Selection of Reference Genes for Real-time Quantitative PCR Normalization in the Process of *Gaeumannomyces graminis* var. *tritici* Infecting Wheat

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Gaeumannomyces graminis var. *tritici* is a soil borne pathogenic fungus associated with wheat roots. The accurate quantification of gene expression during the process of infection might be helpful to understand the pathogenic molecular mechanism. However, this method requires suitable reference genes for transcript normalization. In this study, nine candidate reference genes were chosen, and the specificity of the primers were investigated by melting curves of PCR products. The expression stability of these nine candidates was determined with three programs-geNorm, Norm Finder, and Best Keeper. *TUBβ* was identified as the most stable reference gene. Furthermore, the exopolysaccharide synthase gene (*ExoPG*) was selected to verify the reliability of *TUBβ* expression. The expression profile of *ExoPG* assessed using *TUBβ* agreed with the results of digital gene expression analysis by RNA-Seq. This study is the first systematic exploration of the optimal reference genes in the infection process of *Gaeumannomyces graminis* var. *tritici*.

Keywords : geNorm, housekeeping gene, *Triticum aestivum*, *TUBβ*

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Gaeumannomyces graminis var. *tritici* (*Ggt*), a soilborne pathogenic fungi of wheat roots, causes “take-all disease” on roots of wheat and other cereals and results in severe crop losses worldwide. Take-all disease is an area of intense current interest of research on wheat root diseases yet remains the most important root disease of wheat worldwide. The genetic improvement of resistance in wheat to this pathogen remains a priority, and the study of pathogenic gene expression patterns is crucial for understanding the molecular mechanisms underlying the infection process, providing valuable information for wheat resistance breeding programs.

According to our previous RNA-Seq analysis (Yang et al., 2015), 3,258 DEGs (2,107 up-regulated genes and 1,151 down-regulated genes) were detected during infection process of *Ggt*. Hence these candidate genes were presumed to play important role in infection process. However, how these genes regulate infection process is still unclear. The more accurate and precise expression pattern of every candidate gene would be helpful to understand how it plays a crucial part. RT-qPCR has become a powerful and frequently used experimental method to evaluate gene expression levels due to its high sensitivity, specificity, accuracy, and reproducibility (Bustin, 2002; Bustin et al., 2005; Nolan et al., 2006). Furthermore, in some cases, it is the only method that allows for the detection of low copy numbers of genes of interest and slight variations in gene expression (Bustin, 2010). Regardless of its many advantages,

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this method requires appropriate reference genes to ensure the reliability of the target gene's expression. Recent studies have shown that no single reference gene is universally applicable for all systems. Those genes (e.g., *18S rRNA*, *actin*, tubulin, et al.) involved in basic cellular processes have been frequently used as reference genes. However, many studies have demonstrated that some of these reference genes might not be stably expressed under some conditions (Gutierrez et al., 2008; Schmittgen et al., 2000). For example, *Gapdh*, which is typically used as reference gene, shows the lowest expression stability among different *Aspergillus niger* AB1.13 culture conditions (Bohle et al., 2007). Similarly, the research of Kim and Yun (2011) found that *GAPDH* is an unsuitable reference gene, while *GzUBH* and *EF1A* are most stable reference gene of *Fusarium graminearum* PH-1 strain under different culture conditions. In rust-infected wheat, ADP-ribosylation factor and RNase L inhibitor-like protein (RLI) encoding genes showed the best stability in *Puccinia Strititica*-infected wheat, while the cell division control (CDC) protein and RLI encoding genes showed the best stability in *Puccinia Striiformis*-infected wheat (Scholtz and Visser, 2013). However, in *Puccinia graminis* f.sp. *tritici*-infected wheat, *CDC*, *18S rRNA*, and β -*Tubulin* showed the best stability. Thus, a credible reference gene should be evaluated before performing RT-qPCR studies. Many studies have addressed the selection of reference genes in plants and fungi, but none have done so in *Ggt*, a harmful fungus. This study was conducted to identify reference genes for transcript normalization of pathogenic genes during the infecting process of *Ggt*. The expression profiles of nine reference genes including *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase), *TUB2* (tubulin alpha chain), *G6PDH* (Glucose-6-phosphate-1-dehydrogenase), *GK* (Glucokinase), *EF-3* (Elongation factor 3), *EF2-2* (Elongation factor 2), *TUB β* (Tubulin beta), *ACT* (Actin) and *EF2-1* (Elongation factor) were studied in different stages of *Ggt*-infected wheat, and the expression stability of these genes was evaluated with geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and Bestkeeper (Pfaffl et al., 2004). Furthermore, the expression pattern of target gene *ExoPG* (exopolygalacturonase) was investigated using the selected reference gene, which may be helpful in revealing its role in *Ggt* pathogenicity.

