Analysis of Reference Genes stability and Histidine Kinase Expression under Cold Stress in *Cordyceps militaris*

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Research article

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Abstract

Background: The development of fungal fruiting bodies from a hyphal thallus represents a transition from simple to complex multicellularity that is inducible under low temperature (cold stress). The molecular mechanism has been subject to surprisingly few studies. Analysis of gene expression level has become an important means to study gene function and its regulation mechanism. But identification of reference genes (RGs) stability under cold stress have not been reported in famous medicinal mushroom-forming fungi *Cordyceps militaris.*

Results: Herein, 12 candidate RGs had been systematically validated under cold stress in *C. militaris*. Three different algorithms, geNorm, NormFinder and BestKeeper were applied to evaluate the expression stability of the RGs. Our results showed that *UBC* and *UBQ* were the most stable RGs for cold treatments in short and long time, respectively. 2 RGs (*UBC* and *PP2A*) and 3 RGs (*UBQ*, *TUB* and *CYP*) were the suitable housekeeping genes for cold treatments in short and long time, respectively. Moreover, target genes, two-component-system histidine kinase genes, were selected to validate the most and least stable RGs under cold treatment, which indicated that use of unstable expressed genes as housekeeping genes leads to biased results.

Conclusions: Our results provide a good starting point for accurate reverse transcriptase quantitative polymerase chain reaction normalization by using *UBC* and *UBQ* in *C. militaris* under cold stress and better support for understanding the mechanism of response to cold stress and fruiting body formation in *C. militaris* and other mushroom-forming fungi in future research.

Background

Cordyceps militaris, a famous traditional Chinese medicine, has been used as a healthy food for a long time in China. The C. militaris fruiting body has anti-inflammatory [1], anti-tumor [2], anti-influenza virus [3] and radio-protection [4] functions. Methods for commercial production of fruiting bodies of this fungus have been established in artificial media [5, 6] or with insects, such as silkworm Bombyx mori pupae [7]. The molecular mechanism of mushroom fruiting body formation is a basic biological problem. The essential role of light in fruit body development in C. militaris was demonstrated in previous study [8]. A blue-light receptor gene white collar-1 (wc-1) inactivation results in thicker aerial hyphae, disordered fruit body development in C. militaris [9]. Further study showed that fruiting body primordia could form in the DASH-type cryptochromes gene mutant strain, but the fruiting bodies were unable to elongate normally, and cry-DASH and Cmwc-1 exhibit interdependent expression under light in C. militaris [10].

Compared to light, temperature is another pivotal factor influencing fruit body formation from a hyphal thallus which represents a transition from simple to complex multicellularity [11]. The fruiting body formation of various mushrooms depends on low temperature (cold stress) induction. For example, the fruiting body development was induced by shifting the cultivation condition (25 °C for mycelia) to lower temperature (15 °C) in Flammulina velutipes [12]. In Pleurotus ostreatus the mycelia were cultured at 20 °C for 30 days in a sawdust-medium, and then the temperature was lowered to 15 °C to induce fruit body development [13]. In C. militaris, after the mycelia were cultured under static conditions at 25 °C on potato dextrose broth medium for 10 days, the mycelia were injected into pupae and then the inoculated pupae were kept at 20 C to induce the fruiting bodies [7]. However, the molecular mechanism of mycelial development to fruiting body of mushroom under cold induction has been subject to surprisingly few studies, which will seriously restrict the further development of commercial production of mushrooms fruiting bodies.

Two-component signal transduction is commonly used as a stimulus–response coupling mechanism to allow organisms such as eubacteria, archea, and eukaryotes to sense and respond to changes in many different environmental conditions, especially temperature sensing [14]. They consist of a histidine kinase (HK) and a response regulator. The histidine kinase DesK from Bacillus subtilis is mechanistically the best understood. After a temperature downshift DesK gets auto-phosphorylated at the conserved histidine residue and donates the phosphate group to the conserved aspartate residue of DesR. The phosphorylated DesR activates the transcription of the des gene [15]. However, little is known the role of HK genes in cold stress response and fruiting body formation in C. militaris and other mushroom-forming fungi.

Real-time quantitative polymerase chain reaction (RT-qPCR) is an important tool towards the understanding of the complex signaling networks during organism is submitted to different stimuli due to its high sensitivity, specificity, and reliable quantification [16, 17]. Due to the introduction of errors in primer specificity, complementary DNA (cDNA) synthesis and PCR amplification, the results of RT-qPCR are often inaccurate [18]. Therefore, a stably expressed internal RG is needed for normalization during RT-qPCR analysis. However, validation of suitable RGs for expression analysis under cold stress have not been conducted in C. militaris and many other mushroom-forming fungi. In this study, we systematically identified 12 candidate RGs in C. militaris that were measured using RT-qPCR. Three different algorithms, geNorm, NormFinder and BestKeeper were applied to evaluate the expression stability of the 12 candidate RGs under cold treatment in C. militaris. Moreover, the relative expression levels of two-component-system HK genes were analyzed under cold stress conditions with three different normalization strategies. The RGs screened out in this paper could provide robustness to study the regulatory mechanism of cold induced fruit body formation from mycelia in C. militaris and other mushroom-forming fungi.

