fig. 3B) (Czechowski *et al.*, 2005). There are highly informative differences between the two sets of results and the ranking of the genes appears to depend on the specificity of the database used to assess the stability of their expression. Some genes could be valid references in some cases, but inappropriate in others. For example, both the At5g60390 and At4g38070 genes seem to be stably expressed according to Fig. 3A, but they would be unsuitable references to use for the more limited types of samples examined in the analyses that yielded the data presented in Fig. 3B. Furthermore, since even the more limited types of samples spanned a substantial range, further changes in rankings may occur if samples representing an even narrower range of conditions were analysed. These changes clearly illustrate the inconsistency of using a wide approach to validate reference genes and the impossibility of compiling a list of suitable genes that could be used as references across a wide range of experimental conditions, confirming points raised above, and indicating that it is essential to validate references under every set of specific experimental conditions.

Variability and risks associated with the current flexibility of reference validation requirements

The development of algorithms like geNorm was prompted several years ago by increasing awareness amongst some molecular biologists of the extent to which the use of invalid references could affect qRT-PCR analysis and thus the need to validate references properly. Surprisingly, this awareness has not fully permeated throughout the community of molecular biologists. The importance of this issue does not seem to have been fully realized, and most reviewers do not regard it as a crucial criterion when evaluating the validity of qRT-PCR analyses. While considerable efforts have been made to increase the use of validated references in medical studies (although their use is still not obligatory for publication even in highly-regarded medical journals), the importance of this issue has received little attention in other research fields, for example, plant science. Indeed, genes currently used as references for qRT-PCR analysis in

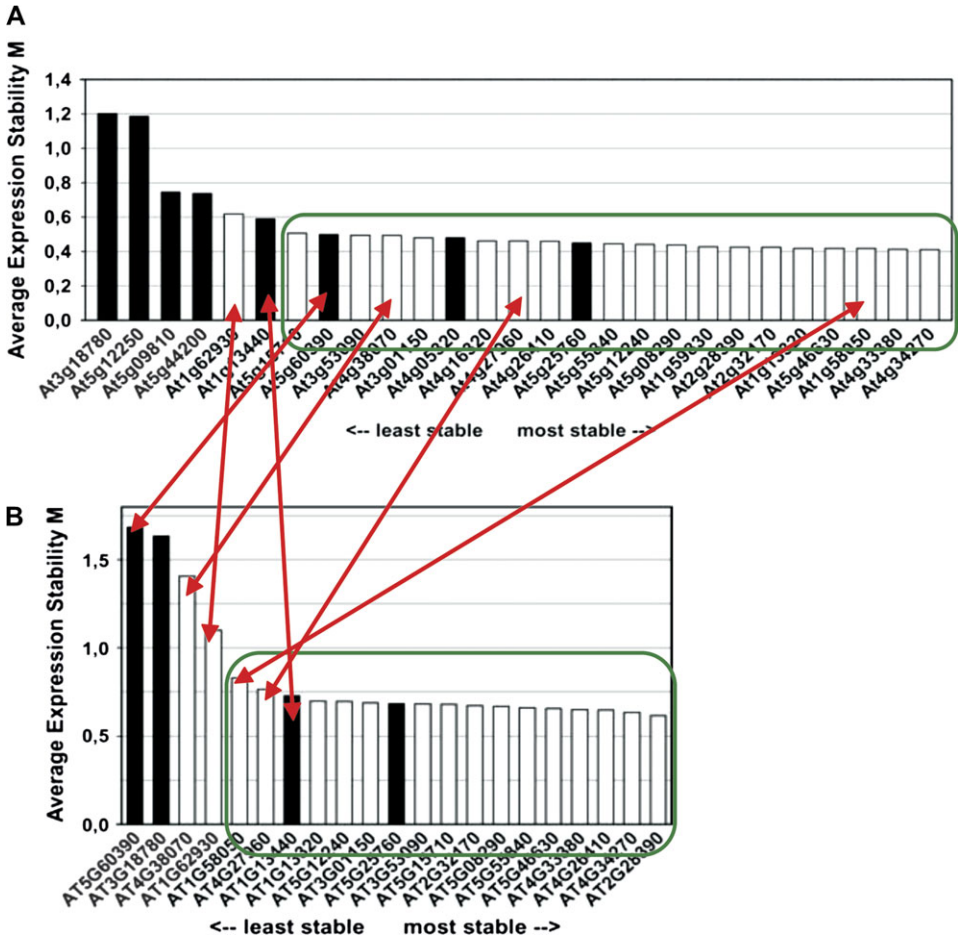

Fig. 3. Ranking of genes by geNorm based on data from microarray public databases (A) or on data generated by qRT-PCR analysis of much more limited types of sample (B). Genes showing high expression stability are grouped in the green squares. Depending on the specificity of the database used to assess the stability of their expression, the ranking of the genes changes. Adapted from Czechowski *et al.*, 2005 and reproduced by kind permission of the American Society of Plant Biologists.

