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ORIGINAL PAPER

Identification and validation of new reference genes for accurate quantitative reverse transcriptase-PCR normalization in the Antarctic plant *Colobanthus quitensis* under abiotic stress conditions

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Abstract

The Antarctic ecotype of *Colobanthus quitensis* is a vascular plant highly adapted to the harsh environmental conditions of Maritime Antarctica which is now facing with the rapid local warming experienced in the Antarctic Peninsula during the last decades. Thus, the identification of the molecular mechanisms leading to the adaptation to this warming trend is a new target for modern cell physiology. The selection of suitable reference genes for quantification of key stress-responsive genes through quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) is important to ensure accurate and reliable results. In this study, we evaluated the expression stability of eleven candidate genes in *C. quitensis* under different abiotic stress conditions using *geNorm* and *RefFinder* tools. The statistical analysis showed that the appropriate reference genes varied depending on the experimental conditions, even if *EF1α* and *PP2Acs* ranked as the most stable reference genes when all stress conditions were considered. To further validate the stability of the selected reference genes, the expression patterns of *C. quitensis* catalase gene (*CqCAT*) was analyzed. The reference genes validated in this study will be useful for improving the accuracy of qRT-PCR analysis for gene expression studies of the Antarctic ecotype of *C. quitensis* and could be extended to other ecotypes adapted to low temperatures.

Keywords *Colobanthus quitensis* · Gene expression validation · qRT-PCR · Reference genes

Laura Bertini and Silvia Proietti have contributed equally to this work

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Introduction

The rise in average Earth's temperatures is one of the main consequences of the increase of atmospheric concentrations of greenhouse gases. The areas along the Antarctic Peninsula are warming faster than many other parts of the world (Turner et al. 2005; Mulvaney et al. 2012). This makes particularly interesting to investigate the genetic and molecular traits of the local plants to shed some light on the molecular basis of adaptation to low temperatures and their ability to cope with climatic changes. During evolution, the climatic conditions of Maritime Antarctic ecosystems allowed spreading and survival of only two highly adapted vascular plants species: the Antarctic hairgrass (*Deschampsia antarctica* Desv., Poaceae) and the Antarctic pearlwort (*Colobanthus quitensis* (Kunth) Bartl., Caryophillaceae) (Longton 1979; Smith 2003), which are also endemic of South and Central America. In particular, *C. quitensis* is present in the Andes of the central Chile as well as in Mexico, while *D. antarctica* occurs from Antarctica to South Central

Chile through the Andes Mountains, neighboring Argentina (Moore 1970). The identification of the mechanisms underlying their stress and cold adaptations is a challenge for modern cell biology and cell physiology. It has been reported that during evolution and following glaciation events the Antarctic ecotype of *C. quitensis* adapted both physiologically and morphologically to cold environment (Alberdi et al. 2002; Pérez-Torres et al. 2004; Bravo et al. 2007; Bascuñán-Godoy et al., 2012). For instance, it has been demonstrated that the photosynthetic machinery evolved to cope with low temperature-induced photoinhibition modifying chloroplasts in order to restore the energy balance (Krause 1994). Moreover, the global warming has also influenced the life of organisms in the Antarctic Peninsula. In particular, it has been proved that the size and number of populations of *C. quitensis* as well as germination ability increased, suggesting an improvement in its reproductive performances as result of longer and warmer growing seasons (Day et al. 1999; Vera et al. 2013; Sanhueza et al. 2017). Furthermore, it has been reported that higher temperature also influences in situ freezing resistance (Sierra-Almeida et al. 2018) and photosynthesis rates (Xiong et al. 2000; Sáez et al. 2018). Recently, ecophysiological traits of Antarctic vascular plants have been unveiled figuring out that response to climate change relies on many factors including metabolic reprogramming as well as morphological modifications (Cavieres et al. 2016). Interestingly, it has been also demonstrated that *C. quitensis* populations show anatomical and physiological adaptation to simulated global warming along a latitudinal gradient (Acuña-Rodríguez et al. 2017). Moreover, many studies have been also proving that the interaction with microorganisms may help *C. quitensis* colonize the unique Antarctic environment (Torres-Díaz et al. 2016; Nibert et al. 2018; Ballesteros et al. 2020). Despite the growing number of physiological studies addressing the ability of *C. quitensis* to adapt to the Antarctic environment and its progressive warming, little is known about the molecular characterization of genes involved in its adaptation to these climate conditions. Changes in photosynthesis and stress response genes were recently reported to be related to extreme environments adaptation (Cho et al. 2018), but more studies are needed to disclose the transcriptomic reprogramming induced by the rapid local warming experienced in the Antarctic Peninsula during the last decades.

