Technical advance

Evaluation of reference genes for quantitative reverse-transcription polymerase chain reaction normalization in infected tomato plants

TIZIANA MASCIA^{1,2}, ELISA SANTOVITO², DONATO GALLITELLI^{1,2} AND FABRIZIO CILLO^{1,*}¹Istituto di Virologia Vegetale, CNR, Via Amendola 165/A, 70126 Bari, Italy²Dipartimento di Biologia e Patologia Vegetale, Università degli Studi di Bari 'Aldo Moro', Via Amendola 165/A, 70126 Bari, Italy

SUMMARY

The quantification of messenger RNA expression levels by real-time reverse-transcription polymerase chain reaction requires the availability of reference genes that are stably expressed regardless of the experimental conditions under study. We examined the expression variations of a set of eight candidate reference genes in tomato leaf and root tissues subjected to the infection of five taxonomically and molecularly different plant viruses and a viroid, inducing diverse pathogenic effects on inoculated plants. Parallel analyses by three commonly used dedicated algorithms, geNorm, NormFinder and BestKeeper, showed that different viral infections and tissues of origin influenced, to some extent, the expression levels of these genes. However, all algorithms showed high levels of stability for *glyceraldehyde 3-phosphate dehydrogenase* and *ubiquitin*, indicated as the most suitable endogenous transcripts for normalization in both tissue types. *Actin* and *uridylylase kinase* were also stably expressed throughout the infected tissues, whereas *cyclophilin* showed tissue-specific expression stability only in root samples. By contrast, two widely employed reference genes, *18S ribosomal RNA* and *elongation factor 1 α* , demonstrated highly variable expression levels that should discourage their use for normalization. In addition, expression level analysis of *ascorbate peroxidase* and *superoxide dismutase* showed the modulation of the two genes in virus-infected tomato leaves and roots. The relative quantification of the two genes varied according to the reference genes selected, thus highlighting the importance of the choice of the correct normalization method in such experiments.

INTRODUCTION

Genomic technologies offer unprecedented possibilities for the understanding of biological mechanisms and the investigation of

molecular bases of natural phenomena. Gene networks, metabolic pathways and complex biological relationships are being unravelled using approaches such as transcriptome analysis and gene expression profiling, which are now affordable by many research groups worldwide. In the field of plant–pathogen interactions, transcriptomic technologies, such as microarray, quantitative real-time polymerase chain reaction (qPCR) and reverse transcription-qPCR (RT-qPCR), have provided new insights into the mechanisms underlying pathogenesis, disease symptom development, resistance and basal defence (Wise *et al.*, 2007).

Although microarray is the most popular strategy of choice for genome-wide gene expression profiling, RT-qPCR is commonly employed at a smaller scale, because it offers high sensitivity, high specificity, good reproducibility and a wide dynamic quantification range. The consistency of the results obtained by PCR-based technologies relies on accurate data normalization, which, in turn, depends on a number of variables that need to be accurately controlled. RNA quantity and quality, presence of inhibitors, RT and PCR performances, and the use of robust methods for data normalization are among these variables (Pfaffl, 2006). The quantification of mRNA transcripts by RT-qPCR is usually performed by two approaches, referred to as 'absolute quantification' and 'relative' or 'comparative quantification'. The former strategy is based on the comparison between the mRNA expression level and a calibration curve of a target reference sequence of known quantity (Bustin, 2000), whereas the latter calculates the amounts of mRNA of interest relative to the amounts of a second RNA transcript, chosen as the reference or 'housekeeping' sequence. Relative quantification does not require nucleic acid sequences with known concentrations, and the reference can be any transcript, as long as its expression is not dependent on variable experimental conditions (Bustin, 2002; Pfaffl *et al.*, 2004).

In recent years, a number of studies have outlined the importance of a considerate application of RT-qPCR approaches to the study of gene expression profiles in plants. A particular focus has been the choice of appropriate reference genes (RGs) with a proven expression stability throughout all organs and treatments

*Correspondence: Email: f.cillo@ba.ivv.cnr.it

being compared (Czechowski *et al.*, 2005; Nicot *et al.*, 2005; Udvardi *et al.*, 2008). Many studies have described gene expression profiling approaches through RT-qPCR for the investigation of molecular mechanisms related to plant responses to stresses and diseases (Wise *et al.*, 2007; Yamaguchi-Shinozaki and Shinozaki, 2006), but only a few have demonstrated *a priori* the suitable expression stability of the proposed RGs in the tested conditions. The choice usually falls on genes commonly considered as good housekeeping genes, although many have been proven to undergo certain degrees of variability after more in-depth evaluation (Nicot *et al.*, 2005; Sturzenbaum and Kille, 2001).

Among the well-studied examples of biotic stresses, virus and virus-like infections evoke extensive metabolic alterations and gene expression reprogramming as a result of their endocellular parasitic activity and pathogenetic properties (Whitham *et al.*, 2003; Wise *et al.*, 2007). It has been demonstrated that diverse viruses can induce divergent alteration of host gene expression profiles as a consequence of differences in molecular and biological features, as well as pathogenetic processes (Whitham *et al.*, 2003). In addition, a single virus can modulate differentially the gene expression profiles in different host organs (Catoni *et al.*, 2009). Therefore, the possibility that the expression levels of known plant housekeeping genes might be altered specifically by viruses, throughout the plant or in specific organs, should be properly addressed when performing host transcript profiling.

The aim of this study was to evaluate eight different tomato genes, putatively suitable as RGs in RT-qPCR experiments, for their expression stability on inoculation with different pathogenic viruses and a viroid. We included in our analysis six endogenous transcripts usually employed as RGs and two additional genes, *uridylate kinase* and *cyclophilin*, that were identified as being expressed at unvarying levels in microarray analysis of differential gene expression profiles in tomato plants infected with different *Cucumber mosaic virus* (CMV) strains (F. Cillo, D. Gallitelli and A. Polverari, unpublished results). Indeed, whole-genome transcriptomic analyses have been indicated as good sources of novel RGs, as they emphasize the stability of transcript accumulation levels in different experimental conditions (Czechowski *et al.*, 2005; Libault *et al.*, 2008).