Materials and Methods

Fungal materials and infection of wheat roots. An isolate of *Ggt* (GGT-007, Henan Academy of Agricultural Sciences, Zhengzhou, China) was isolated by Quan et al.

(2014). Liquid cultures were grown in potato dextrose broth at 25°C with shaking at 180 rpm for 5 d to prepare for wheat root infection and *Ggt* RNA extraction.

Seeds of winter wheat (*Aestivumtriticum* cv 'Zhengmai 366') were supplied by the Wheat Research Institute of the Henna Academy of Agricultural Science. The experiment of accelerating germination and inoculation procedure of wheat root were carried out according to Yang et al. (2015a). The wheat roots infected by *Ggt* samples from three biological replicates were collected at 0.5, 1, 2, 3, 4, 5, 7 d after inoculation. The liquid culture of *Ggt* was control group. The samples were immediately frozen and then stored in liquid nitrogen until analysis.

Extraction of total RNA and synthesis of cDNA. Frozen *Ggt* hypha and infected-wheat roots were ground to a fine powder in liquid nitrogen with a pestle and mortar. About 100 mg of powder was used for RNA extraction. Total RNA was isolated using the RNAiso Plus (TaKaRa, Dalian, China). Purified RNA was quantified with NanoDrop2000 spectrophotometer (Thermo, Wilmington, DE, USA), and the concentration and integrity of RNA was checked by a denaturing 1.0% (p/v) agarose gel. Only RNA samples with 260/280 wavelength ratio between 1.9 and 2.1 were used for cDNA synthesis. cDNA was synthesized with 1 μ g total RNA according the manufacturer of the Prime Script™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) in a total volume of 20 μ l cDNA was stored at -20°C.

Primer design and PCR conditions. The nine candidate reference genes (*PFK*, *GAPDH*, *TUB2*, *G6PDH*, *GK*, *EF-3*, *EF2-2*, *TUB β* , *ACT* and *EF2-1*) and one target gene *ExoPG* were selected from Transcriptome Sequencing Data and Genebank. Their accession numbers are listed in Table 1. Primer sequences were designed using Primer 3.0 (primer3.ut.ee/) with the following parameters: Tm around 60°C and product size ranges 150-250 base pairs, primer sequences with a length of 18 to 22 nucleotides, and a GC content of 45-55%. All the primers' specificities were confirmed using Primer-BLAST searches on nr database of NCBI and analyzed by real-time PCR and electrophoresis on 2% agarose gel.

QRT-PCR reactions were performed in 96-well plates with Step One Plus Real-Time PCR System (Applied Biosystems, CA, USA) and a SYBR® Premix Ex Taq™ Kit (TaKaRa, Tokyo, Japan). The qRT-PCR conditions were as follows: 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 30 s. The amplicons specificities were detected by melting curves analysis (60 to 95°C) after 40 PCR cycles.

Analysis of candidate reference gene expression stability. For every primer pair, a standard curve was used to calculate the gene-specific PCR efficiency from the 5-fold series dilution of *Ggt* cDNA. The correlation coefficients (R^2) and slope value were determined from the standard curve. The following formula was used to calculate the qRT-PCR efficiencies (E). $E = (5^{-1/\text{slope}} - 1) \times 100$ (Radonić et al., 2004).

The expression stabilities of all the candidate genes were evaluated by three Excel-based softwares: geNorm (Vandesompele et al., 2002), BestKeeper (Andersen et al., 2004), and NormFinder (Pfaffl et al., 2004). The geNorm (<http://medgen.ugent.be/~jvdesomp/genorm/>) is based on the mean pairwise variation between all the tested genes to calculate the expression stability value (M) of genes, and BestKeeper (<http://www.gene-quantification.de/bestkeeper.html>) is used to evaluate the expression stability according to an index which is obtained by standard deviation (SD) and percentage covariance (CV). NormFinder (<http://www.mdl.dk/publicationsnormfinder.htm>) is used to rank the genes expression stability according to their intra- and inter-expression variation.