Results

Expression profiling of 12 candidate reference genes in C. militaris

12 genes (namely ACT, TUB, UBC, EF-1α, GAPDH, PP2A, UBQ, PGK, RPS, FBOX, CYP and GTPB) were selected as candidate RGs to determine the most stable RGs under cold treatments. We first calculated the PCR efficiency of 12 pairs of primers according to the previously reported method [19]. As shown in Table 1, RT-qPCR efficiency of the 12 genes ranged from 95.5% (CYP) to 106.2% (EF-1α) which fell within the acceptable range (80–120%) [20]. The cycle threshold (Ct) values of the 12 genes exhibited a high variation ranging and shown in Fig. 1. The Ct values ranged from 16.6 to 28.4 and 16.9 to 29.0 of short time (Fig. 1A) and long time (Fig. 1B), respectively. RPS and PP2A was the most and least transcribed, respectively, across all the tested samples (Fig. 1A and 1B). Three different algorithms (geNorm, NormFinder and BestKeeper) were used to analyze the expression stability of the 12 genes in the next section.

Table 1

Details on Primers Used for Quantitative Reverse Transcriptase Polymerase Chain Reaction Analysis

Gene	Annotation	Accession No.	Primer Sequences (5′-3′)	Amplification Size (bp)	Efficiency (%)
ACT	Actin cytoskeleton protein	XM_006669552.1	F: CAACAACTTCCTGACGGGC R: TCCTTGGGCTTCTGCTGAC	219	99.6
TUB	Tubulin	XM_006669203.1	F: ATGTCGTTCGTCGTGAGG R: AGAGTGGCGTTGTAGGGT	208	97.5
UBC	Ubiquitin-conjugating enzyme	XM_006672277.1	F: ACCATTGACACGAGCCAGTT R: GCCCATGTAAGCCTCCTCA	204	101.3
EF-1α	Elongation factor 1-alpha	XM_006665968.1	F: TATCGGAACTGTGCCTGT R: CGTTACCACGACGGATTT	200	106.2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	XM_006669697.1	F: CATCCACTCCTACACTGCTAC R: CTCAAGACGAACAGTCAGGT	220	102.3
PP2A	Serine/threonine protein phosphatase 2A	XM_006673033.1	F: CCTCCTACAGTCGTCATCAGC R: AGAAATGTCAAAGCGAGA	198	105.7
UBQ	Polyubiquitin	XM_006672469.1	F: TCAAAGAAGATAATGGTAACG R: GTATGGGTTCTCGGAAAGGT	209	102.5
PGK	Phosphoglycerate kinase	XM_006673408.1	F: GCTCAAGCCCGTCGTTTC R: CCCTCCTCCTCAATGTGG	159	98.9
RPS	Ribosomal protein S25	XM_006665399.1	F: AAGTGGTCTAAGGGCAAGG R: TTCTCCTCCAGGTCGGTAA	182	97.8
FBOX	F-box protein	XM_006672810.1	F: CCGATGACAACGACAGCGAC R: GTAGTTGACCGTGGAGATGT	224	101.3
CYP	cyclophilin	XM_006674778.1	F: TTTTCCGCCTTATTCCACC R: TCCAGAGCATCAAATCCCT	197	95.5
GTPB	GTP-binding protein	XM_006674648.1	F: TAAGAAGCCCAAGAAGAAAA R: GTCCCACAGGTTCAGCGT	181	103.4
F, forward; R, reverse					

geNorm Analysis

The geNorm algorithm calculates the value of measurement (M) to evaluate the stability of gene expression. The lowest M value and highest M value has the most stable and unstable expression, respectively. As determined by geNorm (Fig. 2), expression stability (M values) of the 12 genes both in short and long time were within the acceptable range (M < 1.5). The M value ranged from 0.91 (PGK) to 0.34 (UBC and PP2A) in short time (Fig. 2A) and

1.12 (PGK) to 0.25 (TUB and EF-1α) in long time (Fig. 2B). The genes were ranked from the highest M value (least stable) to the lowest M value (most stable): PGK, ACT, GAPDH, TUB, EF-1α, FBOX, RPS, UBQ, CYP, GTPB, UBC and PP2A in short time (Fig. 2A), PGK, UBC, ACT, FBOX, RPS, GTPB, PP2A, CYP, GAPDH, UBQ, TUB and EF-1α in long time (Fig. 2B).