For the purpose of this study, six isolates of five different plant virus species and a viroid were selected as representative members of diverse and distant taxonomic groups showing different pathogenic behaviour and inducing different disease phenotypes in tomato. Quantitative expression measurements obtained by RT-qPCR were analysed by three of the statistical algorithms most commonly used for assessing the appropriateness of RGs, i.e. geNorm (Vandesompele *et al.*, 2002), Norm-Finder (Andersen *et al.*, 2004) and BestKeeper (Pfaffl *et al.*, 2004), all freely available for download from the authors' web-

sites. The results provided in this article indicate the best-performing RGs in our experimental model, and could help in the future selection of suitable RT-qPCR normalization procedures in the study of other pathogen–host interactions.

RESULTS

Assessment of target amplification efficiencies and expression levels of candidate RGs

The stabilities of eight tomato candidate RGs, *actin* (*ACT*), *elongation factor 1- α* (*EF1 α*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *β -tubulin* (*TUB*), *ubiquitin 3* (*UBI*), *uridylate kinase* (*UK*), *cyclophilin* (*CyP*) and *18S ribosomal RNA* (*18S*) (Table 1), were tested under different biotic stress conditions determined by the infection of pathogenic viruses and a viroid. Before quantitative analyses, validation experiments were carried out to confirm equal amplification efficiencies between the eight target genes. It has been proposed that PCR efficiencies for different target DNA species can be considered to be comparable when included in the range of $100 \pm 10\%$, corresponding to a standard curve slope of -3.3 ± 0.33 (Livak, 2001). The amplicons size, which was optimized to 98–120 base pairs, favoured optimal polymerization efficiencies of the selected RG sequences, which ranged between 97.6% and 103.2% (Table 1). The standard curves demonstrated a good linear relation ($R^2 > 0.99$) between the Ct values and the log-copy numbers for all the tested RGs (Table 1), confirming the suitability of primer pairs and target sequences for RT-qPCR-based quantification. Melting curves generated during the PCR amplification showed a single peak, indicating a single amplified product for all target transcripts (not shown).

The Ct values for the mRNAs selected as candidate RGs ranged between 17.70 (*Cyp*) and 36.45 (*EF1 α*), and these two transcripts showed the most and least abundant accumulation levels, respectively, in both tissue types tested (Fig. 1). RT-PCR amplification of *18S* RNA, that is largely overexpressed in plant cells, produced Ct values lower than most transcripts in leaves in spite of an additional 1:10 dilution of the template (Fig. 1A). The average Ct values obtained for all the genes under study did not show remarkable differences between leaf and root tissues, but the range of values was consistently narrower in leaves than in roots (Fig. 1).

geNorm analysis

When using geNorm, a ranking of the tested genes is provided according to the stability measure *M* (average pairwise variation of each combination of candidate RGs), from the most stable (lowest *M* value) to the least stable (highest *M* value). The pairwise variation (*V*) is implemented to calculate the optimal

Table 1 Primer sequences for eight candidate reference genes and real-time reverse transcription-polymerase chain reaction (RT-PCR) amplification efficiency parameters.

Gene name	Gene symbol	GENBANK Acc.	Primer sequence		Amplicon length (bp)	E (%) [*]	R ² †
<i>Actin</i>	<i>ACT</i>	BT013707	For	5'-AGGCAGGATTGCTGGTGATGATGCT-3'	107	101.5	0.998
			Rev	5'-ATACGCATCCTTCTGTCCCATTCGA-3'			
<i>Cyclophilin (peptidyl-prolyl cis-trans isomerase)</i>	<i>CyP</i>	AK326854 (TC174635)‡	For	5'-CTCTTCGCCGATACCACTCC-3'	120	99.4	0.998
			Rev	5'-TCACACGGTGGAAGGTTGAG-3'			
<i>Elongation factor 1-α</i>	<i>EF1α</i>	X53043	For	5'-ATTGGAAATGGATATGCTCCA-3'	100	98.6	0.998
			Rev	5'-TCCTTACCTGAACGCCTGTCA-3'			
<i>Glyceraldehyde 3-phosphate dehydrogenase</i>	<i>GAPDH</i>	U93208	For	5'-ACCACAAATGCTTGTCCCTTG-3'	110	97.6	0.998
			Rev	5'-ATCAACGGTCTTCTGAGTGGCTGT-3'			
<i>β-Tubulin</i>	<i>TUB</i>	DQ205342	For	5'-CCTGACAGCTTCTGCCATGT-3'	106	97.9	0.998
			Rev	5'-CATCTTCAGCCAGTTGGTG-3'			
<i>Ubiquitin 3</i>	<i>UBI</i>	X58253	For	5'-TCGTAAGGAGTGCCCTAATGCTGA-3'	119	103.2	0.998
			Rev	5'-CAATCGCCTCCAGCCTTGTGTAA-3'			
<i>Uridylate kinase</i>	<i>UK</i>	AK322232 (TC196198)‡	For	5'-TGGTAAGGGCACCCAATGTGCTAA-3'	114	99.6	0.998
			Rev	5'-ATCATCGTCCCATTCGGAACCA-3'			
<i>18S rRNA</i>	<i>18S</i>	X51576	For	5'-GGGCATTCTGATTTTCATAGTCAGA-3'	98	98.2	0.998
			Rev	5'-GTTCTTGATTAATGAAACATCCT-3'			

^{*}Measure of the real-time PCR efficiency (calculated by the standard curve method).

†Regression coefficient calculated from the regression line in the standard curve.

‡Corresponding accession number from DFCI Tomato Gene Index.

Fig. 1 Graphical representation of the expression level data relative to the candidate reference genes. Box plots exhibit the expression levels of candidate reference genes in virus-infected leaf tissue samples ($n = 24$) (A), virus-infected root tissue samples ($n = 15$) (B) and all samples combined together (C). Values are given as the cycle threshold (Ct, mean of duplicate samples), and are inversely proportional to the amount of template. Global expression levels of the different genes tested are shown as the 25th and 75th quantiles (horizontal lines), median (emphasized horizontal line) and whiskers. Whiskers go from the minimal to maximal value or, if the distance from the first quartile to the minimum value is more than 1.5 times the interquartile range (IQR), from the smallest value included within the IQR to the first quartile. Outliers, the values smaller (Min) or larger (Max) than 1.5 times the IQR are indicated.

number of genes to be included when the normalization is performed using multiple RGs (Vandesompele *et al.*, 2002).