plants are almost exclusively putative housekeeping genes (Gutierrez *et al.*, 2008*a*). Figure 4 shows the distribution of genes used as references in qRT-PCR analyses that have been published during a 6-month period from July through December 2007 in *The Plant Cell*, *Plant Physiology*, and *The Plant Journal*; three leading plant science journals according to the ISI Web of Knowledge^{SM}. References that had been properly validated in either the same study or a previous study and shown to be suitable for validation under the experimental conditions applied were only used in 3.2% of the 188 studies involving qRT-PCR analyses published in these journals during this period (Gutierrez *et al.*, 2008*a*). In the other 96.8% of the papers, all the genes were merely putatively stably expressed. The choice of such genes as references is inappropriate for several reasons. First, their status as 'housekeeping' genes is generally based on unpublished data acquired using Northern blotting or histochemical analysis; methods known to be largely qualitative. Consequently, transcript normalization using such genes is not consistent with the high accuracy associated with qRT-PCR. Second, these genes are often used as references for samples collected under experimental

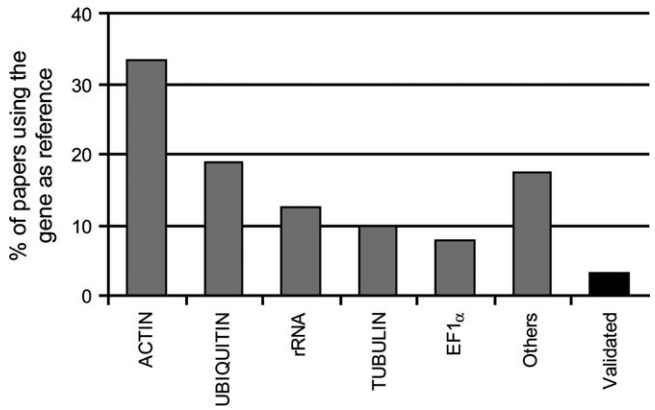

Fig. 4. Distribution of genes used as references from July through December 2007 in three leading plant science journals.

conditions that differ from those in which their expression stability was tested. Such housekeeping genes continue to be frequently used as reference genes, even though their expression has only been shown to be constant under some experimental conditions, and highly variable in other cases

(Volkov *et al.*, 2003; Czechowski *et al.*, 2005; Nicot *et al.*, 2005; Waxman and Wurmbach, 2007; Remans *et al.*, 2008).

The continued use of inappropriate reference genes may have already resulted in the misinterpretation of some published results. Figure 5 illustrates how easily such misinterpretation could result from the use of an inappropriate reference. Using data from our recent article (Gutierrez *et al.*, 2008*b*), 14 genes, commonly used as references in *Arabidopsis* analyses, were assessed for their expression stability in six types of samples: 7-d-old seedlings (S); leaves (L) and roots (R) from 3-week-old (Y for young) and 6-week-old (O for old) plants; and inflorescences (ISL). The ranking by geNorm obtained under these experimental conditions (Fig. 5A) confirms the inconsistency of using a non-systematic approach for reference validation. Indeed, the gene stability assessment by geNorm we previously performed in Gutierrez *et al.* (2008*b*) using a different set of *Arabidopsis* tissues, i.e. flowers and siliques at different stages, provided a different ranking than the one obtained here for the same set of genes (Fig. 5A). For instance, while eF1α was previously shown to be one of the most stably expressed genes and, thus, assessed as being a valid reference, this gene definitely constitutes an unsuitable reference to be used in the set of experimental conditions described above. This comparison reinforces the necessity of validating references under every set of specific experimental conditions, i.e. of adopting a systematic validation.