Results

Identification of reference gene candidates and primer specificity and efficiency. Nine reference gene were selected for RT-qPCR normalization. Gene name, accession number, gene description, primer sequences, melting temperatures value (T_m), amplification efficiency and correlation coefficients are listed in Table 1. The T_m of PCR products ranged from 86.2°C for *EF-3* to 89.63°C for *GAPDH*. Amplification efficiency (E) of PCR reactions ranged from 103.7 for *GAPDH* to 119.82 for *EF-3*. Correlation coefficients (R^2) ranged from 0.9853 for *TUB2* to 0.9999 for *EF-3*. The amplifications were confirmed by the presence of a single band in 1.5% agarose gel electrophoresis and by the single peak melting curve of the PCR products (Fig. 1A, B), and the size of PCR products were consistent with expected size for each primer pairs.

Expression levels of the selected candidate reference genes. Based on average cycle threshold (Ct) values, there were obvious differences in transcript levels among the reference genes during *Ggt* infection. The Ct range and coefficient

Table 1. Candidate reference genes, primers and different parameters derived from RT-qPCR

Gene symbol	Accession number	Gene description	Primer pair (5' to 3') (forward/reverse)	T_m (°C)	Amplification efficiency (%)	R^2
<i>GAPDH</i>	XM_009222105	Glyceraldehyde 3-phosphate dehydrogenase	CGTGATGGGTGTCAACGAGA/ GCTGCTGGGGATGATGTTCT	89.63	103.7	0.9954
<i>TUB2</i>	XM_009228181	tubulin alpha chain	GCCGTTTCTCGGGTCACAG/ CAACCGTCTCATTTGCTCAG	86.8	105.73	0.9853
<i>G6PDH</i>	XM_009230131	Glucose-6-phosphate 1-dehydrogenase	GTCGGGCATCTTCAAGGACA/ TCGTCTCGCACAAAGTTGGA	88.59	104.02	0.997
<i>GK</i>	XM_009229629	Glucokinase	CTTGAACACGGGCGTCAAAAG/ ACACCCCTGAAGTTAGTGCCG	86.8	114.42	0.9989
<i>EF-3</i>	XM_009221076	Elongation factor 3	GGAGGCCAAGGTTGATGTCA/ AGCAGCAGAATATCGGGAGC	86.2	119.82	0.9999
<i>EF2-2</i>	XM_009220540	Elongation factor 2	TGTCGACCGCAACAAGATGA/ TTGCCCTCCTTGTCCTTGTC	87.39	102.97	0.9961
<i>TUBβ</i>	XM_009231745	Tubulin beta	TCCCAACAACATCCAGACCG/ TCCATACCCCTCGCCAGTGTA	86.35	105.32	0.9974
<i>ACT</i>	XM_009225830	Actin	GTGACGAACTGGGACGACAT/ GGCGTTGAAGGTCTCGAAGA	88.74	110.36	0.9974
<i>EF2-1</i>	XM_009218808	Elongation factor	CATCAAGCGCATGTCTTCCG/ GGTCGACCGTGTCTTCCAT	89.04	111.81	0.9975
<i>ExoGP</i>	XM_009227044	Exopolysaccharuronase	TCGACGACTTTCAGCTGCAC/ CCGCAAACTCATTCCACCA	87.25	102.90	0.9965

Fig. 1. Specificity of RT-qPCR and amplicon size. (a) Agarose gel (1.5%) electrophoresis showing amplification of a specific PCR product of the expected size for each gene. (b) Melting curves of one target gene and nine reference genes showing single peaks. M1 and M2 represent D2000 DNA Marker (150 bp-2000 bp) and DNA Marker I (100 bp-600 bp) marker, respectively.

Fig. 2. RT-qPCR Ct values for reference genes. Expression data displayed as Ct values for each reference gene in all Ggt and Ggt-infected wheat root samples. A line across the box is depicted as the median. The box indicates the 25th and 75th percentiles, whiskers represent the maximum and minimum values.

cient of variance for each gene were also obtained from the Ct value. As expected, the average Ct values varied from 20.56 for *GAPDH* to 25.77 cycles for *GK* (Fig. 2). Most of them were expressed abundantly (ranging from 20 to 25). Among them, *GAPDH* was the most abundantly expressed reference gene, while *GK* was the least abundant. According to the coefficient of variance (CV), which can reveal the reference gene's expression stability, *EF-3* had the least variation with a CV value of 4.17%, while *GAPDH* had the most variation with a CV value of 7.43%.

