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Validation of reference genes for real-time quantitative PCR normalisation in non-heading Chinese cabbage

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Abstract. Non-heading Chinese cabbage is an important vegetable crop that includes pak choi, caixin and several Japanese vegetables like mizuna, mibuna and komatsuna. Gene expression studies are frequently used to unravel the genetics of complex traits and in such studies the proper selection of reference genes for normalisation is crucial. We assessed the expression of 13 candidate reference genes including *ACTIN*, *ACTIN-1*, *ACTIN-2*, *GAPDH*, *Tub_α*, *CyP*, *EF1-α*, *18SrRNA*, *UBQ*, *UBC30*, *PPR*, *PP2A* and *MDH*. Their expression stabilities were analysed using two programs, geNorm and NormFinder, in 20 different samples that represent four strategic groups. Results showed that no single gene was uniformly expressed in all tested samples. *ACTIN* and *CyP* are proposed as good reference genes when studying developmental stages. *CyP*, *Tub_α* and *UBC30* are good reference genes when studying different tissues (from flowering to seed set). *CyP* and *Tub_α* are the most stable reference genes under biotic stress treatments using the fungi *Peronospora parasitica* and *Alternaria brassicicola*. *UBC30*, *EF1-α* and *ACTIN* are recommended for normalisation in abiotic stress studies, including hormone, salt, drought, cold and heath treatments. Moreover, at least five reference genes (*ACTIN*, *CyP*, *UBC30*, *EF1-α* and *UBQ*) are required for accurate qRT–PCR data normalisation when studying gene expression across all tested samples.

Additional keywords: *Brassica rapa* ssp. *chinensis*, gene expression, qRT-PCR, reference genes.

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Introduction

The quantification of mRNA (mRNA) transcript levels has become an important research tool in recent years. Changes in mRNA transcript levels are crucial during plant developmental processes, between different tissues and under changing environmental conditions. Real-time quantitative PCR (qRT–PCR) has become the most popular method to quantify mRNA transcription levels and to validate whole-genome microarray data because of its outstanding accuracy, broad dynamic range and high sensitivity not only in the fields of molecular medicine, biotechnology, microbiology and molecular diagnostics but also in plant research (Vandesompele *et al*. 2002; Jian *et al*. 2008; Paolacci *et al*. 2009). Estimating the expression levels of target genes of interest by qRT–PCR depends on endogenous control genes to normalise qRT–PCR; control genes are also called reference genes or housekeeping genes (HKGs) (Wierschke *et al*. 2010; Martínez-Beamonte *et al*. 2011). HKGs play a general role in basic cellular processes, such as cell structure maintenance and primary cellular metabolism and thus, their expression is usually unaffected by external factors. An 'ideal' reference gene for qRT–PCR has a constant and consistent expression level over all samples across different experimental conditions and different tissues. However, several reports demonstrated that there was no single gene with a constant expression level under all the experimental situations tested (Kim *et al*. 2003; Ding *et al*. 2004; Argyropoulos *et al*. 2006). Use of inappropriate reference genes in relative quantification of gene expression profiles may lead to erroneous normalisation and consequently, misinterpretation of the results. Therefore, it is essential to validate the expression stability of reference genes in each experimental system.

In plant research, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-ACTIN (*ACTIN*), tubulin α (*Tub_α*) and *18S rRNA* were considered to have a constant expression level and as a consequence have been widely used as reference genes for normalisation of qRT–PCR data in various experimental conditions (Kim *et al*. 2003; Ding *et al*. 2004; Jian *et al*. 2008; Løvdal and Lillo 2009). However, it has also been reported that the transcript levels of these genes can change significantly under different experimental conditions (Czechowski *et al*. 2005; Terrier and Glissant *et al*. 2005; Basa *et al*. 2009; Chen *et al*. 2010). Recently, many novel reference genes have been identified from Affymetrix GeneChip data and Microarray datasets in *Arabidopsis*. One of the findings was that among them F-box protein (F-box), SAND family protein and mitosis protein YLS8 were more stably expressed than the commonly used reference genes *ACTIN-2*, elongation-factor-1-α (*EF1-α*) and ubiquitin-conjugating

enzyme 10 (*UBC10*) (Remans *et al*. 2008). In a recent paper a *Brassica napus* L. microarray database was analysed, which showed that *EF1*-α and a new unknown protein 1 (UP1) were the most suitable reference genes among the given set of tissues (Chen *et al*. 2010). Furthermore, two commonly used reference genes *ACTIN-7* and *UBC21*, plus two new genes, TIP41-like protein (*TIP41*) and *PP2A* that were selected from a microarray dataset, were identified as the most stable reference gene set for normalisation during *B. napus* embryo maturation (Chen *et al*. 2010). A study in Chinese cabbage showed that *EF1*-α and adenine phosphoribosyl-transferase (*Apr*) were the most stably expressed genes among different tissues (root, stem, heading leaves and lateral sprout) (Qi *et al*. 2010).

The morphological variation present within *Brassica rapa* (L.) Hanelt is enormous. This includes the leaves in crops like heading Chinese cabbages and the leafy types that do not form heads (pak choi, caixin and several Japanese vegetables like mizuna, mibuna and komatsuna), the enlarged roots of turnips, the inflorescences and stems of broccoletto and the seeds of the oil types. When studying the genetic relationship among accessions using AFLP and SSR marker profiling, clusters or groups of accessions were identified that were represented by different crop types, but it was also clear that genetic distance was more defined by geographical origin than by crop type (Zhao *et al*. 2007, 2010). There is no information about selection of reference genes for normalisation of qRT–PCR results for gene expression studies in Chinese cabbage. With the recently released *B. rapa* genome sequence (The *Brassica rapa* Genome Sequencing Project Consortium 2011) and development of gene expression platforms for *B. rapa*, genome-wide large-scale gene expression studies will become available and will be mined to select reference genes for real time PCR studies.