NormFinder Analysis

Next, the expression stability of the 12 candidate RGs was analyzed using NormFinder to confirm the geNorm results. In short time, UBC and UBQ were the most stable; their stability values were 0.141 and 0.244, respectively (Table 2). In long time, CYP and UBQ were the most highly ranked; their stability values were 0.123 and 0.202, respectively (Table 3). Combining the results of geNorm and NormFinder analysis, UBC followed by PP2A, GTPB and UBQ were the most stable RGs in short time (Table 2 Mean Rank); UBQ followed by TUB, CYP and GAPDH were the most stable RGs in long time (Table 3 Mean Rank).

Table 2

Gene expression stability under short time cold stress ranked by geNorm, NormFinder and BestKeeper

Genes	geNorm		NormFinder		Mean Rank[a]	BestKeeper	
	M Value	Rank Order	Stability Value	Rank Order		CV	SD
TUB	0.254	1.5	0.308	4	2	4.934	0.711
EF-1α	0.254	1.5	0.360	6	5	6.359	1.930
UBQ	0.470	3	0.202	2	1	4.066	0.701
GAPDH	0.523	4	0.247	3	4	4.589	0.949
CYP	0.548	5	0.123	1	3	3.560	0.693
PP2A	0.571	6	0.404	7	7	5.381	1.442
GTPB	0.599	7	0.319	5	6	5.670	1.426
RPS	0.655	8	0.489	8	8	3.882	0.704
FBOX	0.701	9	0.543	9	9	2.487	0.644
ACT	0.764	10	0.641	10	10	2.935	0.743
UBC	0.830	11	0.730	11	11	8.127	1.792
PGK	1.121	12	1.733	12	12	7.325	1.786

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UBC	0.830	11	0.730	11	11	8.127	1.792
PGK	1.121	12	1.733	12	12	7.325	1.786

Table 3 Gene expression stability under long time cold stress ranked by geNorm, NormFinder and BestKeeper

Optimal number of reference gene for normalization across the experimental sets

Next, we determine the minimal number of genes for RT-qPCR normalization by estimated pairwise variation (Vn/Vn + 1) in geNorm. Below 0.15 which was a proposed cut-off Vn/Vn + 1 value, adding an additional RG is not required [16]. According to this principle, the Vn/Vn + 1 value was calculated. In short time, the V2/3 value was 0.124 (< 0.15), therefore, UBC together with PP2A would be sufficient for purpose (Fig. 3A and Table 2). In long time, compared with V2/3 (0.188), V3/4 value was 0.125 (< 0.15), suggesting that three RGs, UBQ, TUB and CYP were identified as available for normalization (Fig. 3B and Table 3).

BestKeeper Analysis

We further used BestKeeper to analyze the stability of 12 candidate RGs by calculating the SD (standard deviation) and the CV (coefficient of variation) of their Ct values. It has been reported that any studied gene with the SD lower than 1 can be considered stable, and the most stably expressed gene, exhibiting the lowest CV [21]. As shown in Table 2, in short time, the top two stable genes UBC and PP2A with lower CV values 4.501 and 3.596, respectively. And the SD values of UBC and PP2A were 0.882 (< 1) and 0.872 (< 1), respectively. In long time, the top three stable genes UBQ, TUB and CYP with lower CV values 4.066, 4.934 and 3.560, respectively. And the SD values of UBQ, TUB and CYP were 0.701 (< 1), 0711 (< 1) and 0.693 (< 1), respectively (Table 3). These BestKeeper results also suggested that UBC/PP2A and UBQ/TUB/CYP were stable in short and long time, respectively.

Taken together, our results suggesting that two RGs UBC/PP2A and three RGs UBQ/TUB/CYP were identified as available for RT-qPCR normalization in cold treatments for short and long time, respectively, in C. militaris.

Quantitative effects of best and least ranked reference genes on target gene expression