In geNorm, genes with a high *M* value have a high variance in gene expression, but, in our experimental conditions, candidate genes showed an overall limited variance for each infection and tissue analysed (Fig. 2). In particular, in root tissues, all the *M* values were far below ($M < 0.96$) the acceptable limit of 1.5 (Fig. 2B). In tomato leaves, the most stably expressed genes of the pool (*ACT* and *UBI*) allowed an optimal normalization of RT-qPCR data, and the addition of a third, less stable normalization factor (*GAPDH*) did not increase significantly the statistical reliability of this calculation. Indeed, the *V*_{2/3} value (pairwise variation when the number of normalization factors is increased from two to three) was 0.133 in leaves, abundantly below the default cut-off value of $V = 0.15$ (Vandesompele *et al.*, 2002) (Fig. 2D). Similarly, in samples from root tissues, geNorm identified *UBI* and *CyP* as the best candidate RGs. Even in this case, when including a third housekeeping gene (*UK*) in addition to *UBI* and *CyP*, *V*_{2/3} (0.107) remained below the proposed cut-off value of $V = 0.15$ (Fig. 2D). When all samples were analysed together, gathering RT-qPCR data from different viral infections and from leaf and root samples, the gene expression stability of our pool was calculated in the same ranking order as obtained for leaf samples only (compare Fig. 2A and C), with the most stable RG pair showing $M = 0.33$ and the least stable, *EF1α*, only just below the *M*-value limit of 1.5 (Fig. 2C). In addition, in the case of geNorm analysis of all combined data, the pairwise variation data, calculating $V < 0.15$, supported the use of no more than two RGs, *ACT* and *UBI*, for RT-qPCR normalization (Fig. 2D).

Overall, tomato *18S* and *EF1α* were indicated by geNorm to be unreliable RGs under viral infections, in particular the latter in leaf tissues, where $M > 1.5$ indicated an unacceptable level of expression variability.

NormFinder analysis

NormFinder is another Excel-based visual basic application that assigns stability values to single candidate RGs. The NormFinder algorithm uses a model-based approach for the estimation of expression variation among the candidate genes, taking into account variations among and inside subgroups and avoiding misinterpretations caused by the artificial selection of coregulated genes.

The results of NormFinder analysis are shown in Fig. 3. *UBI*, *ACT*, *GAPDH* and *UK* still occupied the four top positions for higher stability in leaf tissues, and the entire ranking order was repeated with limited differences between the geNorm and NormFinder results, with *18S* and *EF1α* indicated again as the least stable genes. However, NormFinder analysis in leaves did not show such extensive variations in stability values as

observed in geNorm and in root tissues. In roots, *GAPDH*, *ACT*, *UBI* and *CyP* were estimated to be the most stably expressed genes (Fig. 3), an observation only partially in agreement with the ranking order produced by geNorm analysis. Regardless of the ranking order, however, output data from both geNorm and NormFinder showed that *GAPDH*, *ACT*, *UBI*, *CyP*, *UK* and *TUB* had a good level of expression stability in root tissues (Figs 2B and 3B).

BestKeeper analysis

The BestKeeper tool estimates the variability in expression of candidate RGs by calculation of the Ct data variations, performing a comparative analysis based on pairwise correlations of all candidate gene combinations.

Gene expression variation was calculated for the eight candidate genes, and the standard deviation (SD) provided stability estimates (Table 2). In leaf samples, BestKeeper highlighted five RGs, *ACT*, *UK*, *GAPDH*, *UBI* and *TUB* in ranking order, all being characterized by the least overall variation, with SD [$\pm$ Ct] < 1 and SD [$\pm$ x-fold] < 2 (Table 2), which represents an acceptable change in expression (Pfaffl *et al.*, 2004). By contrast, the analysis of virus-infected root samples revealed that only the expression of *UBI* fluctuated in a range compatible with SD [$\pm$ Ct] < 1 and SD [$\pm$ x-fold] < 2, whereas all other seven transcripts overcame the stability threshold indicated at SD [$\pm$ Ct] > 1.

When samples from the two tissues were analysed together, the results ranked *GAPDH*, *ACT* and *UK* as the best RGs. The variation in expression of *CyP*, *18S* and *EF1α* was greater than two-fold (SD [$\pm$ Ct] > 1), showing the highest variability in all virus-infected tissues (Table 2).

Quantification of ascorbate peroxidase and superoxide dismutase expression levels in infected tomato leaf and root tissues

To assess the validity of the data obtained by geNorm, NormFinder and BestKeeper, we applied our findings to the analysis of genes of interest in plant–virus interaction studies. We identified two genes encoding reactive oxygen species (ROS) scavenging enzymes, *ascorbate peroxidase-2* (*APX*) and *iron superoxide dismutase* (*SOD*), which give well-known stress-responsive transcripts. Their expression patterns were quantified in tomato leaf and root tissues 9 days after infection with the following: (i) CMV (strain Fny), which induces severe leaf malformation and growth reduction in young tomato plants, (ii) an inoculum combining CMV-Fny plus the CMV-77satRNA (CMV/satRNA), which co-induce cell death and lethal systemic necrosis, and (iii) *Tomato mosaic virus* (ToMV, strain SP), whose symptoms are similar to those elicited by CMV-Fny infections (not shown). According to the results shown above (Fig. 2A–D), we selected

Fig. 2 Expression stability of the candidate reference genes analysed by geNorm. Ranking is based on the principle that gene pairs that have stable expression patterns relative to each other are appropriate reference genes. Average expression stability values (M) of the eight candidate reference genes are shown for virus-infected leaf tissue samples (A), virus-infected root samples (B) and all samples combined together in a single comprehensive analysis (C); (D) determination of the optimal number of reference genes according to geNorm analysis. V2/3, pairwise variation when the number of normalization factors (reference genes) is increased from two to three. Stepwise inclusion of less stable normalization factors generates the next data points (V3/4 to V7/8). A decrease in the V value indicates a positive effect of an additional gene for a reliable calculation of quantitative reverse transcription-polymerase chain reaction (RT-qPCR) normalization.