In the present study, we selected and validated 13 reference genes particularly for accurate normalisation of qRT–PCR results in non-heading Chinese cabbage. These reference genes include seven widely used reference genes in plant research (*ACTIN*, *ACTIN-2*, *GAPDH*, *Tub_α*, *CyP*, *EF1*-α and *18S rRNA*) and six potential reference genes (*ACTIN-1*, *UBQ*, *UBC30*, *PPR*, *PP2A* and *MDH*) that were identified based on their stability in expression studies comparing plant developmental stages, different tissues or various environmental stimuli including biotic and abiotic stress (Brunner *et al*. 2004; Czechowski *et al*. 2005; Reid *et al*. 2006). The 13 genes were tested in 20 different samples, including three developmental stages, eight different tissues harvested at the mature plant developmental stage (between flowering and seed set) and from seedlings exposed to two biotic stresses and seven abiotic stress treatments, ranging from hormone-, salt-, drought-, till temperature stress treatments. We used the statistical algorithms geNorm (Vandesompele *et al*. 2002) and NormFinder (Andersen *et al*. 2004), which have been widely employed to select the best suitable reference genes from given biological samples (Silver *et al*. 2006; Wierschke *et al*. 2010).

Materials and methods

Seeds of one pakchoi inbred line (*Brassica rapa* ssp. *chinensis* (L.) Hanelt; Suzhou Qing, a non-heading Chinese cabbage) were germinated and grown under controlled conditions in pots in a climate room: 25°C day/20°C night temperature, 12 h light/12 h dark cycles. The plants were used to collect tissues under normal growth conditions and after biotic and abiotic stress treatments. All samples were snapped frozen in liquid nitrogen and kept at −80°C until use.

Developmental stages (Ds)

Three young leaves per plant were harvested and leaves of three plants were pooled for each developmental stage: (i) early stage (third leave present) (Ds1); (ii) before bolting (8 weeks after sowing) (Ds2); and (iii) after bolting (10 weeks after sowing) (Ds3).

Different tissues (Dt)

Eight different tissues including root (Dt1) and stem (Dt2) at the third leaf stage, leaves after bolting (Dt3, same sample as Ds3), flower buds (Dt4), petiole s(Dt5), stamens (Dt6), pistils (Dt7) and seed pods (Dt8), were collected from three plants and pooled.

Biotic stress treatments (Bs)

Two fungi, *Peronospora parasitica* (*P.p*) and *Alternaria brassicicola* (*A.b*), were isolated from the leaves of different susceptible *B. rapa* cultivars in the farm of Nanjing Agricultural University, China. Conidial suspensions were adjusted to 1×10^5 spores mL^{-1} and Tween-20 was added as a surfactant to a final concentration of 0.1%. For each treatment, 53-week-old seedlings were sprayed either with 50 mL pathogen suspension, or demi water (as control). Treated seedlings were placed in a climate chamber (25°C, 85% ± 5% RH, 12 hour light/12 hour dark) and the second leaves from three plants per treatment were harvested and pooled at 48 h after inoculation. The two treatments are referred to as Bs1 and Bs2 respectively.

Abiotic stress treatments (As)

Fifty seedlings (3 weeks old) were sprayed respectively with 50 mL solutions, water (as control), SA (2 mmol L^{-1}, PH = 6.5), ABA (50 µmol), NaCl (200 mM), H_2O_2 (100 µM) and Mannitol (400 mM). For salt (As1) and drought (As2) stress treatments, leaves were harvested at 12 h (for NaCl and Mannitol) after stress treatments. For hormone treatments, leaves were harvested at 6 h after SA treatment (As3), 24 h after ABA treatment (As4) and 24 h after H_2O_2 treatment (As5). In addition, leaves from 50 seedlings (3 weeks old) that were exposed to cold (4°C) and heat shock (40°C) were harvested 2 h after temperature stress treatments (As6 and As7).

RNA isolation, quality control and cDNA synthesis

Total RNA was isolated by RNA simple Total RNA Kit extractions (Bio Teke, Beijing, China). Genomic DNA contaminations were effectively removed using RNase-free DNase I treatment (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions, as melting curve gave single peak and genomic amplification was larger than RNA/cDNA amplification (see Fig. S1, available as Supplementary Material to this paper). RNA integrity was electrophoretically verified by agarose gel and by 260/280 nm absorption ratio 1.9~2.1

(see Fig. S2). The first-strand of cDNA was synthesised by reverse transcribing 1 μg of total RNA in a final reaction volume of 20 μL using the M-MLV reverse transcriptase (Takara, Dalian, China) according to the manufacturer's protocol and diluted 1 : 10 before use in qRT–PCR assays. The concentration and quality of each RNA and cDNA sample was also measured using the nucleic acid analytic apparatus K6000 (Bio Photometer, Eppendorf, Germany).

