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Review article

Traditional and novel references towards systematic normalization of qRT-PCR data in plants

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Abstract

The selection of appropriate controls is the key requirement for target gene normalization through quantitative real-time PCR. The consistent control is selected via a gene validation approach that includes several classic and novel housekeeping references. Our aim in this paper is to present major achievements, largely concerned with finding an invariable internal control in different plant species under a range of experimental conditions. The conflicts in gene normalization using classical references and their replacement with novel references are discussed as leading subject. This review also accentuates different methods used for the amplification of reference genes with special importance on ortholog/paralog approaches, and multi-transcript targeting. Some other issues are also discussed such as the use of quantitative real-time PCR in different areas of plant science for target gene normalization, and some areas where it might be a useful technique in unraveling other issues.

Keywords: Reference gene, Gene normalization, Quantitative RT-PCR, Traditional and Novel reference genes.
Abbreviations: BCMV = Bean common mosaic virus, ESTs = expressed sequence tags, HKGs = housekeeping genes, NF = normalization factor, NCBI = national center for biotechnology information, SSR = simple sequence repeat, SNP = single nucleotide polymorphism, SCAR = sequence characterized amplified regions, qRT-PCR = quantitative real-time PCR, QTL = quantitative trait loci.

Introduction

Cellular and molecular biology techniques have revealed the hidden information of DNA sequences, determined structural and functional architecture, and regulation of a plant by utilizing highly-sensitive tools. Sequences include three main types, broadly defined as intergenic (or junk sequences), regulatory, and coding sequences of genes. Present molecular biology investigates nucleotide sequences, gene families, specificity, regulation and level of expression. Northern blotting, microarray and real-time quantitative PCR (qRT-PCR) are commonly employed for quantification of gene expression under different experimental conditions. qRT-PCR, the most recent technique to explore expression due to its ability of accurate and sensitive quantification of low copy number genes express at a minute scale, has emerged as having the most potential in this regard (Huggett et al., 2005; Radonic et al., 2004). Some low copy number genes express at such minute levels that no method except qRT-PCR can detect expression accurately (Czechowski et al., 2004). The major advantage of qPCR over standard PCR includes the homogeneous or 'closed-tube' format, reducing the chance of cross-contamination, elimination of electrophoresis, dual specificity of primers and probes and, significantly, the ability to quantify the target.

Plant science is an important area of research, yet not heralded enough with respect to microarray and qRT-PCR, except for some work conducted with model plants like rice, wheat, maize, *A. thaliana* etc. So to have a complete molecular world, we need to examine a number of the thousands of plant species important to the environment and agriculture, especially for biotechnology studies i.e. proteomics and genomics. Similar to model organism plants, the genomic database of important plants should be saturated with expressed sequence tags (ESTs), simple sequence repeat (SSR), single nucleotide polymorphism (SNP), and regulatory, structural, and functional gene sequences, which are preliminary requirements for accurate qRT-PCR. A few studies have been conducted on biotic (Takle et al., 2007; Jarošová and Kundu 2010; Barsalobres-Cavallari et al., 2009), abiotic stresses (Dombrowski and Martin 2009; Løvdal and Lillo 2009), and growth and development (Expósito-Rodríguez et al., 2008; Li et al., 2010 etc.) using qRT-PCR. Transgene expression analysis using qRT-PCR, has been completed successfully for maize (Vaitilingom et al., 1999), rice (Ding et al., 2004), tomato (Yang et al., 2005a & b), potato (Randhawa et al., 2009), oil palm (Ismail et al. 2010) and *Eucommia ulmoides* Oliver (Chen et al., 2010). In addition, the kinetic expression pattern of some genes could not be determined because of undetectable transcript levels by Northern analysis, where qRT-PCR was preferred to measure the transcript level e.g. due to low sensitivity of Northern or *in situ* hybridization to measure the expression level of *Or-MYB1*, qRT-PCR was employed in *Oryza*

ramosa. Every task utilizing qRT-PCR requires a gene identified as a reference for relative quantification of the gene of interest, recommended as a reference because of its stable expression from tissue-to-tissue, and condition-to-condition, and subject to the same errors during cDNA preparation as the target gene. Mammalian samples are thoroughly investigated in this respect, and standardized for several aspects of qRT-PCR. However, not much work has been done in plants for quantification of important genes using reference genes as a standard. Due to the variable nature of plant growth and development under different experimental conditions, several factors affect the consistency of reference gene validation and target gene normalization. Hence, a systematic study with respect to various plant species as well as cultivars is required to make it a globally applicable technique. Similarly, all of the preparative steps (RNA preparation, cDNA synthesis, and qPCR amplification) for qRT-PCR are error-prone in nature (Bustin et al., 2005; Huggett et al., 2005; Guenin et al., 2009) and should be normalized due to variation in the sample volume with respect to targeted mRNA species. The ratio of target mRNA level to a reference mRNA level is typically used for this normalization. Endogenous reference genes having putative housekeeping roles in basic cellular processes are frequently used for this purpose (Brunner et al., 2004; Jain et al., 2006; Reid et al., 2006; Cruz et al., 2009). The most common traditional references used for plants as internal standards are 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor-1a (*EF-1a*), polyubiquitin (*UBQ*), actin (*ACT*), α-tubulin and β-tubulin (*TUA* and *TUB*, respectively), translation initiation factor (*IF4a*), ubiquitin-conjugating enzyme (*UBC*) and cyclophilin (*CYP*) genes (Goidin et al., 2001; Bustin 2002; Kim et al., 2003; Andersen et al., 2004; Brunner et al., 2004; Dheda et al., 2004; Radonic et al., 2004; Reid et al., 2006). These genes were adopted from traditional techniques like Northern blotting, with the assumption of highly uniform expression in different cell types under different experimental conditions and applied to a number of research areas: for studies of sea grass subjected to heat stress (Ransbotyn and Reusch 2006); for rice plants under hormone, salt and drought treatment, and in different developmental stages (Jain et al., 2006); for potato under different biotic and abiotic stresses (Nicot et al., 2005); for poplar in different tissues (Brunner et al., 2004); for Arabidopsis under increased metal concentrations (Remans et al., 2008); and, for wheat, barley and oat under viral (*Barley yellow dwarf virus*) infection (Jarosova and Kundu 2010). To date, traditional reference genes are frequently used for target gene normalization and to verify comparative ability of novel references for gene validation. However, traditional references have been shown to vary considerably (Volkov et al., 2003; Czchowski et al., 2005; Nicot et al., 2005; Remans et al., 2008). In comparative studies (Hu et al., 2009; Libault et al., 2008), novel genes outperformed traditional reference genes, indicating that traditional reference genes have some limitations of variable expression and expression over threshold level, while novel genes cover a wide range of absolute expression levels, makes them valuable for normalizing levels of other genes, varied in their expression levels.