To validate the utility of stable RGs on gene expression analysis, three different strategies, the least stable RG, the best ranked RG and multiple stable RGs were selected to normalize the expression of target gene in short and long time, respectively (PGK, UBC and UBC/PP2A for short time; PGK, UBQ and UBQ/TUB/CYP for long time). HK genes in cold stress response were used as target genes. Base on the annotation with C. militaris genome database (National Center for Biotechnology Information accession GSE28001 and AEVU00000000), 9 genes (CmHK1-9, Table S1) were identified as HK homologous genes. Normalization of the 9 HK genes using the three different strategies showed that a significant increase in transcription of HK2/HK3/HK6 and HK1/HK3/ HK5 were observed under cold stress conditions for short and long time, respectively (Fig. 4). The gene expression levels of other HK genes were not shown. When using the best ranked UBC or the multiple stable UBC/PP2A as the housekeeping genes in short time, the upregulated trends for HK2, HK3 and HK6 genes were consistent and the peak points were observed at 1 h (Fig. 4A-C). When using the best ranked UBQ or the multiple stable UBQ/TUB/CYP as the housekeeping genes in long time, the upregulated trends for HK1, HK3 and HK5 genes were consistent and the peak points were observed at 4 d (Fig. 4D-F), respectively. However, using the least ranked PGK as the housekeeping gene in short time, the peak points were observed at 2, 0.5, 2 h of HK2, HK3, HK6, respectively (Fig. 4A-C). In addition, when PGK was applied as the housekeeping gene in our experiment, the transcription patterns of HK genes were higher than those using the best ranked RG and multiple stable RGs as housekeeping genes (Fig. 4A-F), which indicated that normalization using the least stable PGK as housekeeping gene resulted in an overestimated relative expression level of the target genes. Thus, our experimental results suggest that it is feasible to use any one or more of UBC/PP2A and UBQ/TUB/CYP genes as the normalization gene in cold stress for short and long time, respectively, with C. militaris.

Discussion

Compared with microarray and RNA sequencing, RT-qPCR technique has the advantages of high accuracy, high sensitivity, good repeatability and low cost for quantifying gene expression [16, 17]. To ensure the accuracy of RT-qPCR results, it is necessary to analyze the stability of internal RG under a specific experimental condition. A previous study in C. militaris showed that polymerase II large subunit (rpb1) gene was the best RG during all developmental stages examined, while the most common RGs, actin and tub, were not suitable internal controls [22]. But suitable RGs for expression analysis under cold stress have not been reported in C. militaris. In this study, the expression stability of 12 candidate RGs in C. militaris was systematically analyzed by geNorm, NormFinder and Bestkeeper under the cold treatment. Our results showed that UBC and UBQ were identified as the most available for RT-qPCR normalization in cold treatments for short and long time, respectively. While the ACT, a traditionally housekeeping gene, was not suitable cold stress in C. militaris.

A large number of studies have been conducted to identify the suitable RGs under cold treatment conditions among different species, but the most stable RGs are diverse (Table 4). In Dermatophagoides farina, geNorm, NormFinder and BestKeeper analysis showed that TUB was the most stable RGs under cold stress [23]; in Pineapple (Ananas comosus L.), PP2A and CYP were the stable RGs under cold stress [24]; and in Hulless barley (Hordeum vulgare L. var. nudum. hook. f.), PGK was not stable RG under cold stress [25], which were consistent with our results. However, in Hemarthria compressa and H. altissima leaf tissue, EF-1α was the most stable gene under cold stress [26]; and in Atropa belladonna, PGK was a reliable gene for normalizing gene expression under cold stress conditions [27], which were not consistent with our results. In entomopathogenic fungus Beauveria bassiana, both ACT and CYP were the most stably expressed gene sets under a variety of stress conditions (including cold) [28]. However, in our study, ACT was not stable in our experimental conditions. In another mushroom-forming Ascomycota, True morels (Morchella spp.), two candidate internal control genes ACT and GAPDH were not reliable gene for normalizing gene expression under cold stress conditions [29], which was similar to our findings. An interesting result is that, in Arabidopsis

pumila, both UBC and GAPDH under cold stress were the most stable RGs [30] (Table 4). But, in our result, UBC and GAPDH were stable and not stable RGs in short time, respectively. The similar results were also found in Siberian Wild Rye (Elymus sibiricus) that PP2A and ACT presented the highest degree of expression stability for cold stress [31]. But, in our result, PP2A and ACT was stable and not stable RG in short time, respectively. The more interesting result is that, in our study, UBC was the stable RG in short time but not the stable RG in long time. All of these results indicate that the expression stability of the same gene differs in different species. Moreover, the expression stability of the same gene is also not the same under the same treatment at different times.

Table 4: **The screening results of candidate reference genes of different species under cold stress**

Gene	Species	Expression Stability (Stable/Unstable)	Ref.	Expression Stability In This Study
TUB	*Dermatophagoides farina*	Stable	Niu et al., 2019	Stable (Long time)
PP2A	*Ananas comosus*	Stable	Chen et al., 2019	Stable (Short time)
	Elymus sibiricus	Stable	Zhang et al., 2019	
CYP	*Ananas comosus*	Stable	Chen et al., 2019	Stable (Long time)
	Beauveria bassiana	Stable	Zhou et al. 2012	
PGK	*Hordeum vulgare*	Unstable	Cai et al., 2018	Unstable (Short and Long time)
	Atropa belladonna	Stable	Li et al., 2014	
EF-1α	*Hemarthria compressa and H. altissima*	Stable	Lin, et al., 2019	Unstable (Short time)
ACT	*Morchella* spp.	Unstable	Zhang et al. 2018	Unstabl (Short and Long time)
	Beauveria bassiana	Stable	Zhou et al. 2012	
	Elymus sibiricus	Stable	Zhang et al., 2019	
GAPDH	*Morchella* spp	Unstable	Zhang et al. 2018	Unstable (Short and Long time)
	Arabidopsis pumila	Stable	Jin et al., 2019	
UBC	*Arabidopsis pumila*	Stable	Jin et al., 2019	Stable (Short time) Unstable (Long time)