Primer design and qRT–PCR

Specific primer pairs were designed for 10 single genes (*GAPDH*, *Tub_α*, Cyclophilin (*CyP*), *EF1-α*, *18S rRNA*, poly ubiquitin enzyme (*UBQ*), *UBC30*, Pentatricopeptide repeat (*PPR*), *PP2A* and Malate dehydrogenase (*MDH*)) and three orthologous genes (*ACTIN*, *ACTIN*-1 and *ACTIN*-2) of the *ACTIN* gene family. The sequences of the genes were retrieved from *Arabidopsis thaliana* (L.) Heynh. and blasted against EST (expressed sequence tag) libraries (*Brassica*) via nucleotide blast. The *Arabidopsis* consensus sequences were used for comparison with *B. rapa* EST sequences, if available, to reveal the exon-intron structure. When no *B. rapa* L. ssp. *chinensis* (L.) Hanelt EST sequence was available the gene structure was obtained by comparison of the *A. thaliana* sequence with *Brassica napus* L. and *Brassica oleracea* L. EST sequences assuming that the exon-exon boundaries are conserved between *A. thaliana* and *B. napus*/*B. rapa*/*B. oleracea* (see Fig. S3). Sequence comparisons were done by DNASTAR, Lasergene 9.1 (Lasergene, Madison, WI, USA) and primers were designed using Primer Express 2.0 software (PE Applied Biosystems, Foster, CA, USA) under default parameters. Primer sequences and exon-exon junctions of the reference genes are listed in Table 1. Primer specificity and DNA contamination were visualised by separating PCR products from cDNA and DNA on agarose gel (Fig. S1A) and when only a single band was observed, the band was purified to be template for preparing the standard curves (which takes into account primer efficiency) using qRT–PCR (Ramakers *et al*. 2003). The PCR reaction efficiencies (E) were calculated using the equation $E = 10^{(-1/\text{slope})}$ (Lekanne Deprez *et al*. 2002). This calculation method results in efficiencies ranging from 95 to 108%. The standard curve was generated using a dilution series of the Ds1 (leaf developmental stage 1) sample over at least five dilution points (Fig. S4). qRT–PCR was performed in triplicate on a 32-position carousel (Light Cycler) with the Light Cycler-RNA amplification kit SYBR Green I (Roche, Mannheim, Germany) and conducted in 25 μL reaction volumes containing 30ng μL^{-1} cDNA sample, along with an RNA template control in parallel for each gene. The thermal cycling consisted of 95°C for 2 min and 40 cycles of 95°C for 20s, 55°C for 20s and 72°C for 20s. After the PCR a melting curve was generated to check the specificity of the amplified fragment. Data analysis was performed with the Rotor-gene 6 ver. 6.1 software (Applied Biosystems). All the cycle threshold (Ct) values from one gene were determined at the same threshold fluorescence value of 0.2. The single-peak melting curves obtained using the 13 primer pairs to amplify the candidate reference genes are displayed in Fig. S1B.

Data analyses

The 20 samples were divided into four strategic groups (developmental stages, different tissues, biotic and abiotic stress treatments) and were also treated as one group for analysis of candidate reference gene stabilities. Data or mean Ct values obtained from qRT–PCR were transformed to quantities with PCR efficiency derived straight from amplification plots using LinReg (ver. 7.0; Amsterdam, The Netherlands) software (Ramakers *et al*. 2003). The normalised data were imported and analysed by two stability analysis programs for reference genes, geNorm ver. 3.4 (Vandesompele *et al*. 2002) and NormFinder (Andersen *et al*. 2004) for ranking the reference genes.

geNorm determines the gene stability measure (M) value for each gene, based on the average pair-wise variation for a particular gene with all the other tested genes. Thus, genes can be ranked according to their expression stability through the stepwise exclusion of the least stable gene. The genes with an M value were arbitrarily suggested to be lower than 1.5; genes with the lowest M values have the most stable expression. A pair-wise stability measure aims at determining the benefit of adding extra reference genes for the normalisation process. For this, an arbitrary cut off value of 0.15 for pair-wise variation (Vn/Vn + 1) of normalisation factor (NF) (NFn and NFn + 1) is calculated, reflecting the effect of including additional (n + 1) genes.

The second different statistical algorithm software, NormFinder, generates a stability value for each gene, which is a direct measure for its estimated expression variation. It ranks the stability level of each candidate gene and highlights the most stably expressed gene with the lowest stability value by using a model-based variance estimation approach.

Results

Expression profiling of the candidate genes

Melting curve analysis of the amplification products confirmed that the primers amplified a single PCR product (Fig. S3). The standard curves for each of the candidate reference genes were found to have $R^2 \geq 0.995$ (Table 1), indicating a strong linear relationship between the detected Ct values and the corresponding relative amount of cDNA in all the PCR reactions. Based on the slopes of the standard curves, the 13 gene assays were found to have PCR efficiencies ≥90.7% (Table 1). It was apparent that each candidate reference gene had variable Ct values in the wide variety of samples tested, as the Ct values ranged widely from 14.56 (*18S rRNA*) to 39.53 (*PP2A*) across all samples (Table 2). None of the 13 candidate reference genes had a uniform expression over all samples tested. As a consequence, for accurate normalisation of gene expression in different experimental conditions, specific sets of reference genes are needed.

geNorm analysis

The geNorm program conducts sequential elimination of the least stable gene in any given experimental group, resulting in the exclusion of all but the two most stable genes in each strategic group (Table 3). For all 20 samples tested, *ACTIN* and *CyP* were the most stable genes, followed by *UBC30*, *EF1-α* and *UBQ*. In contrast, *PP2A* was the least stable gene tested. The two most stably expressed genes when comparing samples of different developmental stages were *ACTIN* and *CyP* that were also most stable when all 20 samples were analysed together, followed by

Table 1. Primers and PCR efficiency for the 13 selected candidate reference genes