Recently, we performed *de novo* assembly of *C. quitensis* transcriptome (Arthofer et al. 2015). With the aim to study the influence of temperature on *C. quitensis* transcriptomic reprogramming, small plexiglass greenhouses open on the top (Open Top Chambers, OTC) have been installed in December 2012 near the Arctowski base in King George Island, Maritime Antarctic (62°14′S; 58°48′W), in a field where *C. quitensis* grows and expands together with *D. antarctica*. Plants growing inside OTC experienced a mean temperature increase of around 4 °C at midday, mimicking regional warming for remote areas such as polar habitats (Bokhorst et al. 2013). It is worthwhile mentioning that the OTC may influence plant growth also affecting the light quality, air humidity and water content. After one year, *C. quitensis* individuals grown inside OTC (condition IN) and control plants grown in close proximity outside OTC (condition OUT) were harvested. RNA sequencing (RNA-seq) analysis of both samples resulted in a high number of genes differentially expressed among the two experimental conditions (Bertini et al. in preparation). This approach is of great sensitivity for gene expression profiling, representing indeed the gold standard for studying the influence of different experimental conditions on transcriptome remodeling. Nevertheless, RNA-seq, and high-throughput transcriptomic data in general, are often validated using a different approach and quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) is the most extensively used (Chuaqui et al. 2002; Tsukagoshi et al. 2015). Indeed, qRT-PCR has become extremely useful in many research fields contributing to understand signaling and metabolic pathways involved in several biological processes, including the molecular adaptation of different *C. quitensis* haplotypes coming from multiple colonization events of the Maritime Antarctic (Biersma et al. 2020). This method is rapid, sensitive and specific; moreover, it has good reliability to study gene expression, determining changes of the transcripts in different experimental conditions (Gachon et al. 2004; Wong and Medrano 2005; Nolan et al. 2006). Despite its powerful capacity, qRT-PCR presents some pitfalls such as the use of unreliable reference genes for the normalization steps (Huggett et al. 2005). Consequently, qRT-PCR could be underused due to the lack of characterized reference genes that enable robust normalization, especially in less known plant species.

Reference genes are usually housekeeping genes (HKGs) that are stably and constitutively expressed in all plant tissues regardless of growth, developmental stage or environmental conditions, therefore having an expression level unaffected by experimental conditions (Radonic et al. 2004; Huggett et al. 2005). Generally, HKGs encode for proteins involved in basic metabolism or in the cytoskeleton architecture, such as Elongation Factor 1-α (EF1-α), Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH), ubiquitin, α-actin or β-tubulin. Several papers reported the identification of HKGs that can be used for validation and gene expression analyses in different plant species (Czechowski et al. 2005; Paolacci et al. 2009; Dai et al. 2018; Ruduś and Kępczyński 2018; Santos et al. 2018; Zhang et al. 2018). However, there are no universal normalizing genes that can be used for all plant species because many of the genes examined showed a variable expression depending on the experimental conditions and the species tested (Thellin et al. 2009; Artico

et al. 2010; Kozera and Rapacz 2013). Thus, it is of utmost importance to evaluate the stability of reference genes under certain experimental conditions in order to select the most appropriate ones to be used for normalization of gene expression assessment. Therefore, validation studies are fundamental to select new reference genes that are constitutively expressed regardless of experimental condition prior to their use in qRT-PCR analysis.

Several algorithms are now freely available that allow the evaluation of the best candidate reference genes related to a given set of experimental conditions, such as: the comparative Δ *cycle threshold* (Δ*Ct*) method (Silver et al. 2006), *geNorm* (Vandesompele et al. 2002), *NormFinder* (Andersen et al. 2004), *BestKeeper* (Pfaffl et al. 2004) and *qBase* (Hellemans et al. 2007). In addition, *RefFinder* (Kim et al. 2010) that integrates *geNorm*, *NormFinder*, *BestKeeper* and the comparative Δ*Ct* method is very useful to calculate the rank of the selected candidate reference genes. It is important to underline that some of these algorithms have been developed using animal or human systems, so their use in plant science needs to be integrated with more specific analysis.