Fig. 3 Expression stability values of the candidate reference genes analysed by NormFinder. Average expression stability values of the eight candidate reference genes are shown for virus-infected leaf tissue samples (A), virus-infected root samples (B) and all samples combined together in a single comprehensive analysis (C). Error bars represent $\pm$ standard errors.

Table 2 Descriptive statistics of eight candidate reference genes based on their cycle threshold values as calculated by BestKeeper.

Ranking:		1	2	3	4	5	6	7	8
Leaves (n = 24)	Gene name	ACT	UK	GAPDH	UBI	TUB	CyP	18S	EF1α
	Geo Mean [Ct]	21.73	24.38	22.59	22.20	23.35	20.01	24.68	26.65
	Min. max [Ct]	20.92, 22.95	23.38, 25.07	21.54, 23.80	20.65, 23.91	21.77, 25.24	17.90, 23.96	19.21, 27.91	22.87, 36.45
	Std dev [$\pm$ Ct]	0.37	0.45	0.46	0.57	0.60	1.40	2.05	2.44
	Min. max [x-fold]	-1.75, 2.33	-1.99, 1.61	-2.07, 2.31	-2.91, 3.28	-2.98, 3.71	-4.31, 15.45	-44.52, 9.39	-13.76, 889.32
	Std dev [$\pm$ x-fold]	1.29	1.37	1.38	1.49	1.52	2.64	4.15	5.42
Roots (n = 15)	Gene name	UBI	UK	ACT	CyP	GAPDH	TUB	18S	EF1α
	Geo Mean [Ct]	20.40	23.59	20.80	19.29	22.29	22.18	26.97	29.22
	Min. max [Ct]	18.84, 22.18	22.02, 25.50	18.83, 23.17	17.70, 21.41	20.19, 25.07	19.76, 24.95	23.81, 34.16	26.70, 33.36
	Std dev [$\pm$ Ct]	0.89	1.03	1.06	1.07	1.12	1.32	1.75	2.02
	Min. max [x-fold]	-2.95, 3.43	-2.98, 3.75	-3.90, 5.19	-3.02, 4.35	-4.27, 6.89	-5.35, 6.83	-8.93, 145.91	-5.71, 17.72
	Std dev [$\pm$ x-fold]	1.85	2.05	2.08	2.10	2.17	2.50	3.37	4.04
All combined (n = 39)	Gene name	GAPDH	ACT	UK	UBI	TUB	CyP	18S	EF1α
	Geo Mean [Ct]	22.47	21.36	24.07	21.49	22.89	19.73	25.54	27.61
	Min. max [Ct]	20.19, 25.07	18.83, 23.17	22.02, 25.50	18.84, 23.91	19.76, 24.95	17.70, 23.96	19.21, 34.16	22.87, 36.45
	Std dev [$\pm$ Ct]	0.72	0.76	0.76	0.93	0.98	1.23	1.98	2.49
	Min. max [x-fold]	-4.87, 6.05	-5.78, 3.50	-4.15, 2.69	-8.77, 5.10	-6.26, 5.35	-4.09, 18.74	-80.57, 393.51	-26.73, 457.84
	Std dev [$\pm$ x-fold]	1.65	1.69	1.69	1.90	1.97	2.35	3.94	5.62

[Ct], cycle threshold; Geo Mean [Ct], geometric mean [Ct]; Min, max [Ct], extreme values of Ct; Min, max [x-fold], extreme values of expression levels expressed as absolute x-fold over or under coefficient; n, number of samples; Std dev [$\pm$ Ct], standard deviation [Ct]; Std dev [$\pm$ x-fold], standard deviation of the absolute regulation coefficients.

two of the best candidate RGs, *GAPDH* and *UBI*, and the two least stable genes in the tested conditions, *18S* and *EF1 α* , and compared the two RG pairs for their ability to provide reliable relative quantification of *APX* and *SOD* by RT-qPCR.

First, the variations in the expression levels of *18S* and *EF1 α* were estimated relative to *GAPDH* and *UBI*. As shown in Fig. 4A, *18S* expression variations were not significant in mock-inoculated and infected leaves, but very evident in roots infected with CMV/satRNA and ToMV (four-fold and seven-fold upregulation, respectively). *EF1 α* expression levels were particularly reactive to CMV infection in both tissue types, and upregulation was also induced to a lower extent by the other two inocula.

As expected, *18S* and *EF1 α* infection-responsive accumulation levels determined a rather inaccurate estimation of *APX* and *SOD* expression profiles. The differences were less evident in leaf tissues, where the downregulation of *SOD* by CMV and ToMV, but not by CMV/satRNA infection, was evidenced by both RG pairs (Fig. 4B). However, *APX* was upregulated significantly in comparison with mock-inoculated plants only when using *GAPDH* and *UBI* as RGs. This difference was masked by RG instability when normalized against *18S* and *EF1 α* .