In the present study, all of the possible online databases (Science direct, Blackwell Synergy, Oxford journal, Willey Interscience, Springerlink) were searched until December 2010, for research published on validation of reference genes for qRT-PCR, specifically in plant sciences. In this field, ~50 studies related to plants for reference genes were extracted and extensively scrutinized for stability, applicability, and comparative potential of traditional and novel references in plant science areas. Most of the studies on comparative statistics of references for target gene normalization in plants were conducted in 2009 and 2010.

Source and choice of internal controls in qRT-PCR for gene normalization

In most quantitative PCR procedures, the linear range of the amplification is evaluated against control samples or standards. The search for adequate standards for RNA quantitation is much more challenging, and represents the first and most difficult step for target gene normalization using qRT-PCR. To date, only existing literature is the source for reference gene selection. Traditional references are the usual choice for less sensitive techniques like Northern blotting or semi quantitative PCR, where little variation in gene expression do not affect the final results. Such non-detectable variations in gene expression might be amplified by several magnitude by qRT-PCR and leads to erroneous results in target gene normalization. This limitation can be overcome by selecting a set of most common traditional references for validation of invariable control across a number of plant species in qRT-PCR, but their inconsistent expression indicate the need of their replacement with some other novel housekeeping genes (HKGs). However, the confines of genomic data let us depend on the traditional references, despite the developmental or physiological state of the materials being analyzed (Czechowski et al., 2005). Any substitution of traditional references will only be available after screening and identification of novel HKGs. The availability of genomic data i.e. expressed sequence tags databases (EST) for tomato (Coker and Davies 2003), *Arabidopsis* (Czechowski et al., 2004 & 2005), rice (Caldana et al., 2007; Li et al., 2010; Narsai et al., 2010) and wheat (Paolacci et al., 2009), are source of miscellaneous genes for various experimental conditions and might be used in qRT-PCR to fix the difficulties of gene expression variability. Various genes from studies mentioned have been analyzed for validation of transcriptome data using qRT-PCR, and represented important resources for the scientific community (Caldana et al., 2007; Czechowski et al., 2004; Kakar et al., 2008). Microarray analysis might be informative for selection of a valid control in qRT-PCR. Microarray analysis for differential experimental conditions across several tissues/organs in *Arabidopsis* (using the large public collection of data from Affymetrix GeneChip experiments) (Czechowski et al., 2005), and in barley (on the basis of ESTs analysis and relative expression calculation for all the cDNA libraries available through the TIGR Barley Gene index database) (Faccioli et al., 2007) has provided a number of novel references, may substitute the traditional reference genes in future. Existing housekeeping references for quantitation in qRT-PCR have limitations. Considering tissue specificity and treatments applied to plants, the choice of reference gene can be depend on these factors. For example, UBC16 expression in leaves was quite stable under various treatments, whereas its expression in the roots of the lily plants was rather variable (Luo et al., 2010). Several authors have suggested the necessity of selecting valid internal controls before profiling gene expression, even for closely related organisms with different tissues and different treatments of interest. Among the seven reference genes investigated by Dong et al., (2010), α-tubulin was stably expressed in leaves, flowers and fruits, and at a higher level in roots, a lower level in stems. Therefore, it may be used as the internal control when the former three tissues are

concerned. The choice of reference genes in qRT-PCR can also be made on the basis of expression level. Four novel genes designated At1g62390 (*PPR repeat*), At4g38070 (*bHLH*), At5g12240 (*Expressed*) and At5g15710 (*F-box family*) extracted from the ATH1 array of *A. thaliana*, demonstrated high expression stability, but expression level was found to be 1,000- to 10,000-fold lower than commonly used traditional references (*UBQ10, EF-α,* and *GAPDH*). Such stable but low expression genes are the internal references of choice in qRT-PCR experiments because of a smaller difference of threshold cycle number (ΔC_t) between the reference and target transcript, where the calculation of the results will be less influenced by variations in amplification efficiencies, and hence more accurate (Czchowski et al., 2005). Caution must be taken when selecting reference genes, even when the gene is proven to be stable under several conditions and in several related species.

Multi-transcript targeting of references from the same gene family

The simultaneous amplification of two or more reference genes of the same family by a single primer pair designed in the conserved regions has been suggested by some authors as a strategy for reducing the cost of using multiple and independent control genes (Brunner et al., 2004, Reid et al., 2006, Charrier et al., 2002). This approach assumes the balanced expression of different amplified genes of the same family, but this is not always the case. For example, *UBQ5* has been one of the most suitable reference genes in a set of tissue samples in rice, whereas the expression of *UBQ10* was unstable (Jain et al., 2006). A similar situation has been observed for the actin gene family in a set of samples collected at different development stages of soybean, where *ACT2/7* was stably expressed, and *ACT11* showed variable profiling (Jian et al., 2008). In the study of Paolacci et al. (2009), conducted on different experimental conditions of wheat concluded that this approach is not successful, in fact, the primer pairs amplifying the single best gene for each of the four analyzed families performed better than those targeting multiple genes (Actin, α-tublin, Translation elongation factor, and ADP-ribosylation factor), however, it is a strategy known to avoid the additional expense and labor of using multiple internal control genes, where normalization requires two or more stable reference genes (Brunner et al., 2004). *ADP-RF* targeting multi-transcripts, used by Gimenez et al., (2010) in different wheat species was observed to be a good reference gene. Multi-transcript amplification is effective where all the family genes show a similar level of expression, differentially expressed family genes might cause erroneous results in transcript normalization. The expression pattern and stability of two members of the same gene family might vary and normalization results may be doubtful for gene expression based on multi-transcript targeting. Four genes (*MDH*, *GAPDH*, *UBQ*, and *EF1-α*) used to target paralogous transcripts during berry development (*V. vinifera*), were inconsistent in targeting multiple transcripts except *GAPDH,* which was ranked relatively high in terms of stability (Reid et al., 2006).

Reference genes validation among different plant species and cultivated varieties

So far, most of the qRT-PCR reference gene validations conducted specifically on different plant tissues and organs have been in model organisms, where the stability of reference genes across different plant accessions or cultivars is not known. Careful screening of transcriptional differences among different crop varieties may be used for the selection of superior genetic stock against biotic and abiotic stresses using a set of validated controls. Such types of differences have been investigated in the context of natural populations (Jin et al., 2001; Enard et al., 2002; Oleksiak et al., 2002; Stamatoyannopoulos 2004) using microarrays. However, no study has been conducted for confirmation of microarray data using qRT-PCR. Above studies have shown that a significant component of gene expression variation is heritable, and could possibly be used to solve complex phylogenetic problems of germplasm banks and culture collections, assigning relationships between morphologically similar varieties or populations, and helping to generate evolutionary trees in cases where other methods (Morphological and molecular markers) have failed (Ancillo et al., 2007). Gene expression stability should also be validated across different lines, varieties, species and populations. Mallona et al., (2010) conducted a study on two lines of *Petunia hybrid* (Mitchell and V30) for validation of reference genes, and revealed two different sets of genes for each line (Table 1a). *EF1α* was the most reliable gene in the Mitchell line, but results differed in line V30, where *ACT* found as the best candidate gene. Line-associated variability was not in accordance with results where reference gene stability was not very variable regarding cultivar influence in soybean (Jian et al., 2008). Cultivar influence on reference gene stability was also observed by Kim et al., (2003) where *18S rRNA* and tubulin were found as stable references, while *GAPDH* and actin varied among six different rice cultivars. Gene expression levels of ten housekeeping genes used as internal controls were found similar among three different soybean cultivars which differed in sensitivity to photoperiod (Jian et al., 2008). So, to make use of remarkable differences among different plant varieties and populations in respect to gene expression profiles, reference gene(s) are needed to be validated across different cultivars of economically important plant species for a particular set of experimental conditions.