Symbol[A]	*Arabidopsis* orthologue locus	Accession number	Primer sequence 5′–3′ (forward and reverse)	Tm (°C)	Junction	BLASTn[B]	Amplicon size (bp)	R^2	Slope	PCR efficiency (%)
ACTIN	AT5G09810	AF111812	GGAGCTGAGAGATTCCGTTG	60	Exon4	0.0	158	0.999	–3.410	96.50
			GAACCACCACTGAGGACGAT	60						
ACTIN-1	AT2G37620	AF044573.1	CCAACAGAGAGAAGATGACCC	59	Exon3	e-149	95	0.998	–3.150	107.70
			ACTGGCGTAAAGGGAGAGG	59						
ACTIN-2	AT3G18780	BG732274.1	ATCGAGCATGGTGTTGTGAG	60	Exon2	e-140	132	0.997	–3.219	104.00
			GGCCTTTGGGTTAAGAGGAG	60						
GAPDH	AT1G12900	AB331373	TCCACCATTGATTCTTCTCTG	58	Exon5	0.0	108	0.999	–3.293	101.20
			TCAGCCAAATCAACAACTCTC	58						
CyP	AT2G16600	M55018.1	AGGAGGAGATTTCACCGC	58	Exon1	0.0	232	0.999	–3.312	100.40
			TCTCTAACGACATCCATCCC	58						
EF1-α	AT5G60390	AF398148.1	TCTGGAAAAGAGATTGAGAAGG	59	Exon3	e-107	129	0.999	–3.465	94.40
			AACAGCGAAACGACCCAAT	61						
Tub_α	AT5G19780	AC189186.2	TTTGGGTTCTCTCTTGCTAG	59	Exon3 Exon 4	0.0	143	0.999	–3.267	102.30
			CGAGTAGAGAATGAGTTGAG	59						
18S rRNA	AT3G41768	AF513990.1	ATTGACGGAAGGGCACCAC	60	Exon1	0.0	158	0.999	–3.568	90.70
			TCGCTCCACCAACTAAGAAC	59						
UBC30	AT5G56150	U17250.1	TGAAAGAGCAGTGGAGCC	58	Exon4 Exon5	e-117	122	0.999	–3.480	93.80
			GGTCTGTCTTGTAGGTGTGAGC	59						
UBQ	AT2G36170	L21898.1	CAGCCAAGGTACGACCATCT	60	Exon4 Exon5	e-149	165	0.999	–3.380	97.60
			TATTCGTGAAGACGCTGACG	60						
PPR	AT1G62860	FJ455099.1	AAGAGGGTAGATGATGGAATG	56	Exon2	e-151	180	0.999	–3.333	99.50
			TTACAAGTGACGACATTAGGG	55						
PP2A	AT1G69960	AC240932.1	AGGCTACACGTTCGGACAAG	60	Exon5	2e-152	142	0.997	–3.431	95.60
			TGGGGCACTAAACACAGTCA	60						
MDH	AT1G62480	CB331882	CGAGATGACACCACCAAAGAC	61	Exon2	4e-10	157	0.995	–3.278	101.90
			GGTTTCATCTGCTTCTTCGG	60						

[A]All the selected reference genes were named according to the most similar orthologue locus from *A. thaliana*.

[B]E-value was obtained from the *A. thaliana* nucleotide sequence using BLASTn.

Table 2. Ct values (± s.d.) of the 20 analysed samples ordered according to their respective strategic groups