Wider variability was observed in root tissues, depending on the RG pair chosen. As shown in Fig. 4C, by using *GAPDH* and *UBI* as the RG pair, *APX* did not reveal altered expression levels with any of the viral inocula, whereas *SOD* displayed overexpression on ToMV infection. The adoption of *18S* and *EF1 α* as RGs resulted in completely different *APX* and *SOD* expression profiles, as both target genes were downregulated to relative quan-

Fig. 4 Expression patterns of tomato transcripts 9 days post-inoculation with *Cucumber mosaic virus* strain Fny (CMV), CMV-Fny/77-satRNA (C/SAT) and *Tomato mosaic virus* (ToMV), quantified by quantitative reverse transcription-polymerase chain reaction (RT-qPCR). (A) Relative quantities (RQs) of *18S ribosomal RNA* (*18S*) and *elongation factor-1 α* (*EF1 α*) estimated in infected tomato leaves and roots and normalized vs. two reference genes, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *ubiquitin* (*UBI*). (B) Comparison of *ascorbate peroxidase 2* (*APX*) and *iron superoxide dismutase* (*SOD*) expression levels in infected tomato leaves estimated by normalization vs. a stable reference gene pair, *GAPDH* and *UBI* (left), and a variable reference gene pair, *18S* and *EF1 α* (right). (C) Comparison of *APX* and *SOD* expression levels in infected tomato roots estimated by normalization vs. a stable reference gene pair, *GAPDH* and *UBI* (left), and a variable reference gene pair, *18S* and *EF1 α* (right). Expression levels of target transcripts were first normalized vs. two reference gene levels and then made relative to the amount of target mRNA in an individual mock-inoculated sample, the calibrator. RQ values indicate the fold change of gene expression relative to the calibrator (for which RQ = 1). Columns represent mean RQ values from three biological replicates and vertical bars indicate standard errors. The statistical significance of expression differences of individual genes on infection by different viral inocula was analysed by one-way analysis of variance. Differences were assumed to be statistically significant, and indicated with different letters, for $P < 0.05$ (Tukey *post-hoc* test).

tity (RQ) levels between 0.4 and 0.25 in all infected plants (Fig. 4C), an obvious consequence of the virus-dependent over-expression of *18S* and *EF1 α* themselves (Fig. 4A). Thus, we can conclude that transcription levels of *APX* and *SOD* are subjected to complex regulation in virus-infected tomato plants, and expression modulation, when present, shows a generally limited extent and, in some cases, opposite directions within the same plant (e.g. ToMV-induced levels of *SOD* in leaves vs. roots; Fig. 4B,C). This information is distorted or hampered when using highly variable RGs, whose instability has been demonstrated here to cause errors in quantitative estimations of gene expression.

DISCUSSION

Normalization is a very important preliminary phase in the study of gene expression, as the choice of reference control, the expression of which may be influenced by exogenous treatments in plant cells, could cause the misinterpretation of results (Bustin, 2002; Bustin *et al.*, 2009). The importance of this issue does not seem to have been fully realized, and still receives little attention in plant science and in plant pathology in particular. In response to the increasing awareness about the use of invalid references in RT-qPCR analysis, different statistical methods have been developed. A comparison of the different methods of RG selection would allow a better identification of the most reliable internal controls and reduce the risk of artificial selections. In this study, eight candidate RGs were analysed and ranked by three different statistical packages, geNorm, NormFinder and BestKeeper, for their stability in virus- and viroid-infected tomato leaf and root tissues. Data on gene expression stability provided by the three tools were concordant overall, with only minor differences caused by the different algorithms implemented. Some differences between BestKeeper, which, for instance, indicated that *UBI* was the only stable gene in infected roots (Table 2), and the other two applications might be explained by the fact that BestKeeper uses Ct values as input data instead of the raw data (relative quantities) used by geNorm and NormFinder.

GAPDH and *UBI* were indicated to be among the first four most stable transcripts by all statistical methods and, consequently, they can be proposed as RGs of general use for RT-qPCR normalization in tomato-virus interaction studies. Interestingly, *GAPDH* was indicated as one of the best candidate RGs in stability analysis in virus-infected mammalian cells (Radonic *et al.*, 2005; Watson *et al.*, 2007), whereas *UBI* was one of the most stable genes in tomato leaf tissues subjected to different abiotic stresses, such as nitrogen starvation, low temperatures and suboptimal light conditions, which conversely induced a high variability of *GAPDH* expression (Lovdal and Lillo, 2009). *ACT* similarly displayed constant stability in both tissues, failing

to be among the four best RGs only when root tissues were analysed by geNorm. However, its use as RG for normalization in RT-qPCR studies should be carefully tested in experimental conditions differing from our model. Indeed, *ACT* was top ranked in tomato leaves under abiotic stresses (Lovdal and Lillo, 2009), but was one of the least suitable genes in potato leaves under various biotic and abiotic stresses (Nicot *et al.*, 2005), and in virus-infected mammalian cells (Radonic *et al.*, 2005; Watson *et al.*, 2007).

Our results showed that *UK*, one of the two tomato mRNAs selected from previous microarray analysis together with *CyP*, was another gene not responsive to virus and viroid infections. Therefore, a shortlist of candidate RGs to be considered in virus- and viroid-infected plant cells could also include *UK*, which is not usually found among the transcripts used for gene expression normalization. This observation highlights, once more, that microarray analysis is a robust method to identify least variable genes (Czechowski *et al.*, 2005; Libault *et al.*, 2008).

Guenin *et al.* (2009) pointed out the necessity of validating RGs under each set of specific experimental conditions, including different plant tissues. GeNorm, NormFinder and BestKeeper analysis, performed separately on tomato leaf and root samples, indicated that the candidate genes were highly stable in a tissue-specific manner. This was particularly the case for *CyP*, which was the most stably expressed transcript in root tissues according to geNorm and among the top four according to the other two applications. Our work, through the comparative analysis between two different host tissues, clearly emphasizes that candidate RGs should be validated in the light of the cell/tissue types of origin of the tested samples. Indeed, we showed that different tissues were a natural source of alteration of gene expression stability, and thus endogenous controls might be suitable for normalization for a specific plant tissue but not for others.