Paralogous sequences validated as references genes in qRT-PCR

The existence of multiple paralogs of housekeeping genes in plants is a choice of researchers for targeting gene normalization in qRT-PCR (Brunner et al., 2004; Jain et al., 2006; Zhang et al., 2009; Garg et al., 2010; Wan et al., 2010). These paralogous genes (i.e., gene family members) could be redundant and may be involved in specific plant processes, tissues, or developmental stages (Van Lijsebettens et al., 1994; Williams and Sussex 1995; Barakat et al., 2001; McIntosh and Bonham-Smith 2005; Degenhardt and Bonham-Smith 2008). Different paralogs of actin, tubulin and ubiquitin are most commonly utilized for reference gene validation in plants, however, the results indicate that expression profiles and stability vary between paralogous gene copies (Gutierrez et al., 2008; Zhang et al., 2009). Actin is a ubiquitous protein involved in the formation of filaments that are a major component of the cytoskeleton. Interaction with myosin provides the basis of muscular contraction and many aspects of cell motility. The paralogous genes of actin have been used in some studies for target gene normalization in plants (Jian et al., 2008; Gutierrez et al., 2008; Caldana et al., 2007; Hu et al., 2009; Wan et al., 2009) and with special emphasis in rice on eight paralogs of actin by Zhang et al., (2009). The tubulin superfamily includes five distinct families, the alpha-, beta-, gamma-, delta-, and epsilon-tubulins and a sixth family (zeta-tubulin) which is

present only in kinetoplastid protozoa. The alpha/beta-tubulin heterodimer is the structural subunit of microtubules (NCBI) and both of these forms are most commonly used as plant references. The paralogs of the alpha/beta-tubulin superfamily genes have been applied in *A. thaliana* (Gutierrez et al., 2008), *V. vinifera* (Reid et al., 2006), *Glycine max* (Reid et al., 2006) and *Prunus persica* (Tong et al., 2009). Paralogs of ubiquitin were also used in *Oryza sativa* (Jain et al., 2006), *Brachypodium* (Hong et al., 2008), *Cicer arietinum* (Garg et al., 2010) and in *A. thaliana* (Gutierrez et al., 2008). Variations among paralogs of actin, tubulin and ubiquitin were observed during *Arabidopsis* developmental stages in the study by Gutierrez et al., (2008). Remarkable differences between paralogous genes, *UBQ5* and *UBQ10*, were observed in the geNorm-based M values, where expression stability of UBQ5 and UBQ10 was found to be inverse (Jain et al., 2006; Garg et al., 2010), indicating that perhaps these paralogous genes are not suitable references. Paralogs of actin (*ACT11* and *ACT2/7*) and tubulin (*TUB4* and *TUA5*) varied greatly, *ACT11/TUA5* were ranked as the most stable, while *ACT2/7* and *TUB4* as the least stable reference in soybean (Hu et al., 2009). Eight paralogous genes of actin were examined in rice to find out the expression stability in the panicle under drought stress, and all of the genes showed variation in expression stability (Zhang et al. 2009). These results indicate that there is a wide variability of paralogous sequences as control genes in qRT-PCR, even within the same organism. In addition, the design of primers is a key point. Due to the high similarity in the coding sequence between many paralogous genes, it becomes necessary to confirm that only the paralog of interest is amplified by sequencing of the PCR products, or by designing the primers in divergent regions of the UTRs or in a region of lower direct sequence homology. The simultaneous cross-amplification of different paralogous genes that are differentially expressed would render a consensus expression profile that may make the comparisons among species difficult.

Orthologous amplification of important internal controls in qRT-PCR

Plant species for which limited genomic resources are available, orthologous expression of internal controls from other species may present a solution of cross-species amplification. The development of conserved orthologous sequence markers will help to identify candidate genes in one species from the knowledge obtained in other species (Bolot et al., 2009). To illustrate, two stably expressed orthologous genes of *Arabidopsis* (At4g34270 and At4g33380) validated under a large set of experimental conditions, were tested in hybrid aspen (*Populus tremula* X *Populus tremuloides*) for eight different developmental stages and found variable (Gutierrez et al., 2008). Similarly, two stable orthologous sequences (At4g33380 and At4g34270), expressed protein (*EP*) and TIP41-like protein (*TIP41*) from *Arabidopsis* (Czechowski et al., 2005), were quantified under different experimental conditions in rice (Caldana et al., 2007). All samples had M values below 1.5, where *TIP41* was the least stably expressed gene in both (shoot and root) tissues tested, while *EP* showed most stable expression and was categorized as an excellent reference gene, as in *Arabidopsis*. When amplifying orthologous genes across different species, the occurrence of mismatches is likely. Although the identity of the orthologous genes is usually demonstrated through sequencing of the PCR products, mismatches between the primers and the templates cannot be ruled out by this method since, as a result of amplification, the sequence of the amplicons will be complementary to the primer (Gimenez et al., 2010). Previous studies have demonstrated that single mismatches between the primer and the target sequence led to an underestimation of the target gene expression (Bru et al., 2008; Ghedira et al., 2009). Gimenez et al., (2010) have investigated the existence of mismatches between primer sequences and wheat and barley Unigenes. For this purpose, orthologous sequences of unigenes Ta54227 (CDC, cell division control protein, AAA-superfamily of ATPases) and Ta2776 (RLI, 68 kDa protein HP68 similar to *A. thaliana* RNase L inhibitor protein), two stable reference genes from wheat (Paolacci et al., 2009) were tried in barley and rye. The bioinformatics analysis revealed that the wheat-specific primers RLI(b) and CDC(b) did not perfectly match barley sequences.