In Arabidopsis thaliana, Arabidopsis histidine kinase 2 (AHK2), AHK3, and cold-inducible type A Arabidopsis response regulators (ARRs) play roles in cold signaling [32]. In addition, stress-sensing in fungi also depends on a signaling cascade comprised of a two-component phosphorylation relay plus a subsequent MAP kinase cascade to trigger gene expression [33]. Moreover, two-component pathways are important determinants of pathogenicity in animal pathogens, such as Candida albicans [34], Cryptococcus neoformans[35], and plant pathogens including Fusarium oxysporum (tomato) [36], Monilinia fructicola (brown rot of stone fruit) [37] and Botrytis cinerea (bean, tomato, and apple) [38]. In this study, the relative expression levels of two-component-system HK genes were analyzed under cold stress conditions with three different normalization strategies. The results showed that HK genes were significantly up-regulated after cold stress using stable RG(s) in normalization. However, an overestimated relative expression level in the HK genes transcription was obtained from RT-qPCR using PGK in normalization (Fig. 4). The relative expression level of target gene is overestimated due to use unstable gene normalization is also found in other studies [39, 40]. These results indicated that failure to statistically validate RG(s) will lead to inconsistent results. Moreover, our results suggested that the up-regulated HK genes participated in response to cold stress of C. militaris. However, whether or how these HK genes involved in cold-induced fruiting body formation in C. militaris is worthy of further study.

Conclusions

We systematic validation of 12 candidate RGs under cold stress for short and long time, respectively, in C. militaris. Our results showed that two RGs UBC/PP2A and three RGs UBQ/TUB/CYP were identified as available for RT-qPCR normalization in cold treatments for short and long time, respectively. In addition, our results indicate that failure to statistically validate RG(s) leads to inconsistent results. Moreover, the role of up-regulated HK genes in cold-induced fruiting body formation in C. militaris is worthy of further study. All in all, our results provide a good starting point for accurate RT-qPCR normalization in C. militaris under cold stress and better support for understanding the mechanism of response to cold stress and fruiting body formation in C. militaris and other mushroom-forming fungi in future research.

Methods

Strains and culture conditions

A laboratory and commercial strain of C. militaris (CGMCC 3.14242) from China General Microbiological Culture Collection Center was used. The C. militaris was cultured at 25 ℃ in dark with an artificial medium containing 20 g rice, 0.5 g powder of silkworm pupae and 25-mL nutrient solution (glucose 20 g, KH_2PO_4 2 g, $MgSO_4$ 1 g, ammonium citrate 1 g, peptone 5 g, vitamin B_1 20 mg, and 1,000-mL distilled water).

Cold Stress

After cultured for 25 days at 25 ℃ in dark, the C. militaris mycelia were transferred to 20 °C in dark for different treatments under cold stress for different time include short time (0-, 0.5-, 1-, 2-, 4- and 8-hours) and long time (0-, 1-, 2-, 4- and 8-d). After treatment, mycelia samples were immediately frozen in liquid nitrogen and stored at − 80 °C in a deep freezer until RNA isolation. Untreated mycelia samples were used as the control. Each treatment was repeated for 3 times.

RNA Isolation And cDNA Synthesis

RNA isolation and cDNA synthesis were performed as previously described method [41]. Briefly, 0.2 g mycelia were collected and subsequently were disrupted under liquid nitrogen conditions. The RNA Isolation Kit (TaKaRa, China) was used to extract total RNA. Add 2 μg total RNA of each sample into 20 μL reverse transcription reaction system to synthesize cDNA. The cDNA was ten-fold diluted series for determining the amplification efficiency; and ten-fold diluted for conducting the RT-qPCR analysis of cold treatments.

Real-time Quantitative Polymerase Chain Reaction Conditions

RT-qPCR experiments were performed using a previously described method [42]. Briefly, all genes were amplified by initial heating at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 68 °C for 15 s. At the final amplification cycle, the specificity of PCR reactions was checked through the use of melting curve analysis (60–95 °C in increments of 0.5 °C every 5 s). Negative controls were included to ensure the suitability of the assay conditions. Each experiment described above was repeated independently at least for 3 times.