In this paper, we report the validation of eight specific reference genes suited for quantitative analysis of *C. quitensis* grown under different abiotic stresses (i.e. nitrogen supply, salinity and OTC). In particular, we selected Translation Elongation Factor 1-α and 1-β (*EF1α* and *EF1β*, respectively), Glucose 6-Phosphate Dehydrogenase (*G6PDH*), Cyclophilin (*CYP*), Phospholipase A2 (*PLPA2*), catalytic subunit of Protein Phosphatase 2A (*PP2Acs*), Phosphoglycerate Kinase (*PGK*) and F-box family protein (*FBX*). In addition, to test the reliability of the selected reference genes, the expression level of the gene coding for catalase from *C. quitensis* (*CqCAT*) was examined. Ultimately, our data provide a useful set of reference genes that can be used in qRT-PCR to analyze gene expression in *C. quitensis* under different experimental conditions in order to disclose how plants react and adapt to environmental stressors, including abiotic stress as well as selective pressure due to multiple colonization events.

Materials and methods

In vivo plant growth under control and warmer conditions

Field activity was carried out in the area close to Henryk Arctowski Antarctic Research station, King George Island, Maritime Antarctica (62° 14′S, 58° 48′W). Permits for entrance and plant collection in the Antarctic Specially Protected Area (ASPA) 128 were provided by The Chilean Antarctic Institute (INACH) and by the Italian National Agency for New Technologies, Energy and Sustainable Economic Development—Technical Antarctic Unit (ENEA-UTA). *Colobanthus quitensis* plants were grown in the field for 15 months inside OTC (December 2012–March 2014), which determined an increase of about 4 °C in the internal temperature during midday. Leaf samples from individuals grown either in warm conditions (inside OTC, samples IN) or in natural conditions (outside OTC, samples OUT) were harvested and pooled. Freshly collected leaves were soaked in RNAlater® solution (2:10, w/v) (Sigma-Aldrich, Saint Louis, Missouri, USA), and stored at -80 °C until RNA isolation.

Plant growth and treatments

Entire plants of *C. quitensis* with underground portion were extracted near to the Henryk Arctowski Polish Antarctic station in King George Island, and transported to Universidad de La Frontera, Temuco, Chile. Plants were propagated and maintained in a greenhouse, using plastic pot with soil/peat/vermiculite mixture (3:1:1) and fertilized with a Phostrogen® (Bayer-garden, UK). Before treatment application, plants were exposed to nutrient drainage for one month, suppressing addition of fertilization product and supplied only with distilled water. For the experiment, plants were separated into individuals and their roots were rinsed with distilled water. Homogeneous size plants (aerial and subterranean portions) were selected and transferred to plastic pots containing vermiculite as substrate. Ammonium fertilization assay was applied after establishment as described in Rabert et al. (2017). Briefly, plants were watered by saturating the substrate with a modified Hoagland nutrient solution at pH 5.6 every 72 h for the next five months. The following treatments were applied: 0, 8 and 32 mmol L^{-1} of N-NH_4^+ (samples N0, N1, N2, respectively). The treatment without ammonium application (0 mmol/L mmol L^{-1} of N) was prepared as followed: 2 mmol L^{-1} KH_2PO_4, 1 mmol L^{-1} K_2SO_4, 2.05 mmol L^{-1} KCl, 3 mmol L^{-1} $Ca(H_2PO_4)2H_2O$, 2 mmol L^{-1} $MgSO_47H_2O$, 1 mmol L^{-1} $CaSO_4$, 0.025 mmol L^{-1} H_3BO_3, 0.002 mmol L^{-1} $MnSO_4$, 0.002 mmol L^{-1} $ZnSO_4$, 0.05 µmol L^{-1} $CuSO_45H_2O$, 0.05 µmol L^{-1} H_2MoO_4, and 0.02 mmol L^{-1} Fe-EDTA. For ammonium treatments, the solution without N-NH_4^+ was used as basal solution and ammonium application was supplied as $(NH_4)_2H_2PO_4$ and $CO(NH_2)_2$ at required concentrations. At the end of treatments, plants were collected and stored at − 80 °C. Meanwhile, for salinity assay, after establishment, plants were irrigated for the next two months every 48 h at field capacity of substrate with a distilled water or modified Hoagland nutrient solution at pH 5.6 consisting in: 2 mmol L^{-1} $(NH_4)_2H_2PO_4$, 2 mmol L^{-1} $CO(NH_2)_2$, 2 mmol L^{-1} K_2HPO_4, 1 mmol L^{-1} K_2SO_4, 2.05 mmol L^{-1} KCl, 3 mmol L^{-1} $Ca(H_2PO_4)_2H_2O$, 2 mmol L^{-1} $MgSO_47H_2O$, 1 mmol L^{-1} $CaSO_4$, 0.025 mmol L^{-1} H_3BO_3, 0.002 mmol L^{-1} $MnSO_4$,