Internal controls used in plants to accurately normalize the target genes

Novel genes or genes selected via microarray analysis

SAND protein was first identified in *Saccharomyces cerevisiae* (Tizon et al., 1996), then was later identified in several other organisms like *Caenorhabditis elegans*, *Drosophila melanogaster* and the plant *A. thaliana* on a sequence-similarity basis. The SAND protein gene (At2g28390) was selected as a stably expressed gene for different experimental series from ATH1 data, and was later used in qPCR to assess expression stability and absolute expression level. It was categorized as one of the most stable genes, outperforming all of the traditional genes (Czechowski et al., 2005). It has been used in several studies for reference gene validation and target gene normalization, and is categorized as one of the most stable genes for various experimental conditions (Remans et al., 2008; Expósito-Rodríguez et al., 2008; Reid et al., 2006; Mallona et al., 2010; de Almeida et al., 2010). Its overall stable expression might make it a genuine reference gene in future studies for target gene normalization using qRT-PCR. Ribosomal proteins are ubiquitous in the plant kingdom, and are the structural constituents of the ribosomes. Several types of ribosomal proteins (*RPL2, RPL13D, RPL8, RPL7, RPS13, RPS1* and *RPL1 8a-1*) have been successfully used for reference gene validation in qPCR (Nicot et al., 2005; Tong et al., 2009; Expósito-Rodríguez et al., 2008; Mallona et al., 2010; Luo et al., 2010; Paolacci et al., 2009; Løvdal and Lillo, 2009) and in most studies it has been considered a stable reference for gene normalization (Nicot et al., 2005; Løvdal and Lillo, 2009; Tong et al., 2009; Mallona et al., 2010). However, in some cases it was found unsuitable for gene expression studies especially in aquatic plants, water lily (Luo et al., 2010), and in wheat (Paolacci et al., 2009), under different developmental stages. The stability of *RPL2* has been semi-quantitatively confirmed in the tomato cultivar VFN8 (Nebenfuhr et al., 1998) for detection of transgenic tomato. In sugarcane, the relative expression of *RPL35-4* was reported to be stable (Calsa et al., 2007). Cytoplasmic ribosomal protein L2 was used as internal control in potato (Nicot et al., 2005), and in *Bupleurum chinense* (Dong et al., 2010) considered as the most stable of the reference genes, with the lowest *M* value as well as expression level, which are preliminary choice of references in qPCR. The *TIP41* (At4g34270) protein gene was selected as one of the novel genes stably expressed from the ATH1 array under different experimental conditions, and was also reported with the lowest CV (0.141) value among the five novel and traditional

references tested. *TIP41* was identified as novel and the most stable reference when compared to other novel as well as traditional references, and also presented a potential replacement of traditional references which were outperformed by the novel genes (Czechowski et al., 2005). *TIP41* ranked among the top four references (*GAPDH, UBC, TIP41* and *actin*) in a 2003 study of mesocarp tissue during berry development, while it ranked as the most variable gene in a 2004 data set as well as in a combined data set of 2003 and 2004 (Reid et al., 2006). In tomato developmental processes, *TIP41* gene overall ranked as the most stably expressed gene among all of the novel and traditional references used in the study (Expósito-Rodríguez et al., 2008). The *TIP41* gene in combination with *CAC* (Clathrin adaptor complex, At5g46630) and another expressed protein (At4g33380) as a gene-triplet was recommended for accurate normalization of gene expression measures encircling the complete developmental process in tomato (Expósito-Rodríguez et al., 2008). The highest stability of *CAC* (At5g46630), *SAND* (At2g28390) and *TIP41* (At4g34270) concluded by Expósito-Rodríguez et al., (2008), were in good agreement with the results of Czechowski et al., (2005) guided by microarray expression data. The results of Migocka and Papierniak (2010) were also found in good agreement with Czechowski et al., (2005), as most novel genes (*TIP41, CACS, F-box protein*, and At4g33380) outperformed commonly used traditional reference genes. *TIP41* (At4g34270) was found unsuitable in shoot and root of *A. thaliana* under increased metal concentrations (Remans et al., 2008), but increased expression stability was observed when using data input was averaged per treatment and organ, compared to data input using expression levels of individual samples. In the case of soybean, it was not considered overall the most stable, but considered stable when photoperiod and light quality were considered (Hu et al., 2009), and it was found to be an unsuitable reference gene in shoot and root tissues of different rice cultivars (Caldana et al., 2007). Regulatory (At1g13320 and At3g25800) and catalytic subunits (At1g59830) of Ser/Thr protein phosphatase 2A (*PP2A*) have been successfully used in qPCR for gene normalization. Czechowski et al., (2005) first identified them as stably expressed genes among five genes selected from ATH1 data for different experimental series in *A. thaliana*. The catalytic subunit (At1g59830) of *PP2A* used in tomato (Løvdal and Lillo, 2009) and cotton (Artico et al., 2010) appeared to be one of the stably expressed genes in a combined data set, while it was observed as inappropriate for gene normalization in individual data sets. The regulatory subunit (At1g13320) tried in coffee was found stable for drought stressed roots and also across different cultivars (Cruz et al., 2009). The Clathrin adaptor complex subunit (At5g46630/*AP47*) gene selected as a stably expressed gene from the ATH1 array for different experimental conditions in *A. thaliana*, proved its efficiency in qPCR for reference gene validation (Czechowski et al., 2005). The gene was also tried in grape berry (Reid et al., 2006), tomato (Expósito-Rodríguez et al., 2008), *A. thaliana* (Remans et al., 2008), coffee (Cruz et al., 2009) and water lily (Luo et al., 2010) for target gene normalization. The identified and quantitatively validated At5g46630 (Clathrin adaptor complex subunit) gene by Czechowski et al., (2005) was eliminated from the study of Remans et al., (2008) due to inefficient amplification. The At5g46630 gene was expressed at the level of 20- to >1,000-fold lower than *UBQ10* and *EF-1α* in *A. thaliana*, and crossed the cycle threshold for fluorescence detection (C_T) (Czechowski et al., 2005). This might be for reasons of inefficient amplification of this gene in the study of Remans et al., (2008). In the case of cotton, it was observed to be the most stably expressed gene only for fruit developmental stages (Artico et al., 2010). The orthologs of At5g46630 were found to be good references with respect to transcript normalization for all experimental conditions tested in tomato (Expósito-Rodríguez et al., 2008), coffee (Cruz et al., 2009), water lily (Luo et al., 2010) and in cucumber for different metals and organs (Migocka and Papierniak 2010).

The Histone 3 (*H3*) gene was identified being among the most stably expressed 100 genes from microarray data of *A. thaliana* (Czechowski et al., 2005), but was not found suitable as a reference in qRT-PCR for target gene normalization (Paolacci et al., 2009; Luo et al., 2010; Long et al., 2010; Maroufi et al., 2010). However, under *in vitro* conditions, H2B was regarded as stable in *Eucalyptus globules* (de Almeida et al., 2010), and H3 in the longan tree (Lin et al., 2010).