Strategic groups	Sample codes	*ACTIN* CT ± s.d.	*ACTIN-1* CT ± s.d.	*ACTIN-2* CT ± s.d.	*GAPDH* CT ± s.d.	*Cyp* CT ± s.d.	*EF1-α* CT ± s.d.	*Tub_a* CT ± s.d.	*18S rRNA* CT ± s.d.	*UBC30* CT ± s.d.	*UBQ* CT ± s.d.	*PPR* CT ± s.d.	*PP2A* CT ± s.d.	*MDH* CT ± s.d.
Developmental stages (Ds)	Ds1	20.17 ± 0.01	32.58 ± 0.25	28.09 ± 0.13	20.15 ± 0.09	19.1 ± 0.36	21.45 ± 0.14	27.12 ± 0.10	25.58 ± 0.16	26.77 ± 0.08	24.29 ± 0.24	28.64 ± 0.07	27.87 ± 0.13	22.64 ± 0.14
	Ds2	18.64 ± 0.17	29.41 ± 0.15	25.35 ± 0.24	18.32 ± 0.19	18.08 ± 0.01	20.00 ± 0.14	25.85 ± 1.37	14.56 ± 0.16	25.66 ± 0.39	22.53 ± 0.06	28.68 ± 0.22	25.74 ± 0.1	19.08 ± 0.05
	Ds3	19.64 ± 0.31	30.81 ± 0.05	25.91 ± 0.38	20.49 ± 0.20	18.81 ± 0.14	21.91 ± 0.1	30.13 ± 0.06	19.68 ± 0.80	25.68 ± 0.79	25.5 ± 0.13	31.37 ± 0.39	26.38 ± 0.37	18.65 ± 0.04
Different tissues (between flowering and seed set) (Dt)	Dt1	23.20 ± 0.19	33.08 ± 0.13	28.59 ± 0.02	31.88 ± 0.41	20.26 ± 0.40	23.25 ± 0.34	27.40 ± 0.57	20.55 ± 0.04	26.46 ± 0.5	24.49 ± 0.11	32.25 ± 0.12	39.53 ± 0.53	24.96 ± 0.13
	Dt2	19.27 ± 0.19	29.87 ± 0.74	24.96 ± 0.22	20.06 ± 0.01	18.79 ± 0.32	22.55 ± 0.42	26.52 ± 0.24	19.34 ± 0.23	24.38 ± 0.13	24.69 ± 0.26	30.37 ± 0.16	26.34 ± 0.48	16.14 ± 0.16
	Dt3	19.64 ± 0.31	30.81 ± 0.05	25.91 ± 0.38	20.49 ± 0.20	18.81 ± 0.14	21.91 ± 0.1	30.13 ± 0.06	19.68 ± 0.80	25.68 ± 0.79	25.5 ± 0.13	31.37 ± 0.39	26.38 ± 0.37	18.65 ± 0.04
	Dt4	17.53 ± 0.10	27.29 ± 0.40	23.08 ± 0.39	18.76 ± 0.34	16.52 ± 0.03	19.10 ± 0.00	24.10 ± 0.48	16.92 ± 0.09	22.65 ± 1.09	20.64 ± 0.07	30.00 ± 0.44	23.56 ± 0.44	14.85 ± 0.03
	Dt5	18.35 ± 0.12	26.57 ± 0.16	25.20 ± 0.25	19.83 ± 0.05	17.14 ± 0.26	21.53 ± 1.16	25.12 ± 0.77	18.31 ± 0.40	23.13 ± 0.25	23.91 ± 0.26	30.26 ± 0.01	26.1 ± 0.21	16.79 ± 0.19
	Dt6	18.99 ± 0.09	28.44 ± 0.22	22.50 ± 0.10	24.42 ± 0.23	18.09 ± 0.78	22.00 ± 0.12	24.06 ± 0.41	19.47 ± 0.10	22.16 ± 0.42	24.28 ± 0.24	30.92 ± 0.39	25.38 ± 0.49	16.56 ± 0.34
	Dt7	18.15 ± 0.18	29.68 ± 0.13	23.54 ± 0.17	20.47 ± 0.03	17.97 ± 0.22	18.80 ± 0.07	24.06 ± 0.56	17.94 ± 0.07	22.88 ± 0.11	20.94 ± 0.22	28.83 ± 0.08	24.28 ± 0.34	17.74 ± 0.23
	Dt8	18.08 ± 0.08	28.05 ± 0.20	24.36 ± 0.02	21.26 ± 0.09	17.78 ± 0.23	19.62 ± 0.25	25.15 ± 0.28	18.44 ± 0.39	23.8 ± 0.84	21.59 ± 0.38	31.08 ± 0.26	26.18 ± 0.60	14.79 ± 0.22
Biotic stress (Bs)	Bs1	23.91 ± 0.27	32.59 ± 0.01	29.25 ± 0.79	23.73 ± 0.29	21.45 ± 0.28	23.34 ± 0.22	27.27 ± 0.26	20.92 ± 0.56	27.58 ± 0.41	24.54 ± 0.47	30.13 ± 0.01	37.19 ± 0.06	27.2 ± 0.19
	Bs2	21.65 ± 0.55	32.79 ± 0.43	26.74 ± 0.47	23.22 ± 0.09	19.88 ± 0.06	22.58 ± 0.55	25.51 ± 0.77	18.43 ± 0.14	25.15 ± 0.57	23.17 ± 0.09	28.76 ± 0.30	34.47 ± 0.11	22.9 ± 0.16
Abiotic stress (As)	As1	21.63 ± 0.31	28.13 ± 0.04	26.13 ± 0.27	21.40 ± 0.31	18.42 ± 0.52	21.34 ± 0.31	25.04 ± 0.02	19.75 ± 0.11	24.43 ± 0.34	22.49 ± 0.73	32.71 ± 0.48	34.21 ± 1.75	23.57 ± 0.34
	As2	20.85 ± 0.31	28.69 ± 0.36	26.11 ± 0.16	25.18 ± 0.28	18.81 ± 0.44	21.17 ± 0.39	25.77 ± 0.84	19.92 ± 0.59	23.91 ± 0.68	23.21 ± 0.06	32.61 ± 0.08	34.38 ± 0.23	25.96 ± 0.49
	As3	23.33 ± 0.33	32.25 ± 0.31	29.72 ± 0.29	26.39 ± 0.29	21.44 ± 0.13	24.64 ± 0.13	25.43 ± 0.36	20.34 ± 0.19	27.67 ± 0.64	24.54 ± 0.34	29.39 ± 0.42	36.62 ± 0.03	24.69 ± 0.59
	As4	20.38 ± 0.16	28.11 ± 0.05	25.88 ± 0.73	23.18 ± 0.10	18.81 ± 0.29	22.65 ± 0.19	25.56 ± 0.31	20.11 ± 0.34	24.71 ± 0.33	23.62 ± 0.83	32.18 ± 0.34	34.38 ± 0.46	23.32 ± 0.65
	As5	20.49 ± 0.42	28.33 ± 0.02	26.59 ± 0.26	23.54 ± 0.22	20.53 ± 0.10	22.79 ± 0.22	26.07 ± 0.65	20.26 ± 0.32	25.05 ± 0.50	24.73 ± 0.70	32.22 ± 0.54	34.35 ± 1.29	22.56 ± 0.5
	As6	21.98 ± 0.43	31.34 ± 0.35	27.73 ± 0.28	22.84 ± 0.29	19.83 ± 0.32	22.12 ± 0.16	27.00 ± 0.26	20.73 ± 0.51	25.34 ± 0.15	22.51 ± 0.23	32.62 ± 0.37	37.75 ± 0.04	23.12 ± 0.22
	As7	20.00 ± 0.42	27.90 ± 0.31	25.23 ± 0.22	19.32 ± 0.39	17.68 ± 0.34	21.75 ± 0.28	23.95 ± 0.23	19.47 ± 0.38	24.81 ± 0.09	23.28 ± 0.84	30.45 ± 0.04	35.68 ± 0.25	24.85 ± 0.49