0.002 mmol L^{-1} $ZnSO_4$, 0.05 µmol L^{-1} $CuSO_4$ 5H2O, 0.05 µmol L^{-1} H_2MoO_4, and 0.02 mmol L^{-1} Fe-EDTA. The following treatments were applied: 0, 250, 500 and 750 mmol L^{-1} of NaCl (samples S0, S1, S2, S3, respectively). The application of salinity treatments was gradual. In the first month of treatment, every two days, the irrigation was alternated between distilled water and Hoagland solution plus NaCl at required concentrations. In the second month of treatment, the Hoagland solution plus NaCl was applied every two days. At the end of this period, plants were collected and stored at − 80 °C until use.

Total RNA isolation and complementary DNA synthesis

Total RNA was extracted using the Nucleospin® RNA Plant kit (Macherey–Nagel, Düren, Germany), starting from 100 mg of finely grinded leaf samples, according to the manufacturer's instructions. RNA concentration was estimated by reading spectrophotometric absorbance at 260 nm, whereas the OD_{260}/OD_{280} nm and OD_{260}/OD_{230} nm absorption ratios were calculated to evaluate RNA quality and purity (spectrophotometer UV-30 SCAN, ONDA). RNA integrity was also verified by agarose gel electrophoresis (Online Resource 1), whereas the absence of DNA contamination was tested using 100 ng of total RNA as template in a PCR reaction using *EF1α* specific primers for amplification.

Complementary DNA (cDNA) was synthesized using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) starting from 1 µg of RNA as template and using the oligo-dT primer for first strand synthesis.

Candidate reference genes selection and primers design

Sequence information of the candidate reference genes were obtained from the Shotgun Transcriptome Assembly of *C. quitensis* leaves (National Center of Biotechnology Information (NCBI) Sequence Read Archive (SRA), accession SRX814890). In particular, the following accession numbers are related to samples IN (SRR1720758, SRR1720760, SRR1720762) while the subsequent accession numbers are related to samples OUT (SRR1720763, SRR1720765, SRR1720767). Eleven candidate reference genes were selected for analysis of their expression profile, based on the stability of their RPKM (Reads Per Kilobase per Million mapped reads) values obtained from the transcriptomic analysis of both IN and OUT samples (Table 1 and Online Resource 2): Translation Elongation Factor 1-α and 1-β (*EF1α* and *EF1β*, respectively), Polypyrimidine Tract Binding protein (*PTB*), Glucose 6-Phosphate Dehydrogenase (*G6PDH*), Cyclophilin (*CYP*), Porphobilinogen Deaminase (*PBGD*), TATA-box Binding Protein (*TBP*), catalytic subunit of Protein Phosphatase 2A (*PP2Acs*), Phosphoglycerate Kinase (*PGK*), F-box family protein (*FBX*) and Phospholipase A2 (*PLPA2*). The raw data relative to the RPKM values of all the contigs obtained from the Shotgun Transcriptome Assembly of *C. quitensis* leaves are supplied in Online Resource 3. Blast hits can be accessed in Dryad: https://doi.org/10.5061/dryad.jp1mk. For primer pairs design, we searched for the contigs coding for each of the selected candidate genes inside the transcriptome data (Table 1) and we aligned them using the freely available ClustalW bioinformatics tool (https://embnet.vital-it.ch/software/ClustalW.html). Then, primers were designed inside the most conserved region using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/). The specificity of each selected primer pair was observed via standard PCR on synthesized cDNA using the BIOTAQ DNA polymerase (Bioline, London, UK), and each amplification product was verified by 1.5% agarose gel electrophoresis.