Two of the applications employed, geNorm and BestKeeper, also specify a gene expression stability threshold value above which a transcript should be considered as definitely unreliable as an RG. This threshold value is $M = 1.5$ in geNorm, a value exceeded only by *EF1 α* in leaf samples, and $SD = 1$ in BestKeeper, exceeded by all RGs other than *UBI* in roots and by *CyP*, *EF1 α* and *18S* in the analysis combining all data. Thus, *EF1 α* and *18S* were not confirmed as stable normalization factors in our conditions. Traditionally employed housekeeping genes, such as those involved in basic cellular processes, have been used extensively for relative transcript quantification, but it has been pointed out that their expression level is not totally independent of the experimental conditions, as proposed previously (Dheda *et al.*, 2004). Indeed, it is of note that these commonly employed RGs for RT-qPCR were ranked as the most variable under viral infection by all three statistical packages. Both genes were very stably expressed in potato leaves under con-

ditions of biotic and abiotic stress (Nicot *et al.*, 2005), as well as in rice plants subjected to various environmental conditions (Jain *et al.*, 2006). However, *EF1 α* showed poor stability in tomato tissues from different developmental stages (Exposito-Rodriguez *et al.*, 2008) or under light, but not cold, stress (Lovdal and Lillo, 2009), whereas *18S* was the least reliable of 10 candidate RGs in mammalian cells in the context of various virus infections (Watson *et al.*, 2007). An explanation of the inappropriateness of these two genes as reference for RT-qPCR studies in virus-infected tomato tissues may reside in their specific role in ribosomal activity and translation mechanisms, a metabolic process highly altered by the presence of replicating viruses (Hull, 2001).

Several studies have described the activation of oxidative burst mechanisms and the accumulation of ROS in plant–virus incompatible (Dangl *et al.*, 1996; Grant and Loake, 2000) and compatible (Clarke *et al.*, 2002; Diaz-Vivancos *et al.*, 2008; Love *et al.*, 2005) interactions. Plants defend themselves by generating antioxidative mechanisms that scavenge ROS and avoid cell destruction. Enzymatic antioxidants include numerous gene families, such as *APX* and *SOD* among others, the former detoxifying hydrogen peroxide, the latter the superoxide anion. Our data, in a comparative analysis of *APX* and *SOD* expression in tomato leaf and root tissues, showed that modulation of these two genes is strictly virus- and tissue-specific. Upregulation of *APX* and downregulation of *SOD*, in CMV-infected, but also ToMV-infected (*SOD* only), tomato leaves, as reported in our work, was an outcome observed in other plant–virus compatible interactions (Clarke *et al.*, 2002). It is noteworthy that, as we have shown for *SOD* in ToMV-infected tomato plants, the same virus can modulate gene expression, inducing downregulation in leaves whilst inducing upregulation in roots. This observation, never reported previously to our knowledge, confirms the notion that individual plant organs sense and respond to pathogenic virus infections, activating different gene networks (Catoni *et al.*, 2009). For the purposes of this work, we concluded that, for the correct quantification of *APX* and *SOD*, it was crucial to chose two internal controls whose mRNA levels were not influenced by viral infections.

This work defines, for the first time, the appropriateness of candidate genes for use as RT-qPCR references in tomato plants infected with taxonomically, molecularly and pathogenetically different viruses and a viroid. Combining and analysing together the results provided by three easily accessible statistical packages, this study emphasizes the suitability of two genes, *GAPDH* and *UBI*, as the most stably expressed transcripts in all infected tomato tissues. It also indicates other genes that could be used acceptably to the same purpose, and suggests a robust statistical procedure that could be followed by other researchers for obtaining information on RGs useful in different biological contexts and tissues.

EXPERIMENTAL PROCEDURES

Plants and viruses

Seedlings of tomato (*Solanum lycopersicum* cv. UC82) were grown in the glasshouse and used for infection with six viruses and a viroid. With the exception of *Tomato yellow leaf curl virus* (TYLCV), which was always transferred by grafting, the virus and viroid inocula were first prepared in tomato or tobacco, as specified below, and then transferred to tomato plants at the two-leaf stage for the purpose of the test. Mechanical inoculations were carried out using sap from systemically infected tomato leaf tissues at 12–15 days post-inoculation (dpi) ground in 20 vol of 100 mM phosphate buffer, pH 7.2.

The strain CMV-Fny (Owen and Palukaitis, 1988) and the inoculum combining CMV-Fny plus CMV-77satRNA were obtained by co-inoculation of the corresponding *in vitro* transcripts and purified from freshly inoculated tomato seedlings, according to published methods (Cillo *et al.*, 2007). The *Potato virus Y* isolate, denoted PVY^C-to (Mascia *et al.*, 2010), was maintained on and purified from *N. tabacum* cv. Samsun as described. Dried leaf tissues infected with the *Tomato mosaic virus* Brazilian isolate ToMV-SP (Moreira *et al.*, 2003) were inoculated onto and purified from tomato seedlings as described (Asselin and Zaitlin, 1978). Local isolates of *Tomato spotted wilt virus* (TSWV) (Finetti Sialer *et al.*, 2002) and *Potato spindle tuber viroid* (PSTVd) (Di Serio, 2007) were rescued from frozen and dried tissues, respectively, and maintained on tomato plants. TYLCV infection was obtained by grafting 3-cm shoots cut from plants carrying an isolate from Apulia (southern Italy) (Finetti Sialer *et al.*, 2001) onto healthy tomato cv. UC82 plants at the four-leaf stage. To avoid soil residues that could interfere with RT-PCR amplification of RNA samples extracted from roots, all plants were grown in plastic pots containing a sterile sand substrate watered daily with a nutrient solution (Hoagland and Arnon, 1950) and kept in a controlled environment in the glasshouse at 22 (± 2 °C) with a 16-h photoperiod. Plants infected by PSTVd were grown at 30 °C.

RNA extraction

Mock-inoculated plants and plants infected with CMV-Fny, CMV-Fny/77-satRNA, PVY and ToMV were sampled for RNA extraction from leaves and roots, whereas plants infected with TSWV, PSTVd and TYLCV provided only leaf tissues for RNA extraction, because of the poor developing conditions of roots in the sand substrate.

Samples for RNA extraction consisted of second true leaf and root tissues of infected and mock-inoculated tomato plants at 9 dpi, i.e. before the appearance of disease symptoms. Systemically infected leaves were used from plants infected with TYLCV at 30 dpi, after symptom appearance.

Total RNA was extracted from about 100 mg of leaf and from all available root tissues (approximately 20–50 mg) using TRIzol reagent (Invitrogen Ltd, Paisley, Renfrewshire, UK), according to the manufacturer's instructions. RNA preparations were subjected to on-column DNase digestion (RNase-free DNase set and RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany) and eluted in ribonuclease-free water. Nucleic acid quality was estimated by visual analysis on 1.2% agarose gel electrophoresis according to standard procedures (Sambrook and Russell, 2001). RNA concentrations were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, USA), and only RNA samples with an A_{260}/A_{280} ratio in the range 1.75–2.0 were used to minimize the effects of PCR inhibitors.