Table 3. Expression stability values of reference genes ranked by geNorm and NormFinder for the four strategic groups and all 20 samples together (ranking in parentheses)

Gene	geNorm					NormFinder				
	Total	Developmental stages	Different tissues	Biotic stress	Abiotic stress	Total	Developmental stages	Different tissues	Biotic stress	Abiotic stress
ACTIN	0.61 (1)	0.12 (1)	0.56 (4)	0.01 (1)	0.46 (2)	0.09 (1)	0.20 (3)	0.17 (1)	0.05 (5)	0.26 (3)
ACTIN-1	1.21 (8)	0.36 (5)	0.52 (3)	0.71 (12)	0.51 (3)	0.90 (9)	0.34 (7)	0.46 (3)	0.74 (13)	0.32 (5)
ACTIN-2	1.11 (7)	0.53 (7)	0.82 (7)	0.43 (8)	0.65 (7)	0.85 (8)	0.33 (6)	0.56 (6)	0.40 (7)	0.57 (10)
GAPDH	1.02 (6)	0.20 (2)	1.03 (9)	0.31 (7)	0.70 (8)	0.68 (5)	0.29 (5)	0.69 (9)	0.56 (10)	0.50 (8)
CyP	0.61 (1)	0.12 (1)	0.45 (2)	0.12 (5)	0.54 (4)	0.50 (4)	0.05 (1)	0.41 (2)	0.02 (3)	0.32 (6)
EF1-α	0.74 (3)	0.23 (3)	0.66 (5)	0.22 (6)	0.28 (1)	0.39 (2)	0.28 (4)	0.51 (4)	0.40 (8)	0.32 (4)
Tub_α	0.92 (5)	0.77 (9)	0.31 (1)	0.01 (1)	0.60 (6)	0.85 (7)	0.87 (11)	0.53 (5)	0.04 (4)	0.34 (7)
18S rRNA	1.43 (10)	1.54 (12)	1.22 (11)	0.58 (10)	0.57 (5)	1.17 (12)	3.44 (13)	1.03 (12)	0.62 (11)	0.18 (1)
UBC30	0.72 (2)	0.34 (4)	0.31 (1)	0.061 (2)	0.28 (1)	0.46 (3)	0.52 (8)	0.62 (8)	0.00 (1)	0.21 (2)
UBQ	0.86 (4)	0.66 (8)	0.91 (8)	0.07 (3)	0.74 (9)	0.79 (6)	0.59 (9)	0.81 (10)	0.00 (2)	0.54 (9)
PPR	1.31 (9)	0.84 (10)	0.77 (6)	0.08 (4)	0.95 (12)	1.05 (10)	0.97 (12)	0.60 (7)	0.11 (6)	0.91 (13)
PP2A	1.90 (12)	0.44 (6)	1.54 (12)	0.50 (9)	0.80 (10)	2.51 (13)	0.05 (2)	2.21 (13)	0.47 (9)	0.68 (11)
MDH	1.56 (11)	0.92 (11)	1.12 (10)	0.65 (11)	0.86 (11)	1.16 (11)	0.68 (10)	0.83 (11)	0.73 (12)	0.68 (12)

GAPDH, *EF1-α* and *UBC30*. Furthermore, the genes *ACTIN*, *CyP* and *GAPDH* with lowest M values displayed less variation (from 0.1 to 0.3) than M values of other genes. *UBC30* and *Tub_a* were the most stable genes when gene expression in different tissue samples was compared, followed by *CyP*. For the biotic stress treated samples, *CyP* and *Tub_a* showed lowest M values. The *UBC30* and *EF1-α* genes displayed highest stability under abiotic stress conditions, followed by *ACTIN*.

The geNorm software was also used to calculate the pair-wise variation (Vn/Vn + 1) for the determination of the optimal number of control genes between the sequential normalisation factors (NF) (NFn and NFn + 1) (Fig. 1). Data obtained from all 20 samples were analysed together and showed that the V value with the inclusion of a fifth gene (V5/6) was 0.146, which was lower than the arbitrary cut off value of 0.15. This means that the most suitable set of reference genes should contain at least five genes for accurate normalisation, i.e. *ACTIN*, *CyP*, *UBC30*, *EF1-α* and *UBQ*. When comparing developmental stages, *ACTIN* and *CyP* formed the optimal set of reference genes, whereas in different tissue samples, *UBC30*, *Tub_a* and *CyP* were considered as the most suitable set of reference genes. *CyP* and *Tub_a* were the most stable reference genes in the biotic stress samples. For the abiotic stress treated samples, the gene set *UBC30*, *EF1-α* and *ACTIN* was selected for most optimal normalisation. Generally, these results show that different experiments (here comparing developmental stages, tissues or stress treatments) require sets of different reference genes for normalisation.

NormFinder analysis

The NormFinder program was used as a different means for further validation of the data. There were clear differences in