The least (*TUB6*) and one of the two most (*APT1*) stably expressed genes were used to normalize the expression of the second most stably expressed gene (*NDUFA8*) in the six types of samples (Fig. 5B). When normalized using *APT1*, *NDUFA8* shows a constant expression pattern, in accordance with its status as a co-most stably expressed gene, but when normalized with *TUB6* the *NDUFA8* expression pattern varies considerably, solely due to the effects of the inconsistency of *TUB6*'s expression. If *TUB6* had not previously been shown to be an inappropriate reference, the *NDUFA8* expression pattern would have been misinterpreted. Such misinterpretation, which can have major effects on the conclusions drawn from a published study, would be avoided if the systematic validation of references was considered to be an essential component of qRT-PCR analysis.

Conclusions: towards a systematic validation of references in the plant field?

The use of putative housekeeping genes is acceptable for qualitative analyses, in which semi-quantitative techniques are applied. Transcripts of such genes, which are expressed at relatively high levels in all cells, make ideal positive controls for determining whether or not genes of interest are expressed in given types of samples under given conditions. However, the advent of RT-PCR, especially qRT-PCR, has placed the emphasis on quantitative changes, and should have prompted a re-evaluation of the use of these reference genes (Bustin *et al.*, 2005). Unfortunately, awareness of the need for re-evaluation has been patchy, and double standards are currently applied in the requirements for reference validation in publications describing qRT-PCR analysis. More alarmingly, semi-quantitative RT-PCR, rather than qRT-PCR, is still widely used to assess gene expression quantitatively in published analyses. We believe that this lack of confidence in qRT-PCR is due to disappointments arising from the conflicting results sometimes obtained using this technique due to the failure to apply a robust normalization strategy. Making the systematic validation of reference genes obligatory for reported analyses would greatly improve the accuracy and consistency of RT-PCR analyses published in plants. Moreover, the adoption of such a convention would allow the accuracy of a powerful technique, which has often been lost due to inappropriate normalization, to be recovered.

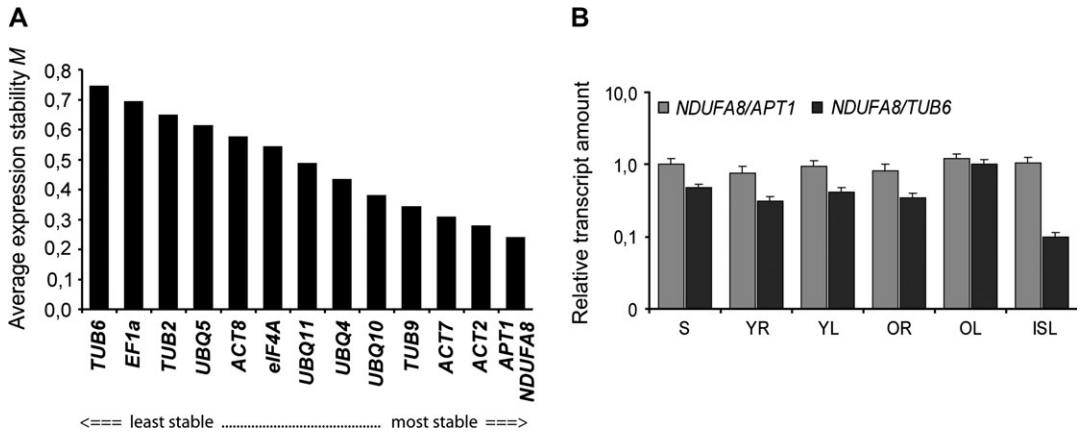

Fig. 5. Normalizing with an improper reference strongly impacts on the expression pattern of a target gene. (A) GeNorm ranking of 14 genes, commonly used as reference, based on qRT-PCR performed on six types of samples. (B) The expression pattern of *NDUFA8* is strongly affected by the normalization with *TUB6*. ISL, inflorescence; OL, old leaf; OR, old root; S, seedling; YL, young leaf; YR, young root. From data published in Gutierrez *et al.*, 2008*b*.

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 Research* **64,** 5245–5250.

Bustin SA, Benes V, Nolan T, Pfaffl MW. 2005. Quantitative real-time RT-PCR: a perspective. *Journal of Molecular Endocrinology* **34,** 597–601.

Caldana C, Scheible WR, Mueller-Roeber B, Ruzicic S. 2007. A quantitative RT-PCR platform for high-throughput expression profiling of 2500 rice transcription factors. *Plant Methods* **3,** 7.