Primer design, reverse transcription and qPCR analysis

Eight candidate RGs were selected (Table 1). Primers pairs used for the amplification of each tomato putative RG were designed with the support of the IDT's PrimerQuest software, freely available at <http://eu.idtdna.com/Scitools/Applications/Primerquest/>, and are listed in Table 1.

RNA samples were denatured at 95 °C for 3 min in the presence of 10 pM random hexamers, and quickly cooled on ice. A total reaction volume of 20 µL containing 1 µg of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Before being used for RT-qPCR, the sequences of the amplification products of each RG were determined by an external sequencing service and confirmed as identical to those deposited in the sequence database.

Quantitative RT-PCRs were performed using a StepOne Real Time PCR System (Applied Biosystems). The cycling profile consisted of 95 °C for 10 s, followed by 40 cycles of 3 s at 95 °C and 10 s at 60 °C, as recommended by the manufacturer, using 2X Fast SYBR Green PCR Master Mix (Applied Biosystems) including the ROX fluorochrome internal check, 300 nM forward and reverse primers, 1 µL of a 1:10 dilution (approximately 5 ng) of reverse-transcribed RNA and nuclease-free water in a total volume of 10 µL. A 1:100 dilution of cDNA was used for 18S amplification to allow RT-qPCR measurements comparable with those of the mRNA RGs, which are normally expressed in smaller amounts. Three different biological replicates were used, i.e. reverse-transcribed RNA extracted from two individual plants pooled together, and each cDNA sample was amplified in duplicate for each primer pair on a 48-well optical plate with appropriate negative controls. Immediately after the final PCR cycle, a melting curve analysis was performed to verify primer specificity.

For *APX* (*S. lycopersicum* cytosolic ascorbate peroxidase 2, GENBANK ID AY974805) amplification, the selected forward and

reverse primers were 5'-GTGACCACTTGAGGGACGTGTTGT-3' and 5'-ACCAGAACGCTCCTGTGGCATCTT-3', respectively. For *SOD* (*S. lycopersicum* plastidial iron superoxide dismutase, GENBANK ID AJ579656) amplification, the selected forward and reverse primers were 5'-CTGGGAATCTATGAAGCCCAACGGA-3' and 5'-CAAATTGTGTTGCTGCAGCTGCCTT-3', respectively. RT-qPCRs of *APX* and *SOD* were set up from the same leaf and root cDNA samples as used for candidate RG experiments, and all PCR experimental conditions were as described above.

RNA expression levels for each sample were recorded as Ct at the PCR apparatus default fluorescence threshold ($\Delta Rn = 0.4$). No calibration was performed between different plates, which were poured at the same occasion for each experiment. For each individual biological replicate, data represented by the mean Ct value of duplicate amplifications were entered separately, in order to take into account any variation in expression between biological replicates that was not caused by the treatments.

The PCR efficiency for each primer pair and amplicon was derived from the slope of the regression line fitted to a subset of baseline-corrected data points in the log-linear phase using StepOne software (Applied Biosystems). The regression line was obtained by interpolating values from triplicates of five serial $\log_{10}$ dilutions of input cDNA amount and the relative Ct values. Relative quantities of RNA accumulation were calculated as RQ values using the comparative cycle threshold (Ct) ($2^{-\Delta\Delta Ct}$) method corrected for PCR efficiencies, as described previously (Cillo *et al.*, 2009).

ACKNOWLEDGEMENTS

We thank Professor P. Palukaitis (Scottish Crop Research Institute, Dundee, UK), Dr M. Eiras (Instituto Biológico, São Paulo, Brazil) and Dr F. Di Serio (CNR, Istituto di Virologia Vegetale, Bari, Italy) for providing some of the viral strains and the viroid used in this work. This work was supported by the Italian Ministry of University and Research (MIUR) project GenoPom, the grant MIUR PRIN 2006 and a grant from Ministry for University and Scientific and Technological Research (MURST) within the framework of Centre of Excellence in Comparative genomics (CEGBA), University of Bari.

REFERENCES

- Andersen, C.L., Jensen, J.L. and Orntoft, T.F. (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245–5250.
- Asselin, A. and Zaitlin, M. (1978) Characterization of a second protein associated with virions of tobacco mosaic virus. *Virology*, **91**, 173–181.
- Bustin, S.A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **25**, 169–193.