Expression stability of candidate reference genes. Ct data were obtained from qRT-PCR. Ct values were used directly for stability calculations for BestKeeper, while Ct

Fig. 3. Average expression stability values (M) and Pairwise variation (V) calculated by geNorm to determine the optimal number of reference genes. The average pairwise variations V_n/V_{n+1} was analyzed between the normalization factors NF_n and NF_{n+1} to indicate the optimal number of reference genes required for RT-qPCR data normalization in different samples.

values were converted to relative quantities for geNorm and NormFinder.

Gene expression stability has a negative correlation with its M value. It was calculated by geNorm software based on the average pairwise variation between all the tested reference genes. The most stable reference gene has the lowest M value. According to the Fig. 3A, all the reference genes can be recognized as stable because they all had an M value less than the geNorm threshold of 1.5. During the process of pathogen infections of wheat roots, *TUBβ* and *EF2-1* had the highest expression stability (i.e., the lowest M values, 0.028 and 0.03), whereas *GAPDH* had the lowest stability (M value = 0.051). The other six genes were in between.

To select the optimal number of reference genes for credible normalization, pairwise variation (V_n/V_{n+1}) between two sequential normalization factors NF_n and NF_{n+1} was calculated by geNorm (Fig. 3B). The result showed all pairwise variation to be smaller than the recommended threshold (0.15). Therefore, one reference genes would be sufficient for proper gene expression normalization throughout the infecting period.

NormFinder, an Excel-based software tool, evaluates the most stable reference genes according to the stability value

Table 2. Ranking of candidate reference genes in order of their expression stability as calculated by Norm Finder

Rank	Gene	Stability value
1	<i>TUBβ</i>	0.004
2	<i>EF2-1</i>	0.009
2	<i>TUB2</i>	0.009
3	<i>G6PDH</i>	0.013
4	<i>ACT</i>	0.018
5	<i>EF2-2</i>	0.02
6	<i>GFKA</i>	0.021
7	<i>EF3</i>	0.027
8	<i>GAPDH</i>	0.032

Table 3. Statistics results by Best Keeper software for ten selected genes based on Ct values

Rank	Gene	Coeff. of correlation	P-value	Coeff. of variation
1	<i>TUBβ</i>	0.991	0.001	4.00
2	<i>TUB2</i>	0.984	0.001	4.45
3	<i>EF2-1</i>	0.983	0.001	3.47
4	<i>G6PDH</i>	0.98	0.001	4.45
5	<i>GAPDH</i>	0.961	0.001	5.51
6	<i>ACT</i>	0.95	0.001	3.98
7	<i>EF2-2</i>	0.943	0.001	4.72
g	<i>GK</i>	0.927	0.001	3.54
9	<i>EF3</i>	0.891	0.003	3.43

based on their intra- and inter-expression variation. Those with lower average stability values are regarded as more stable expression genes (Table 2). *TUBβ* was regarded as the most stable reference gene.

BestKeeper is used to rank the candidate genes based on their coefficient of correlation (r) relative to the BestKeeper index, by calculating the Ct set standard deviation (SD) and coefficient of variance (CV). The results (Table 3) revealed the best correlations were obtained for *TUBβ* (0.991) with the P value of 0.001, while the lowest correlations were obtained for *EF3* (0.891), with a P value of 0.003. Therefore, *TUBβ* was ranked as the most stable reference gene, which was in accordance with the results obtained from geNorm and NormFinder. In general, the results of Best Keeper analysis showed few differences from those obtained from geNorm and Normfinder.

Validation of the selected reference gene. To verify the reliability of the selected reference gene, we analyzed the expression pattern of one different expression transcript GGTG_09200 obtained from transcriptome sequencing. Transcript GGTG_09200 code *ExoPG* belongs to a group

Fig. 4. The expression quantification of *ExoPG* normalized by *TUBβ* and *GAPDH* as internal controls, respectively. Error bars represent standard error of the mean.

of plant cell wall degrading enzymes (PCWDEs), which can damage plant tissue, cell walls and cause leaks of cytoplasm (Kang et al., 2000; Valette-Collet et al., 2003).

The most appropriate reference gene (*TUBβ*) and the most inappropriate reference gene (*GAPDH*) were selected for qRT-PCR normalization of *ExoPG*, respectively. *ExoPG* is important in the infection process of plant pathogenic fungi, and it might be activated to degrade pectin and hemicellulose to obtain nutrients or to weaken the wall to allow hyphal growth in the root. The normalization of qRT-PCR using *TUBβ* generated similar expression patterns with a low expression in earlier stages of infection process and the increase seven days after infection (Fig. 4), which agrees with the results of transcriptome sequencing. When the least stable reference gene *GAPDH* was used for normalization, the *ExoPG* gene was expressed at high levels at the beginning of infection.