Selection Of Candidate Reference Genes

In this study, 12 genes (namely ACT, TUB, UBC, EF-1α, GAPDH, PP2A, UBQ, PGK, RPS, FBOX, CYP and GTPB) were selected as candidate reference genes. Our gene panel contained (1) traditional housekeeping genes such as tubulin (TUB), actin (ACT), polyubiquitin (UBQ), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and translation elongation factor-1α (EF-1α); (2) genes chosen on the basis of stable expression in several RNA sequencing and quantitative PCR experiments, such as cyclophilin (CYP) [43] and ubiquitin-conjugating enzyme (UBC) [44]; and (3) novel stable reference genes in cold stress such as phosphoglycerate kinase (PGK) and serine/threonine protein phosphatase 2A (PP2A). Details of these genes are provided in Table 1. These genes were annotated with a C. militaris genome database (National Center for Biotechnology Information accession GSE28001 and AEVU00000000) [45] and used to design primers. The specific primers for two-component-system histidine kinase (HK) genes were listed in Table S1.

Data Analysis To Select The Internal Reference Gene

The expression stability of the candidate reference genes was analyzed using geNorm [16], NormFinder [46] and BestKeeper [21] as previously described.

Abbreviations

ACT: actin cytoskeleton protein; cDNA: complementary DNA; Ct: cycle threshold; CV: coefficient of variation; CYP: cyclophilin; EF-1α: elongation factor 1-alpha; FBOX: F-box protein; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GTPB: GTP-binding protein; HK: histidine kinase; M value: measurement value; PGK: phosphoglycerate kinase; PP2A: serine/threonine protein phosphatase 2A; RGs: reference genes; RPS: ribosomal protein S25; RT-qPCR: real-time quantitative polymerase chain reaction; SD: standard deviation; TUB: tubulin; UBC: ubiquitin-conjugating enzyme; UBQ: polyubiquitin; Vn/Vn+1: pairwise variation

Declarations

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Authors' contributions

Conceptualization, YL and BL; methodology, YL; software, BL; validation, YM and GL; writing—original draft preparation, YL; writing—review and editing, HY and LL; project administration, YL and GL. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

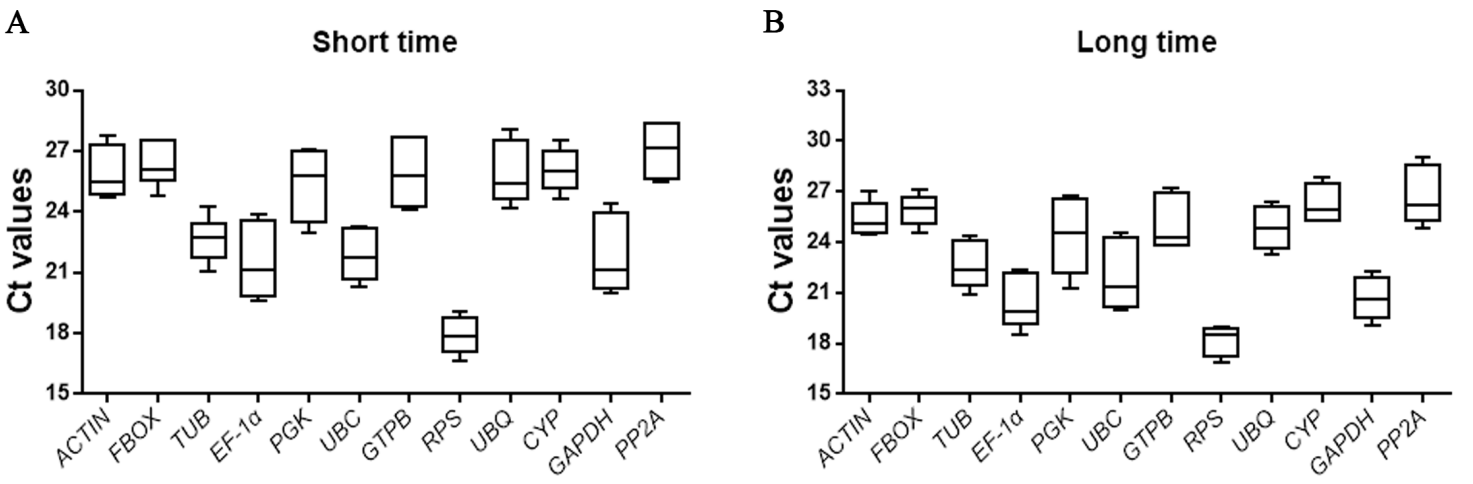

Figure 1

The range of cycle threshold (Ct) values of the 12 candidate reference genes in C. militaris under cold stress for short time (A) and long time (B).