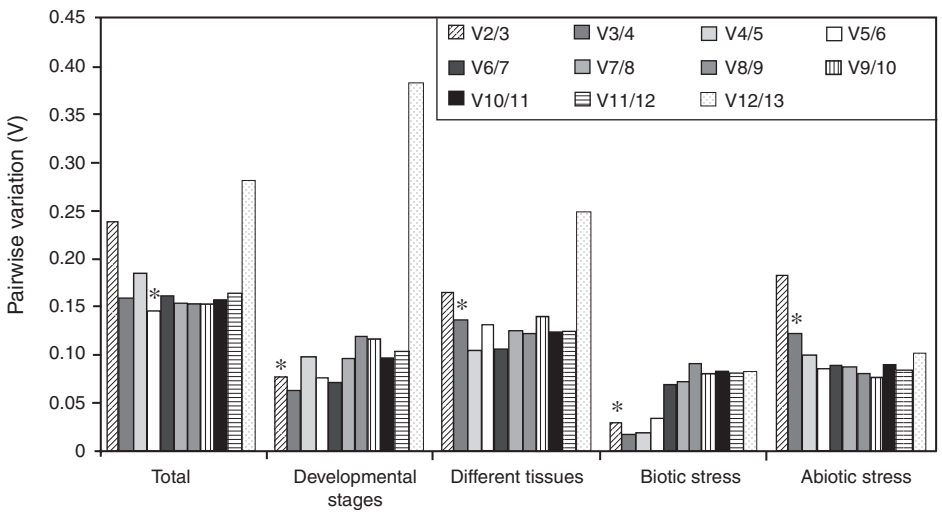

Fig. 1. Pairwise variation (V) measure of the candidate reference genes. When Vn/Vn + 1 <0.15, then the optimal number of reference genes is N. The optimal positions are indicated with an asterisk for the four strategic groups and all 20 samples together.

ranking the putative reference genes based on expression stability when comparing the two programs. However, both programs identified the same genes as the most and the least stable reference genes (Table 3). For example, *ACTIN* was ranked as best reference gene when all tested samples were considered, by both programs geNorm and NormFinder, whereas *Cyp* was evaluated as the most stable reference gene when comparing different developmental stages only. An exception was *18S rRNA* that ranked at the top in abiotic stress treatment comparisons when data were analysed using NormFinder, whereas it ranked sixth using geNorm.

Furthermore, to present expression levels of each of the reference genes, the average of expression levels of *18S rRNA* was used as reference to calculate the relative expression level (Fig. 2). The results showed that *ACTIN-1* was the gene with the lowest average expression level, whereas *Cyp* had the highest average expression level.

Discussion

Real-time quantitative PCR has become a widespread approach to analyse gene expression in plant species. However, no single gene has a constant expression level under all the tested experimental situations. Consequently, normalising the gene expression with one reference gene under different experimental conditions will lead to biased results. In order to obtain more reliable results from qRT–PCR experiments, it is crucial to select one or a set of suitable reference genes.

The present study analysed the gene expression of 13 candidate reference genes in a set of non-heading Chinese cabbage samples using geNorm and NormFinder. This comparison showed some discrepancies in the ranking of the candidate reference genes and in the identification of the best ones calculated by the two programs. However, there was substantial agreement if the grouping of the genes with the most and least stable expression was considered (Table 3). For example, *UBC30*, *Tub_α* and *CyP* were the most stably expressed genes in different tissues and after biotic stress treatments when calculated using geNorm software,whereas in NormFinder analysis, also *CyP* and *UBC30* were identified as the two most stable genes. Programs gave different output for *ACTIN*, which ranked fourth when comparing expression in different tissues according to the geNorm software, although it was the best reference gene according to the NormFinder software; however in abiotic stress, this gene ranked second and third when analysed with geNorm and NormFinder respectively. The essential difference between geNorm and NormFinder is that with the software geNorm expression stability for each gene is determined by pair-wise comparison with all other reference genes across all experimental conditions, whereas NormFinder calculates the expression stability of a gene per see, as a direct measure for its estimated expression variation, without considering other genes tested.

18S rRNA is a commonly accepted reference gene. However, many reports have demonstrated that the expression of this gene varies under different experimental conditions (Vandesompele *et al*. 2002; Jain *et al*. 2006; Paolacci *et al*. 2009). This may partly be explained by the fact that housekeeping genes are not only implicated in the basal cell metabolism but also participate in other cellular functions (Singh and Green 1993). In this study, for all strategic groups except for the abiotic stress group, *18S rRNA* ranked as the least stable gene. However, when comparing different abiotic stress samples, it ranked 5th using geNorm and first using NormFinder. We have no explanation for this observation, but will not suggest this gene.

In grape, the genes *GAPDH*, *ACTIN*, *EF1_α* and *SAND* were proposed as most relevant reference genes for normalisation of qRT–PCR during berry development (Reid *et al*. 2006). A combination of *UBQ5* and *EF1_α* was found as the most stable set of reference genes when comparing different developmental stages in rice (Jain *et al*. 2006). In poplar, a combination of *UBQ*, *Tub_α* and *UBC* was suggested as reference gene set when comparing gene expression in 10 different tissues (Brunner *et al*. 2004). We found that *ACTIN* and *CyP* were the optimal combination when comparing gene expression profiles from different developmental stages in non-

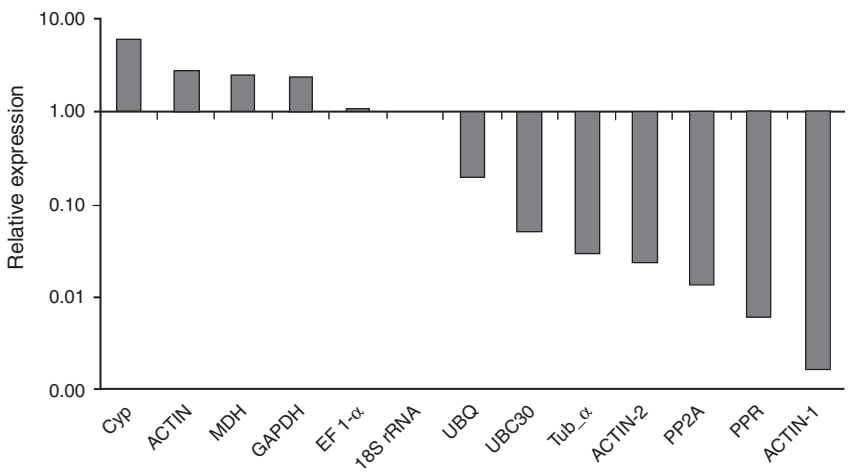

Fig. 2. The expression level of the reference genes relative to *18S rRNA* in 20 different samples. The mean Ct values of the 20 different samples were used for the relative expression analysis.

heading Chinese cabbage, whereas *ACTIN*, *CyP*, *UBC30*, *EF1*-α and *UBQ* were the most stable reference genes when comparing gene expression profiles of all 20 samples together.