Hu et al., (2009) efficiently normalized their qRT-PCR data using a set of three novel reference genes (*SKIP16*/At1g06110, *UKN1*/At3g13410, and *UKN2*/At4g33380) where most of the novel references performed better than most commonly used traditional references across all the experimental conditions. An expressed protein (At4g33380) was identified stable in the ATH1 array by Czechowski et al., (2005) for a range of experimental conditions. The exact function of this protein is still unknown. Its expression was observed to be variable under *in vitro* adventitious rooting of *Eucalyptus globules* (de Almeida et al., 2010) and in *Arabidopsis* plants exposed to Cd and Cu stress (Remans et al., 2008), while Migocka and Papierniak, (2010) considered it a reliable (but not most suitable) reference gene for cucumber plants under metal stress. Another set of two novel housekeeping genes, translation elongation factor 2 (*TEF2*), and RNA polymerase subunit (*RPII*) was constantly and abundantly found stable in all peach samples for transcription normalization (Tong et al., 2009). A flax (*Linum usitatissimum*) ortholog of novel reference gene, *NDUFA8* (NADH-ubiquinone oxidoreductase 19-kDa subunit), was found variable for different developmental stages of *A. thaliana* (Gutierrez et al., 2008). Heat shock proteins (Hsps) play a crucial role in protecting plants against abiotic stresses (Sabehat et al., 1998; Visioli et al., 1997; Wang et al., 2004). Among the five conserved families of Hsps (Hsp70, Hsp60, Hsp90, Hsp100, and the small Hsp), the small Hsps are the most prevalent in plants (Visioli et al., 1997; Wang et al., 2004), and have been successfully applied in qPCR for reference gene validation. HSP90 was identified among the top 25 genes at the whole-genome level that displayed the most constant expression across various developmental stages in rice (Jain et al., 2009). An ortholog of *HSP90* also showed constant expression stability across various organs/developmental stages in chickpea (Garg et al., 2010). *YLS* encodes for the mitosis protein YLS8, however, the actual function of this gene is not known. *Yls8* was shown to be highly stable in *Arabidopsis* exposed to increased metal concentrations (Remans et al., 2008), and constant in different peanut tissues (Brand and Hovav, 2010), while *YSL8* expression was found less stable compared to other novel references (*PP2A, F-box protein*, etc.) in the study of Migocka and Papierniak (2010), which was focused on heavy metal stress in different cucumber organs. Several novel genes identified through microarray analysis in *A. thaliana* (Czechowski et al., 2005), soybean (Libault et al., 2008), wheat (Paolacci et al., 2009; Long et al., 2010), and rice (Jain et al., 2009; Narsai et al., 2010) for several experimental conditions, may also be applied in qRT-PCR to solve the problem of reference gene validation and

target gene normalization, specifically in plants (Table 1b). Out of the thousands of novel references identified through microarray analysis, only some have been tried in a small number of plant species, provided an unambiguous solution to replace the traditional reference genes used.

Traditional genes or genes selected from semi-quantitative techniques

In case of plant species, the abundance, stability and housekeeping nature of traditional references are strongly supported when genomic databases from wheat (Paolacci et al., 2009), and *Oryza sativa* (Narsai et al., 2010) were screened to find a stable reference gene from different experimental conditions, the sequences identified as stable reference gene candidates included most of the known traditional housekeeping genes which are frequently used in qPCR.

Among recent studies on qRT-PCR, nine traditional references (*ACT, TUB/TUA, CYP, GAPDH, 18/25S rRNA, UBQ, UBC, EF-1α*, and *IF-4α*) were most commonly considered for reference gene validation in plants. Out of nine, the most prevalent six housekeeping genes (*ACT, TUB/TUA, GAPDH, 18/25S rRNA, UBQ*, and *EF-1α*) were widely applied for reference gene validation in which elongation factors were frequently quoted as being stably expressed genes. In particular, elongation factor 1 alpha (*EF1α*) (At5g60390, At1g56070, Ta659) and elongation factor 1 beta (*EF-1β*) (At5g19510, At2g18110) are used in qRT-PCR where EF1α is an essential enzyme in protein synthesis that aids the binding of aminoacyl-tRNA to ribosomes during the elongation phase of protein synthesis, while *EF-1β* catalyzes the exchange of GDP bound to the G-protein, EF1α, for GTP, an important step in the elongation cycle of the protein biosynthesis. Elongation factors were used 33 times and found stable 58% of the time, for a range of experimental conditions (Nicot et al., 2005; Ransbotyn et al., 2006; Jain et al., 2006; Caldana et al., 2007; Li et al., 2009; Tong et al., 2009; Migocka and Papierniak 2010). Elongation factors were also found suitable for analysis of transgene expression in transgenic *E. ulmoides* root lines overexpressing *IPPI* or *FPPS1* genes, which are involved in isoprenoid biosynthesis (Chen et al., 2010).

The Translation elongation factors have preferentially been used in most of the studies for transcript normalization, but there are only relatively few reports of eukaryotic translation initiation factor genes being used as reference genes (Martin et al., 2008; Paolacci et al., 2009; Garg et al., 2010; Huis et al., 2010). Heterogeneity in the results was observed when five different genes of eukaryotic translation initiation factors (*IF1, IF3E, IF3H, IF4F* and *IF5A)* behaved asymmetrically in different tissues of flax (Huis et al., 2010). Translation initiation factors were found only 27% suitable for transcript normalization (Martin et al., 2008; Garg et al., 2010; Huis et al., 2010), and considered most of the time unsuitable as reference genes in qPCR (Paolacci et al., 2009; Guitierrez et al., 2008). Cyclophilins, or peptidyl-prolyl cis-trans isomerases, are enzymes belonging to the superfamily of immunophilins and assist in protein folding (Fischer et al., 1989; Takahashi et al., 1989) as a chaperone for protein trafficking as well as the nucleolytic degradation of the genome (Montague et al., 1997). In most of the studies regarding plants, it was found unsuitable for transcript normalization (Brunner et al., 2004; Dong et al., 2010; Huis et al., 2010). In none of the studies it was considered overall as a stable reference gene for all of the experimental conditions, except soybean (Jian et al., 2008), salt-stressed potato (Nicot et al., 2005), and the V30 line of *Petunia hybrida* (Mallona et al., 2010) where it was considered a stable reference gene. Ubiquitin-conjugating enzyme (*UBC*) is another type of traditional housekeeping enzyme, used less frequently as a reference gene in plant biology, however, its expression level was found suitable for relative transcription normalization in soybean (Jian et al., 2008), B*ranchypodium distachyon* (Hong et al., 2008), *Brachiaria brizantha* (Silveira et al., 2009), and in phytohormone treated leaves of water lily (Luo et al., 2010). Its unsuitability for reference gene validation was also observed in several studies (Martin et al., 2008; Garg et al., 2010; Dombrowski and Martin 2009). *18S rRNA* and *25S rRNA* are most frequently used in plants as reference genes, however, not despite frequent citation as stable in expression level, and in most of the studies they were observed as the least stable housekeeping genes. There are some obstacles prevalent with ribosomal RNA when used as a reference gene. The primary reason is that its high abundance compared to target mRNA transcripts make it difficult to subtract the baseline value in qRT-PCR data analysis (Vandesompele et al., 2002; Nicot et al., 2005; Tong et al., 2009; Boava et al., 2010), their inability to amplify when reverse transcription is carried out using oligo-dT primer as they lack poly-A tails (Sturzenbaum & Kille 2001; Jain et al., 2006), and its regulated synthesis (Vera et al., 1993). Plants have different types of rRNAs and their relative contribution may vary considerably depending on the tissue, developmental stage, and metabolic state (Phillips et al., 2009). According to Takle et al., (2007) rRNAs are highly conserved in different bacterial species, even inside eukaryotic chloroplasts and mitochondria. Nonspecific amplifications could occur due to plant material, as well as to bacteria naturally present in the phyllosphere. Ribosomal RNAs (*18*, *25*, *26*, and *28S rRNA*) were tried 19 times and were not considered the most stable reference genes (Kim et al., 2003; Martin et al., 2008; Jarošová and Kundu 2010; Garg et al., 2010).