Czechowski T, Bari RP, Stitt M, Scheible WR, Udvardi MK. 2004. Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *The Plant Journal* **38,** 366–379.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology* **139,** 5–17.

Dallas PB, Gottardo NG, Firth MJ, Beesley AH, Hoffmann K, Terry PA, Freitas JR, Boag JM, Cummings AJ, Kees UR. 2005. Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR – how well do they correlate? *BMC Genomics* **6,** 59.

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. *Analytical Biochemistry* **344,** 141–143.

Gutierrez L, Mauriat M, Guenin S, *et al.* 2008*b*. 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 Biotechnology Journal* **6,** 609–618.

Gutierrez L, Mauriat M, Pelloux J, Bellini C, Van Wuytswinkel O. 2008*a*. Towards a systematic validation of references in real-time RT-PCR. *The Plant Cell* **20,** 1734–1735.

Hansen MC, Nielsen AK, Molin S, Hammer K, Kilstrup M. 2001. Changes in rRNA levels during stress invalidates results from mRNA blotting: fluorescence *in situ* rRNA hybridization permits renormalization for estimation of cellular mRNA levels. *Journal of Bacteriology* **183,** 4747–4751.

Hendriks-Balk MC, Michel MC, Alewijnse AE. 2007. Pitfalls in the normalization of real-time polymerase chain reaction data. *Basic Research in Cardiology* **102,** 195–197.

Holland MJ. 2002. Transcript abundance in yeast varies over six orders of magnitude. *Journal of Biological Chemistry* **277,** 14363–14366.

Huggett J, Dheda K, Bustin S, Zumla A. 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes and Immunity* **6,** 279–284.

Johnson ML, Redmer DA, Reynolds LP. 1995. Quantification of lane-to-lane loading of poly(A) RNA using a biotinylated oligod(T) probe and chemiluminescent detection. *Biotechniques* **19,** 712–715.

Kakar K, Wandrey M, Czechowski T, *et al.* 2008. A community resource for high-throughput quantitative RT-PCR analysis of transcription factor gene expression in *Medicago truncatula*. *Plant Methods* **4,** 18.

Nicot N, Hausman JF, Hoffmann L, Evers D. 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. *Journal of Experimental Botany* **56,** 2907–2914.

Nolan T, Hands RE, Bustin SA. 2006. Quantification of mRNA using real-time PCR. *Nature Protocols* **1,** 1559–1582.

Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30,** e36.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnology Letters* **26,** 509–515.

Quackenbush J. 2002. Microarray data normalization and transformation. *Nature Genetics* **32,** Supplement, 496–501.

Reid KE, Olsson N, Schlosser J, Peng F, Lund ST. 2006. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biology* **6,** 27.

Remans T, Smeets K, Opdenakker K, Mathijsen D, Vangronsveld J, Cuypers A. 2008. Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. *Planta* **227,** 1343–1349.

Solanas M, Moral R, Escrich E. 2001. Unsuitability of using ribosomal RNA as loading control for Northern blot analyses related to the imbalance between messenger and ribosomal RNA content in rat mammary tumors. *Analytical Biochemistry* **288,** 99–102.

Spanakis E. 1993. Problems related to the interpretation of autoradiographic data on gene expression using common constitutive transcripts as controls. *Nucleic Acids Research* **21,** 3809–3819.

Tyler L, Thomas SG, Hu J, Dill A, Alonso JM, Ecker JR, Sun TP. 2004. Della proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. *Plant Physiology* **135,** 1008–1019.

Udvardi MK, Czechowski T, Scheible WR. 2008. Eleven golden rules of quantitative RT-PCR. *The Plant Cell* **7,** 1736–1737.

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 Biology* **3,** RESEARCH0034.

Volkov RA, Panchuk II, Schoffl F. 2003. Heat-stress-dependency and developmental modulation of gene expression: the potential of housekeeping genes as internal standards in mRNA expression profiling using real-time RT-PCR. *Journal of Experimental Botany* **54,** 2343–2349.

Warner JR. 1999. The economics of ribosome biosynthesis in yeast. *Trends in Biochemical Sciences* **24,** 437–440.

Waxman S, Wurmbach E. 2007. De-regulation of common housekeeping genes in hepatocellular carcinoma. *BMC Genomics* **8,** 243.