Previously, most studies have recommended *ACTIN* expression as a reliable normalisation factor (Jian *et al*. 2008). However, in another study in *B. rapa* where expression in tissue samples of Chinese cabbage were compared, *ACTIN* was not most stably expressed (Qi *et al*. 2010). Jian *et al*. (2008) indicated that different paralogues from the same gene family can have varying expression levels in different developmental stages of soybean. Our results showed that *ACTIN* was more stably expressed than *ACTIN-1* and *ACTIN-2* in all four strategic groups tested and in all 20 samples analysed together. A previous observation in *Arabidopsis* and *Brachypodium distachyon* showed that *UBC* had a very stable expression pattern (Czechowski *et al*. 2005; Hong *et al*. 2008). Our study also showed that the *UBC30* exhibited very good expression stability when comparing its expression in 20 samples and in biotic and abiotic stress treated samples. The data presented in Czechowski *et al*. (2005), were screened for the 13 tested candidate reference genes of this study. This revealed that the transcript levels of the five genes *PP2A*, *UBQ*, *PPR*, *Tub*_α and *ACTIN*, were very stable in developmental series and additional *EF1*-α was identified as the best reference gene in abiotic stress comparisons (Czechowski *et al*. 2005). The *EF1*-α gene was also most stably expressed together with *UBC30* and *ACTIN* in our abiotic stress samples (Table 3). When we analysed only the subgroup of hormone treated samples from the abiotic treatment group, *GAPDH* was identified as the best ranking reference gene (results not shown) by both programs. Furthermore, when cold and heat shock samples were analysed, *GAPDH* ranked first by geNorm (data not shown). However, when salt and drought stressed samples were analysed, *GAPDH* ranked last by both programs (data not shown). This last finding is in sharp contrast with another study in heading Chinese cabbage, where *GAPDH* ranked as most stable reference gene during drought stress (Qi *et al*. 2010).

Recently, Hong *et al*. (2008) also showed that *EF1*-α was the most stably expressed gene when comparing Heat/Cold treated *Brachypodium distachyon*, which was confirmed in our study by its stable expression in the abiotic stress group when analysed by geNorm. Qi *et al*. (2010) reported that the gene *CyP* was not the best reference gene under drought and downy mildew stress in Chinese cabbage. However, *CyP* was identified as the most stable reference gene under two temperature treatments (20/25°C) in seagrass (Ransbotyn and Reusch 2006). In our study, *CyP* is a stable reference gene in pakchoi for most strategic groups, except for the abiotic stress group.

The present study indicates that none of the genes tested had a uniform expression profile in all 20 samples (tissues, developmental stages, stress responses), which stresses the fact that more reference genes need to be included when diverse sets of samples are compared, as suggested by the geNorm software. Thus, before studying gene expression by qRT–PCR, it is necessary to consider a suitable set of reference genes.

We also tested these genes in different *B. rapa* accessions that represent different morpho types. Both in yellow sarson (annual oil crop) and fodder turnip, the primers amplified PCR products with similar efficiency (data not shown) compared with the non-heading Chinese cabbage, analysed in this paper. In another project conducted in our laboratory, the expression of all the 13 reference genes was tested in seven different tissue types, including whole plant of seedling, seed, stem, leaf (young and old), turnip/root and flowers from two turnip genotypes (gene bank accessions CGN06678 and CGN07223), harvested at five different developmental stages. The results indicated that *ACTIN* and *EF1*-α were the most stable expressed genes in these tissues/developmental stages (data not shown). We also conducted a gene expression profiling experiment using a Nimble gene 300K array (http://www.ggbio.com/ggb/sub_contents.php?menu_id=2&sub_menu_id=0) using 3-week-old Chinese cabbage seedlings grown under short (8 h light) or long (16 h light) daylengths with RNA extracted from leaves at 9 and at 21 h after dawn (see Table S1, available as Supplementary Material to this paper). The stability of the 13 reference genes used in this study corresponded well with the results of the microarray experiment comparing growth under different daylengths with leaves sampled at different times of the day. *MDH* and *GAPDH* were least stably expressed with expression levels differing at least 2-fold, whereas *ACTIN* and *Cyp* were very stable expressed similar to the results of this study.

The results presented in this paper provide valuable information for future selection of reference genes in gene expression studies in *B. rapa* crops, particularly non-heading Chinese cabbage. We have identified distinct sets of genes appropriate for qRT–PCR in studies on plant developmental stages, different tissues, biotic and abiotic stresses. We recommend at least five reference genes (*ACTIN*, *CyP*, *UBC30*, *EF1*-α and *UBQ*) for accurate qRT–PCR data normalisation, when studying gene expression across diverse types of samples. The genes *ACTIN* and *CyP* are the best choices when studying expression in different developmental stages, whereas the genes *CyP*, *Tub*_α and *UBC30* are preferred when comparing gene expression in different tissues. When studying the effect of biotic stresses, *CyP* and *Tub*_α are recommended and *UBC30*, *EF1*-α and *ACTIN* are proposed as reference genes when studying abiotic stress. We conclude that different studies need different sets of reference genes.

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