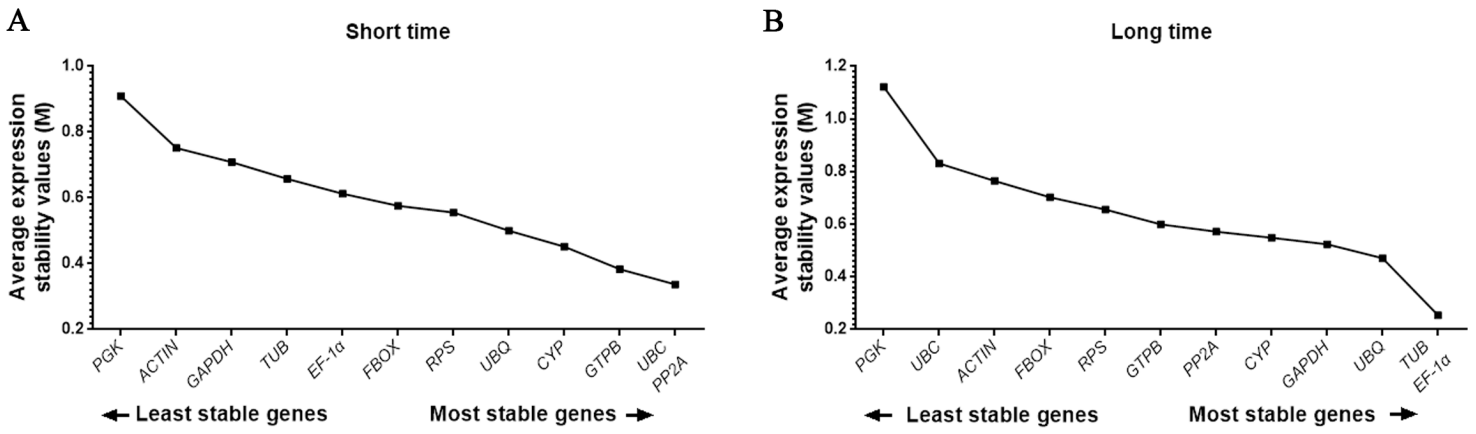

Figure 2

geNorm analysis of the average expression stability values (M) of the 12 candidate reference genes under cold stress for short time (A) and long time (B). A higher M value indicates more unstable expression.

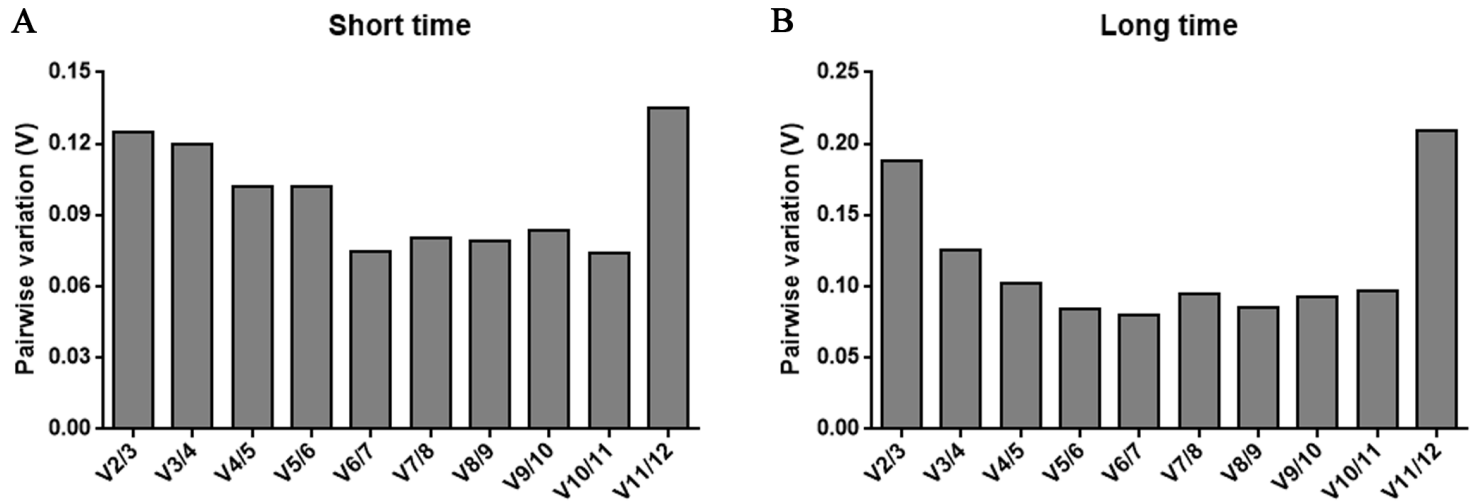

Figure 3

geNorm analysis of the pairwise variation (V) of the 12 candidate reference genes under cold stress for short time (A) and long time (B). The pairwise variation Vn/Vn+1 measured the effect of adding additional reference genes on the normalization factor for cold stress treatment in C. militaris.