Contradictory results were obtained when two different rRNA species were compared, however, 18S rRNA always were considered stable in such studies (Jarošová and Kundu 2010; Garg et al., 2010) and used effectively as a standard in *Arabidopsis* to verify genes, differentially expressed and validated through microarray analysis under low-oxygen stress (Klok et al., 2002). In all of the studies its expression level was found over several orders of magnitude of mRNA transcripts and in most of the studies it was considered the least stable reference gene for transcript normalization (Dombrowski and Martin 2009; Tong et al., 2009; Wan et al., 2010). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is an abundant glycolytic enzyme associated with a high-energy demand by cells during growth and cell differentiation (Carvalho et al., 2010). *GAPDH* is a frequently used reference gene among plants, and found stable for several plants and experimental conditions (Iskandar et al., 2004; Reid et al., 2006; Argyropoulos et al., 2006; Hong et al., 2008; Barsalobres-Cavallari et al., 2009; Løvdal and Lillo, 2009; Garg et al., 2010; Carvalho et al., 2010). It was considered one of the most stable reference genes, among data created for two consecutive years regarding different berry development stages (Reid et al., 2006), where most of the reference genes showed high variability in the gene expression level. Gene expression stability of *GAPDH,* among different studies was observed less (35%) (Fig. 1), and most of the time it was considered the least stable reference gene (Jain et al., 2006; Tong et al.,

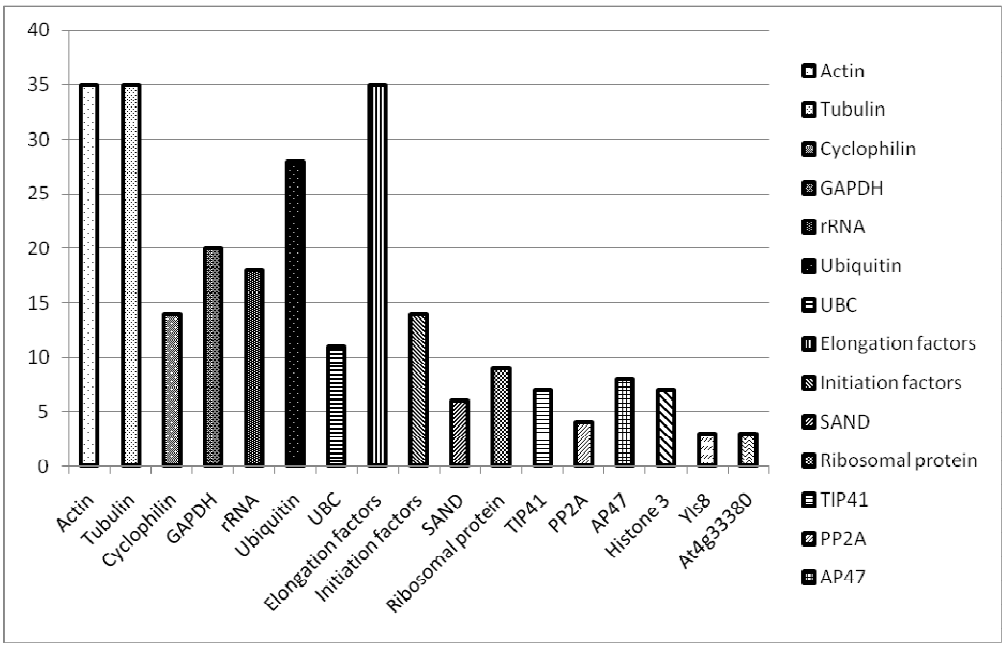

Fig 1. Reference genes (traditional and novel) used for transcript normalization specifically in plants from January 2000 through December 2010 published in important scientific journals.

2009; Expósito-Rodríguez et al., 2008; Dombrowski and Martin 2009). Three important traditional housekeeping genes (actin, tubulin, and ubiquitin) are used more often in comparison to other traditional references for transcript normalization in plants. The following housekeeping references were found stable over a wide range of experimental conditions across several plant species i.e. *ACT* (Guitierrez et al., 2008; Jian et al., 2008; Hu et al., 2009; Luo et al., 2010; Maroufi et al., 2010), *UBQ* (Brunner et al., 2004; Guitierrez et al., 2008; Martin et al., 2008; Hong et al., 2008; Jain et al., 2009; Boava et al., 2010), and α-tubulin/β-tubulin (Wan et al., 2009; Dong et al., 2010; de Almeida et al., 2010; Jarošová and Kundu, 2010). Comparatively, α-tubulin found more suitable than β-tubulin for transcript normalization (Brunner et al., 2004; Wan et al., 2009; de Almeida et al., 2010), was successfully used to normalize *CesA* (cellulose synthase) gene expression levels from barley (*Hordeum vulgare*) in vegetative and floral tissues at different developmental stages (Burton et al., 2004), and for two, up- and downregulated transcripts in salt-stressed leaf and root tissue of barley (Ozturk et al., 2002). Actin has also successfully been applied for transcript normalization of auxin binding protein (*ABP1*) in sunflower (Thomas et al., 2003), and for C-repeat binding factor (*cbf*) gene in barley (Svensson et al., 2006). However, in all of the comparable studies regarding reference gene validation, no study reported actin, tubulin or ubiquitin found > 40% stability across all the plant species, indicating that there are no universal reference genes for all plant species. Validation is thus essential goal for any selected housekeeping gene used as reference gene in gene expression analysis.