Discussion

At present, qRT-PCR has significantly improved the detection and quantification of expression profiles of genes of interest due to its high throughput, specificity, and accuracy (Bustin, 2002; Nolan and Bustin, 2006). However, it is important to select appropriate reference genes for gene expression normalization because of the relative expression of target genes can be influenced obviously by reference genes selected. Some commonly used reference genes have come under scrutiny or were doubted in recent studies for their variable expressions when tested with different experimental treatments (Ana et al., 2011; Fang et al., 2006; Hacquard et al., 2011; Huang et al., 2012; Teste et al., 2009; Yan and Liu, 2006).

Root and crown rot of wheat, also called “Take-all”, and caused by fungus *Ggt*, affects the quality and yield of wheat and other Gramineae crops. We might effectively improve the disease resistance of wheat through genetic improvement by understanding its intrinsic characteristic essentially of pathogenic (rewrite whole sentence). While up to now, the pathogenesis is unclear. Taking advantage of transcriptome data (Yang et al., 2015), a precious sequence resource for non-model fungus, and many pathogenicity-related genes will be identified with comprehensive functional analysis in the near future. In this study, we performed a selection and validation of internal reference genes for further accurate gene expression analysis using RT-qPCR in the infection process of *Ggt*.

Although some previous studies have successfully used one or two of the three excel-based program geNorm, NormFinder and BestKeeper to select reference genes (Ana et al., 2011; Huang et al., 2012), we suggest that taking into consideration the stability ranking of all three programs would provide a certain degree of confirmation of the results. Although there were differences in the rank of the candidate reference genes according to the three programs, all three analytical programs used in this study found *TUBβ* to be the most stably expressed reference gene in the infection process of *Ggt*. *ACT* is a commonly used housekeeper gene for RT-qPCR expression normalization in fungi, however, it was found to be a relatively unstable reference gene in this study. This finding is consistent with the results of Ana et al. (2011) and Huang et al. (2012). Moreover, *TUBβ* is regarded as the least stable reference gene during the infection process of *Hemileia vastatrix* isolate 1065 (Vieira et al., 2011). In contrast, *TUBβ* was regarded as the most stably expressed reference gene during the infection processes of *Ggt* in our study. And the results of Yan and Liu (2006) and Huang et al. (2012) all show *TUBβ* is the most stably expressed housekeeper gene in infection processes of *Phytophthora parasitica* and *Puccinia Striformis* f. sp. *tritici*, which might provide more accurate expression measurement of target genes.

In the infection progress of fungi, Phytopathogenic fungi expose plant cell walls to cell wall degrading enzymes (CWDEs), CWDEs like *ExoPGs* are needed for initial penetration and believed to be important pathogenicity factors (D'Ovidio et al., 2004; ten Have et al., 1998; Weste, 1970). To validate the reference gene candidates selected by the three programs, the transcript level of *ExoPG* gene was asserted during the infection process of *Ggt*. There was an obvious difference between the relative expression levels normalized by the most stable reference gene and the least one. And the relative expression level of *ExoPG* gene was

consistent with the results of RNA-seq (Yang et al., 2015). These results suggest that the incorrect use of reference genes may introduce bias into the analysis and lead to the misinterpretation of data.

In summary, nine candidate reference genes from *Ggt* evaluated in the infection process of *Ggt* using geNorm, NormFinder and BestKeeper programs, the results reveal TUB β was the most appropriate reference gene for RT-qPCR normalization across the infection process of *Ggt*. This study constitutes the first systematic exploration in *Ggt* to identify optimal reference genes for RT-qPCR normalization and useful to understand the functions of candidate target genes.