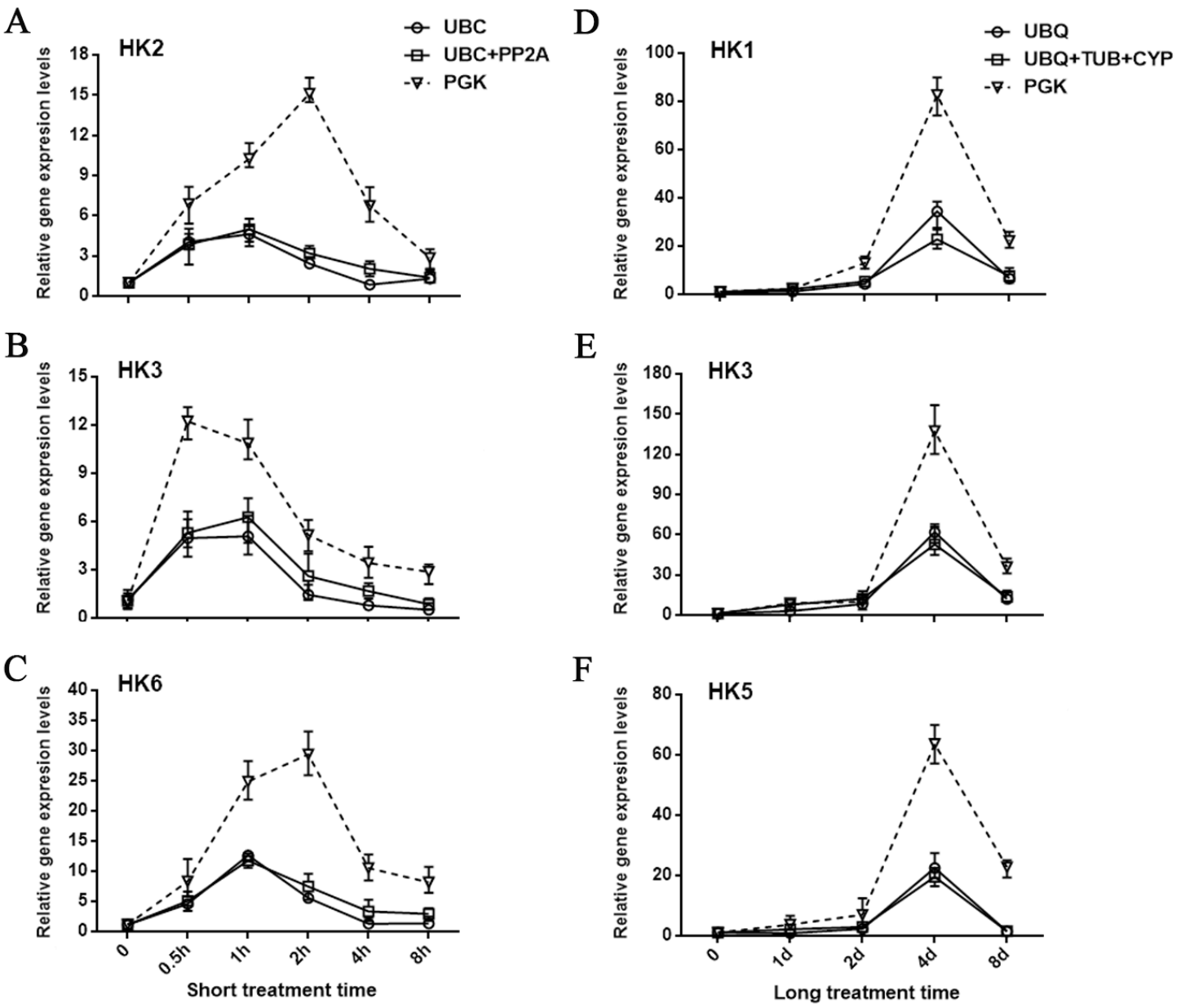

Figure 4

Relative expression levels of HK genes during cold stress conditions using the least stable gene, best ranked gene, or multiple stable reference genes for normalization. The least stable reference gene PGK, best ranked reference gene UBC, and multiple reference genes UBC/PP2A were used for normalization to analysis of HK2 (A) HK3 (B) and HK6 (C) expression levels in short time. The least stable reference gene PGK, best ranked reference gene UBQ, and multiple reference genes UBQ/TUB/CYP were used for normalization to analysis of HK1 (D) HK3 (E) and HK5 (F) expression levels in long time. The average CT value of multiple reference genes is used to analysis of HK gene expression levels in the strategy of multiple reference genes were used for normalization.

Supplementary Files

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- TableS1HKprimer.doc