Tools of gene normalization

Since the search for suitable reference genes is both time-consuming and cost intensive, several computer programs based on different statistical approaches have been developed to assist the selection of a stable reference gene among several HKGs. Statistical algorithms such as geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), Stability index (Brunner et al., 2004), ΔCt approach (Silver et al., 2006) and BestKeeper (Pfaffl et al., 2004) are commonly employed to select a least variable reference gene for normalization of qRT-PCR data in a given set of biological samples (Artico et al., 2010). However, geNorm is the software used most in plant science followed by NormFinder. BestKeeper is also used to determine the best-suited reference gene by pairwise correlation analysis of raw Ct values (Pfaffl et al., 2004). According to Lyng et al., (2008), this approach may be useful to narrow down a search if no specific genes are known to be plausible candidates, whereas more advanced statistics, such as those provided by geNorm and NormFinder, are needed to rank the genes if several of them are identified as good candidates; moreover BestKeeper can analyze a maximum of 10 reference genes. This geNorm calculates a gene expression stability measure (M) for each gene based on the average pairwise expression ratio between it and each of the other genes being studied. geNorm then performs a stepwise exclusion of the least stable gene and recalculates M until only two genes are left, these being the most stably expressed. In some studies, NormFinder was used preferentially over geNorm because it considers intra- and inter-group variations for the normalization factor (NF) (Artico et al., 2010), thus the best combination of reference genes (to reach the appropriate NF) is not necessarily the one containing the most stable genes according to a gene-by-gene comparison (Cruz et al., 2009). The mathematical model of gene expression used by NormFinder enables estimation of both the overall variation of the candidate normalization genes, and the variation between subgroups of the sample set. This approach has been reported to perform in a more robust manner than geNorm, and has been shown to be less sensitive to the presence of co-regulated genes (Andersen et al., 2004), therefore the choice of candidate reference genes without functional relationships is less critical than in geNorm. Very useful information given by geNorm is the ideal number of reference genes which should be included in an NF, whereas NormFinder

indicates only the best single gene and the best combination of two genes. As shown by several studies (Remans et al., 2008; Vandesompele et al., 2002; Maroufi et al., 2010; Artico et al., 2010), the normalization based on multiple reference genes gives more accurate evaluation of gene expression, especially when no single optimal reference gene is available. However, inter-variability of reference genes under different experimental conditions was noted when geNorm and NormFinder were applied to select stable genes from the same data set. Luo et al., (2010) reported on different sets of genes using geNorm and NormFinder in leaves and roots of water lily exposed to different stresses and phytohormones.

A stability index has not been widely adopted to measure the stability of references across different samples, as it produce different results from most commonly used methods (geNorm and NormFinder) for reference gene stability like *elF1β* was the most stable HKG followed by *CYP2*; and the most variable was *UBQ10* followed by *G6PD,* while according to 'Stability index' *ACT11* was the most stable HKG followed by *CYP2*, while *TUA* and *elF1α* became the least stable in all of the 21 sample pools in soybean (Brunner et al., 2004). An explanation might be that the 'Stability index' did not take the PCR efficiency into account, which played an important role in the data analysis (Jian et al., 2008). Some studies that have utilized both geNorm and NormFinder have reported minor changes in gene stability ranking (Cruz et al., 2009; Willems et al., 2006; Perez et al., 2007), while others have observed relatively substantial changes i.e. up to 15 places between the two methods (Langnaese et al., 2008; Spinsanti et al., 2008). Some additional statistical models have been developed for identifying optimal reference genes, but either they are not freely available, or their application is complex (Szabo et al., 2004; Haller et al., 2004), or they do not take the PCR efficiency into account (Brunner et al., 2004).

Versatile application of qRT-PCR in the plant kingdom
For validation of Northern blot data

Some studies further validated the results of traditional techniques like Northern blotting, using more sensitive techniques like qRT-PCR. The results of Paolacci et al., (2009) for the quantitative expression pattern of cold- and heat-responsive genes (*wcor14* and *TaHSP101B*) was in perfect agreement with those obtained by Tsetanov et al., (2000); Campbell et al., (2001) and Gulli et al., (2007), respectively. Several other genes for abiotic stresses have also been defined using less sensitive methods i.e. Northern blot and semi qRT-PCR, should be reconsidered for their accurate measurement under these conditions using qPCR. A number of abiotic stress responsive genes have been defined by using a semi-quantitative RT-PCR technique including heat (Zhang et al., 2005; Magiri et al., 2006), drought (Medini et al., 2009; Liu et al., 2010), chilling (Liu et al., 2010), and salt (Liu et al., 2010).

To detect transcript level of key enzymes in biosynthetic pathways

qRT-PCR has been successfully used by Chen et al., (2009), to detect transcript level of genes encoding enzymes at key flux control points (i.e., *PAL*, *CHS*, *CHI*, and *CHR*) and at a branch-point enzyme (i.e., *IFS*) in the phenylpropanoid pathway in soybean sprouts treated with chitosan (a natural biopolymer derived by deacetylation of chitin). Normalization of enzyme transcripts was performed using the *Actin* gene selected from a group of housekeeping genes (β-*tubulin*, *EF*-1a, *GAPDH* and *Actin*) because of its stable expression, analyzed through *Best-Keeper* (Pfaffl et al., 2004) program.

Fungal biomass determination in plants

An accurate level of fungal biomass in an infected plant can be determined using qRT-PCR. Conventional methods like visual scoring of disease symptoms, analysis of mycotoxins and species-specific PCR (Demeke et al., 2005; Mach et al., 2004; Nicholson et al., 1998) or the assays, detect distinct groups of species producing the same class of toxins (e.g., trichothecens or fumonisins) (Bluhm et al., 2002; Mirete et al., 2004; Mulfinger et al., 2000; Ward et al., 2008) are commonly applied to assess the tolerance of new lines. All of these methods are indirect methods and do not necessarily determine the accumulated fungal biomass. Brunner et al., (2009) developed a qRT-PCR-based assay for trichothecene-producing *Fusarium* species, and to adapt this method for resistance assessment of wheat lines artificially infected with *Fusarium graminearum* and *Fusarium culmorum*. The plant gene *EF-G*, encoding for the wheat translation elongation factor, was included in the measurements as a reference gene to limit the impact of total DNA yield on the determination of the *Fusarium* content of a sample, and thereby represents a possible step towards more standardized qPCR analyses of *Fusarium* infections. TaqMan assays have been used extensively in barley pathogen studies, in particular detection and quantification of toxin-producing *Fusarium* spp. (Strausbaugh et al., 2005; Leisova et al., 2006; Sarlin et al., 2006).

Transcript profiling of defense-associated genes

The qRT-PCR technology clearly has not yet been completely exploited in plant–microbe interaction studies. Wen et al., (2005) conducted a study to quantify transcript levels of defense-associated genes coding for 1,3-β-D-glucanases (*pG101*), phenylalanine ammonia lyase (*gPAL1*), and chalcone synthases (*CHS17*) in roots, stems, and leaves of 1- and 2-week-old bean seedlings following infection with *R. solani*, and to compare them to levels estimated in tissues of nonpathogenic binucleate *Rhizoctonia* spp. (np-BNR) protected and infected or not infected with *R. solani*, using qRT-PCR. Housekeeping gene *Actin* was used for the normalization of defense-associated genes. The method accurately quantified the level of defense-associated transcripts in *Phaseolus vulgaris* cv. Contender UT15. qRT-PCR was also used efficiently in another study of McMaugh and Lyon (2003), to analyze the expression of class II chitinase in Bermudagrass following infection with the root pathogen *Ophiosphaerella narmari*.