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References

- Andersen, C. L., Jensen, J. L. and Ørntoft, 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.
- Bohle, K., Jungebloud, A., Göcke, Y., Dalpiaz, A., Cordes, C., Horn, H. and Hempel, D. C. 2007. Selection of reference genes for normalisation of specific gene quantification data of *Aspergillus niger*. *J. Biotechnol.* 132:353-358.
- 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. 2010. Why the need for qPCR publication guidelines? - The case for MIQE. *Methods* 50:217-226.
- Bustin, S. A., Benes, V., Nolan, T. and Pfaffl, M. W. 2005. Quantitative real-time RT PCR-a perspective. *J. Mol. Endocrinol.* 34:597-601.
- D'Ovidio, R., Mattei, B., Roberti, S. and Bellincampi, D. 2004. Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant-pathogen interactions. *Biochim. Biophys. Acta.* 1696:237-244.
- Fang, W. and Bidochka, M. J. 2006. Expression of genes involved in germination, conidiogenesis and pathogenesis in *Metarhizium anisopliae* using quantitative real-time RT-PCR. *Mycol. Res.* 110:1165-1171.
- Gutierrez, L., Mauriat, M., Guénin, S., Pelloux, J., Lefebvre, J. F., Louvet, R., Rusterucci, C., Moritz, T., Guerineau, F., Bellini, C. and Van Wuytswinkel, O. 2008. The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant Biotechnol. J.* 6:609-618.
- Hacquard, S., Veneault-Fourrey, C., Delaruelle, C., Frey, P., Martin, F. and Duplessis, S. 2011. Validation of *Melampsora larici-populina* reference genes for in planta RT-quantitative PCR expression profiling during time-course infection of poplar leaves. *Physiol. Mol. Plant Pathol.* 75:106-112.
- Huang, X. L., Feng, H. and Kang, Z. S. 2012. Selection of reference genes for quantitative real-time PCR normalization in *Puccinia Striiformis* f.sp. *tritici*. *J. Agric. Biotechnol.* 20:181-187 (in Chinese).
- Kang, Z. S., Huang, L. L. and Buchenauer, H. 2000. Cytochemistry of cell wall component alteration in wheat roots infected by *Gaeumannomyces graminis* var. *tritici*. *J. Plant Dis. Protect.* 107:337-351.
- Kim, H. K. and Yun, S. H. 2011. Evaluation of potential reference genes for quantitative RT-PCR analysis in *Fusarium graminearum* under different culture conditions. *Plant Pathol. J.* 27:301-309.
- Nolan, T., Hands, R. E. and Bustin, S. A. 2006. Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* 1:1559-1582.
- 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.
- Quan, X., Xue, B. G., Yang, L. R. and Wu, C. 2014. Isolation and variety identification of *Gaeumannomyces graminis* causing wheat take-all in Henan Province. *Acta Phytopathol. Sinica* 44:139-146 (in Chinese).
- Radonić, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W. and Nitsche, A. 2004. Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* 313:856-862.
- Scholtz, J. J. and Visser, B. 2013. Reference gene selection for qPCR gene expression analysis of rust-infected wheat. *Physiol. Mol. Plant Pathol.* 81:22-25.
- Schmittgen, T. D. and Zakrajsek, B. A. 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J. Biochem. Biophys. Methods* 46:69-81.
- Teste, M. A., Duquenne, M., François, J. M. and Parrou, J. L. 2009. Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*. *BMC Mol. Biol.* 10:99.
- ten Have, A., Mulder, W., Visser, J. and van Kan, J. A. 1998. The endopolygalacturonase gene Bcpg1 is required for full virulence of *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* 11:1009-1016.
- Valette-Collet, O., Cimerman, A., Reignault, P., Levis, C. and Boccara, M. 2003. Disruption of *Botrytis cinerea* pectin

- methylesterase gene *Bcpme1* reduces virulence on several host plants. *Mol. Plant-Microbe Interact.* 16:360-367.
- 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:research0034.1-0034.11.
- Vieira, A., Talhinhos, P., Loureiro, A., Duplessis, S., Fernandez, D., Silva Mdo, C., Paulo, O. S. and Azinheira, H. G. 2011. Validation of RT-qPCR reference genes for in planta expression studies in *Hemileia vastatrix*, the causal agent of coffee leaf rust. *Fungal Biol.* 115:891-901.
- Weste, G. 1970. Extra-cellular enzyme production by various isolates of *Ophiobolus graminis* and *O.graminis* var. *avenae*. *J. Phytopathol.* 67:189-204.
- Yan, H. Z. and Liou, R. F. 2006. Selection of internal control genes for real-time quantitative RT-PCR assays in the oomycete plant pathogen *Phytophthora parasitica*. *Fungal Genet. Biol.* 43:430-438.
- Yang, L. R., Xie, L. H., Xue, B. G., Goodwin, P. H., Quan, X., Zhang, C. L., Liu, T. G., Lei, Z. S., Yang, X. J., Chao, Y. E. and Wu, C. 2015. Comparative transcriptome profiling of the early infection of wheat roots by *Gaeumannomyces graminis* var. *tritici*. *PLoS ONE* 10:e0120691.