- Bustin, S.A. (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* **29**, 23–39.
- Bustin, S.A., Benes, V., Garson, J.A., Helleman, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611–622.
- Catoni, M., Miozzi, L., Fiorilli, V., Lanfranco, L. and Accotto, G.P. (2009) Comparative analysis of expression profiles in shoots and roots of tomato systemically infected by tomato spotted wilt virus reveals organ-specific transcriptional responses. *Mol. Plant–Microbe Interact.* **22**, 1504–1513.
- Cillo, F., Pasciuto, M.M., De Giovanni, C., Finetti-Sialer, M.M., Ricciardi, L. and Gallitelli, D. (2007) Response of tomato and its wild relatives in the genus *Solanum* to cucumber mosaic virus and satellite RNA combinations. *J. Gen. Virol.* **88**, 3166–3176.
- Cillo, F., Mascia, T., Pasciuto, M.M. and Gallitelli, D. (2009) Differential effects of mild and severe Cucumber mosaic virus strains in the perturbation of MicroRNA-regulated gene expression in tomato map to the 3' sequence of RNA 2. *Mol. Plant–Microbe Interact.* **22**, 1239–1249.
- Clarke, S.F., Guy, P.L., Burritt, D.J. and Jameson, P.E. (2002) Changes in the activities of antioxidant enzymes in response to virus infection and hormone treatment. *Physiol. Plant.* **114**, 157–164.
- Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K. and Scheible, W.R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiol.* **139**, 5–17.
- Dangl, J.L., Dietrich, R.A. and Richberg, M.H. (1996) Death don't have no mercy: cell death programs in plant–microbe interactions. *Plant Cell*, **8**, 1793–1807.
- Dheda, K., Huggett, J.F., Bustin, S.A., Johnson, M.A., Rook, G. and Zumla, A. (2004) Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques*, **37**, 112–114, 116, 118–119.
- Diaz-Vivancos, P., Clemente-Moreno, M.J., Rubio, M., Olmos, E., Garcia, J.A., Martinez-Gomez, P. and Hernandez, J.A. (2008) Alteration in the chloroplastic metabolism leads to ROS accumulation in pea plants in response to plum pox virus. *J. Exp. Bot.* **59**, 2147–2160.
- Di Serio, F. (2007) Identification and characterization of Potato spindle tuber viroid infecting *Solanum jasminoides* and *S. rantonnetii* in Italy. *J. Plant Pathol.* **89**, 297–300.
- Exposito-Rodriguez, M., Borges, A., Borges-Perez, A. and Perez, J. (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. *BMC Plant Biol.* **8**, 131.
- Finetti Sialer, M.M., Di Franco, A., Vovlas, C. and Gallitelli, D. (2001) First report of Tomato yellow leaf curl virus in Apulia (southern Italy). *J. Plant Pathol.* **83**, 148.
- Finetti Sialer, M.M., Lanave, C., Padula, M., Vovlas, C. and Gallitelli, D. (2002) Occurrence of two distinct tomato spotted wilt virus subgroups in southern Italy. *J. Plant Pathol.* **84**, 145–152.
- Grant, J.J. and Loake, G.J. (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiol.* **124**, 21–29.
- Guenin, S., Mauriat, M., Pelloux, J., Van Wuytswinkel, O., Bellini, C. and Gutierrez, L. (2009) Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. *J. Exp. Bot.* **60**, 487–493.
- Hoagland, D.R. and Arnon, D.I. (1950) The water culture method for growing plants without soil. In: *California Agricultural Experimental Station Circular*, Vol. 347, pp. 1–32. Berkeley, CA: University of California.
- Hull, R. (2001) *Matthews' Plant Virology*, 4th edn. San Diego, CA: Academic Press.
- Jain, M., Nijhawan, A., Tyagi, A.K. and Khurana, J.P. (2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* **345**, 646–651.
- Libault, M., Thibivilliers, S., Bilgin, D.D., Radwan, O., Benitez, M., Clough, S.J. and Stacey, G. (2008) Identification of four soybean reference genes for gene expression normalization. *Plant Genome*, **1**, 44–54.
- Livak, K. (2001) Relative quantitation of gene expression. In: *ABI Prism 7700 Sequence Detection System, User Bulletin #2*, pp. 1–36. Foster City, CA: Applied Biosystems.
- Lovdal, T. and Lillo, C. (2009) Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. *Anal. Biochem.* **387**, 238–242.
- Love, A.J., Yun, B.W., Laval, V., Loake, G.J. and Milner, J.J. (2005) Cauliflower mosaic virus, a compatible pathogen of Arabidopsis, engages three distinct defense-signaling pathways and activates rapid systemic generation of reactive oxygen species. *Plant Physiol.* **139**, 935–948.
- Mascia, T., Finetti Sialer, M.M., Cillo, F. and Gallitelli, D. (2010) Biological and molecular characterization of a recombinant isolate of Potato virus Y associated with a tomato necrotic disease occurring in Italy. *J. Plant Pathol.* **92**, 131–138.
- Moreira, S.R., Eiras, M., Chaves, A.L.R., Galleti, S.R. and Colariccio, A. (2003) Caracterização de uma nova estirpe do Tomato mosaic virus isolada de tomateiro no Estado de São Paulo. *Fitopatol. Bras.* **28**, 602–607.
- Nicot, N., Hausman, J.F., Hoffmann, L. and Evers, D. (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. *J. Exp. Bot.* **56**, 2907–2914.
- Owen, J. and Palukaitis, P. (1988) Characterization of cucumber mosaic virus. I. Molecular heterogeneity mapping of RNA 3 in eight CMV strains. *Virology*, **166**, 495–502.
- Pfaffl, M.W. (2006) Relative quantification. In: *Real-Time PCR* (Dorak, T., ed.), pp. 63–82. New York: International University Line.
- Pfaffl, M.W., Tichopad, A., Prgomet, C. and Neuvians, T.P. (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **26**, 509–515.
- Radonic, A., Thulke, S., Bae, H.G., Muller, M.A., Siebert, W. and Nitsche, A. (2005) Reference gene selection for quantitative real-time PCR analysis in virus infected cells: SARS corona virus, Yellow fever virus, Human Herpesvirus-6, Camelpox virus and Cytomegalovirus infections. *Viol. J.* **2**, 7.
- Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. New York: Cold Spring Harbor Laboratory Press.

- Sturzenbaum, S.R. and Kille, P.** (2001) Control genes in quantitative molecular biological techniques: the variability of invariance. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.* **130**, 281–289.
- Udvardi, M.K., Czechowski, T. and Scheible, W.R.** (2008) Eleven golden rules of quantitative RT-PCR. *Plant Cell*, **20**, 1736–1737.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F.** (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034.
- Watson, S., Mercier, S., Bye, C., Wilkinson, J., Cunningham, A.L. and Harman, A.N.** (2007) Determination of suitable housekeeping genes for normalisation of quantitative real time PCR analysis of cells infected with human immunodeficiency virus and herpes viruses. *Virology*, **4**, 130.
- Whitham, S.A., Quan, S., Chang, H.S., Cooper, B., Estes, B., Zhu, T., Wang, X. and Hou, Y.M.** (2003) Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. *Plant J.* **33**, 271–283.
- Wise, R.P., Moscou, M.J., Bogdanove, A.J. and Whitham, S.A.** (2007) Transcript profiling in host–pathogen interactions. *Annu. Rev. Phytopathol.* **45**, 329–369.
- Yamaguchi-Shinozaki, K. and Shinozaki, K.** (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.* **57**, 781–803.