Food adulteration

Protein and conventional PCR-based methods are commonly employed to detect food adulteration. Bryan et al., (1998) developed a simple PCR-based method to detect the presence of hexaploid wheat in durum wheat pasta, however, qRT-PCR was also used successfully by Alary et al., (2002), for quantification of common wheat adulteration of durum wheat pasta since Spanish, Italian, and French regulations enforce a 3% maximal level of common wheat contamination in pasta and semolina.

Transgene expression analysis

The success of any transformed plant depends on the expression of the alien gene in sufficient quantity that can lead to the fulfillment of requirement for which it is transformed, like improvement of agronomic traits (insect and herbicide resistance). The first and essential step in this direction is the estimation of the transgene copy number and determination of the expression levels of transformed gene in genetically

modified crops. Many drawbacks like the inaccurate assessment of transgene copy number and their expression levels have been eliminated as the fluorescent based methods (qRT-PCR) made the transgene quantification easy and accurate at both the DNA and RNA level. Several species-specific internal controls like *LAT52* for tomato (Yang et al., 2005a; Randhawa et al., 2009), *SPS* in rice (Ding et al., 2004), *Sad1* in cotton (Yang et al., 2005b), *SRK* for *Brassicaceae* family members (Randhawa et al., 2008), and *ST-LS1* gene for potato (Randhawa et al., 2009) were identified and validated successfully as a endogenous references for detection of the transgene. Among all of the applications of qRT-PCR in plant science, transgene analysis is most widely explored with improved results and statistical tools (Yuan et al., 2007). Commonly used housekeeping references were also applied to quantify the level of transgene. Chen et al., (2010) selected *ACTα* and *EF1α* genes from a set of 10 housekeeping genes (*ACTα*, *ARPT*, *CYP*, *EF1α*, *EIF1α*, *GAPD*, *rbcL*, *TUBα*, *TUBβ*, and *UBQ* to evaluate transgenic *E. ulmoides* Oliver root lines overexpressing *IPPI* or *FPPS1* genes, which are involved in isoprenoid biosynthesis. In another experiment conducted by Assem and Hassan (2008), ribosomal RNA (rRNA) was used as an internal control for transgenic maize.

Other applications

qRT-PCR has been successfully explored to detect the relationships between the level of gene expression and phenotypes with respect to a particular locus in hybrid performance (Rosas et al., 2010). For this purpose, expression levels of *CYC* and *RAD* were determined by qRT-PCR, using ubiquitin (*UBI*) as reference gene, to establish a more quantitative mapping between expression and morphology for each genotype, generated by crossing *Antirrhinum majus* to lines carrying *cyc* and/or *rad* mutant alleles. Travella et al., (2006) efficiently used this technique for detection of SNPs and in RNA interference-based gene silencing in hexaploid wheat (*Triticum aestivum*) where two genes, *Phytoene Desaturase* (*PDS*) and *Ethylene Insensitive* 2 (*EIN2*) were isolated through RT-PCR and constructed RNAi plasmids were cotransformed. The qRT-PCR was efficiently used to screen the T_1 and T_2 generations, to determine the RNAi-mediated gene silencing of both of the genes using *GAPDH* as a standard reference gene, where both genes result in the reduction of transcripts by up to 93% for *PDS*, and 99% for *EIN2*. Crismani et al., (2006) used the technology efficiently to validate the meiotically-regulated transcripts, selected from Wheat Genome Array across a sub-staged meiotic time series of whole wheat anthers, where overall expression patterns of microarray and Q-PCR were similar, confirming a high degree of reproducibility between the two platforms. Ibraheem et al. 2011 successfully analyzed the effect of drought and salinity on the expression level of 5 rice sucrose transporter genes and found that OsSUT2, which facilitates transport of sucrose from photosynthetic cells, is essential for rice plants to cope with drought and salinity stresses. In the past few years, the development and application of molecular techniques for the detection of infectious diseases has drastically changed the diagnosis and monitoring of plant pathogens. It is a useful tool for examining pathogen gene expression inside infected host tissues. In this regard, qRT-PCR used successfully to provide accurate solution to detection, differentiation and quantification of the viral load in plant and aphid samples (Schneider et al., 2004; Olmos et al., 2005; Capote et al., 2006). The technique has been successfully used for the analysis of potato pathogen *Pectobacterium atrosepticum* (Takle et al., 2007). Several methods are now developed for allelic discrimination assays through multiplexed Real-Time PCR using different fluorescent probes (Lee et al., 1999), as in maize for QTL controlling pollen shed (Salvi et al., 2001), and to differentiate two bacterial species by targeting a unique conserved region (Bowers et al., 2010) in a non-electrophoretic way. A method has been developed based on the use of real time PCR (Holland et al., 1991; Heid et al., 1996) to discriminate between plants that are homozygous or heterozygous for dominant SCAR markers (Vandemark and Miklas 2002, 2005). This method was used to simultaneously genotype for both *SBD51300* (Miklas et al., 2000b) and *SW13690* (Melotto et al., 1996) SCAR markers, which are linked to the independent *bc-12* and *I* alleles, conditioning resistance to *Bean common mosaic virus* (BCMV), respectively.

Concluding remarks

The qRT-PCR approach can be used to validate the results of any primary differential gene expression screening method (cDNA arrays, DD-PCR, serial analysis of gene expression, and subtraction hybridization), once the sequence of the candidate gene is known. The expression analyses of such genes require a standard housekeeping reference with invariable expression. In qRT-PCR, reference genes are selected on the basis of good performance in other species, while they may show remarkable variability under different experimental conditions even for the same plant. Yet, few studies have been conducted on the actual use of references that have been validated previously. Moreover, no studies have been conducted for target gene normalization using novel genes, selected recently through microarray analysis. Traditional internal controls found many reference genes inappropriate in most of past studies hence, replacement is required with appropriate novel genes to increase the efficiency in target gene normalization. However, the qPCR technology is not yet widespread in plant sciences except for the comparative analysis of traditional and few novel internal controls under different experimental conditions for model plant species. To expand the use of this technology in plants we must further gather the basic information (DNA sequences and differential gene expression) needed to make it a universal platform. Its earlier application in important fields of plant research like transgenics, allelic discrimination for resistance to biotic stresses, and differential gene performance against abiotic stresses, make it an efficient technology that can be used to solve other issues in plant biology like phylogenetics, apomixes, and hybrid performance.

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