Quantitative reverse transcriptase-PCR analysis

Quantitative Reverse Transcriptase-PCR (qRT-PCR) reactions were performed in 96-well plates on a Bio-Rad CFX96 Real-Time PCR thermal cycler (Bio-Rad, Hercules, California, USA), using the SYBR Green detection system. The reaction mixture (10 µL) contained 1 µL of four-fold diluted cDNA, 5 µL Sso Advanced SYBR Green Supermix (Bio-Rad, Hercules, California, USA) and 0.5 µM of each gene specific primer, except for *FBX* (0.3 µM) and *PTB* (0.4 µM) primers. The cycling conditions were the following: initial denaturation step at 95 °C for 3 min, followed by 44 cycles at 95 °C for 10 s and primer annealing at different temperature (Table 2) for 30 s. Next, the melting curves ranging from 70 to 95 °C (with constant increase of 0.5 °C every 5 s) were evaluated in order to check the PCR specificity. Each assay included no-template controls (NTCs) for each primer pair. Primers efficiency (*E*) was calculated by generating standard curves for each oligonucleotide pair with at least five serial fourfold dilution points and the slope of the amplification curve was used to calculate $E = 10^{(-1/slope)}$. Output data were processed using the CFX Manager™ Software (Bio-Rad, Hercules, California, USA). All qRT-PCR reactions were run in three technical and biological replicates.

Data analysis for expression stability of candidate reference genes

geNorm and *RefFinder* algorithms were used to calculate and rank the stability of the candidate reference genes. The *geNorm* software (Vandesompele et al. 2002) determines the expression stability of control genes based on non-normalized expression levels. A stability parameter *M* is calculated as the average pairwise variation between all tested

Table 1 RPKM (Reads Per Kilobase per Million mapped reads) values of the contigs coding for the candidate reference genes reported as a mean of three biological replicates

Candidate gene	CONTIG (number)	RPKM samples IN	RPKM samples OUT	Log_2 RPKM samples IN /RPKM samples OUT
EF1α	862	70.74019167	100.8321064	− 0.511353054
	3341	198.5549167	286.807178	− 0.530543045
	4476	122.1658251	177.2007537	− 0.536543981
	5314	107.045916	142.9830141	− 0.417614014
	5089	74.96893677	96.96511546	− 0.371172869
	17,109	1.78327137	2.407327832	− 0.43290636
EF1β	4274	53.45635144	75.2910722	− 0.494117432
	5514	75.33954911	103.3499805	− 0.456058812
	13,868	61.40295338	79.98037179	− 0.381337939
PTB	62,834	3.715959488	4.357018251	− 0.229606383
	31,075	2.829066811	4.345267745	− 0.619118828
G6PDH	3151	2.321976923	3.875666939	− 0.739090962
CYP	20,377	2.655739018	4.067105126	− 0.614888903
	65,365	2.81899944	3.565244825	− 0.338817958
PBGD	37,630	6.114630421	9.299065018	− 0.604820364
	17,795	17.25396793	26.35603883	− 0.611205378
	16,934	11.34602226	19.75512624	− 0.800040466
TBP	14,864	8.799990585	9.034062862	− 0.037872972
PP2Acs	15,052	8.758543519	13.45463468	− 0.619340334
	46,109	5.63549563	8.088929746	− 0.521406333
	47,270	3.879037436	5.188586943	− 0.41964299
PGK	8304	62.48562769	89.8218579	− 0.52354217
	454	134.6894722	205.8503027	− 0.611958481
	136,667	0.244312261	0.221258268	0.142994902
FBX	13,951	2.231265905	3.448470082	− 04.628093998
	78,694	1.427962785	2.411099761	− 0.755732964
	61,416	2.563994055	3.849890321	− 0.586424429
PLPA2	59,935	12.944207	18.92022641	− 0.547622769
	17,683	2.058252333	3.45581448	− 0.747605909
	19,898	11.48461689	19.47157196	− 0.761666627

The values corresponding to each replicate are available in Online Resource 2

reference genes, with a recommended cut-off value of 1.5. The genes are ranked according to their *M* value and those with the lowest *M* value are the most stable ones. *RefFinder* (Kim et al. 2010) is a freely available software platform, which integrates four different algorithms, namely the above mentioned *geNorm, NormFinder* (Andersen et al. 2004), *BestKeeper* (Pfaffl et al. 2004) and the comparative ΔCt method (Silver et al. 2006), to analyze the stability of reference genes comprehensively. It uses the cycle threshold (*Ct*) value as input data and ranks the reference genes according to the four mentioned algorithms independently. Moreover, it assigns a weight to each reference gene and calculates the geometric mean (*Geomean*) of their weights to obtain the recommended comprehensive ranking order, identifying the best reference gene or pair of genes.

The *NormFinder* software (Andersen et al. 2004) uses a model-based approach to estimate expression variation. The software automatically calculates a stability value for all reference genes by organizing samples in groups. This approach considers as best genes those having the least intra- and inter-group variation in expression, so that the genes with the lowest value are the best normalizers.

The *BestKeeper* software (Pfaffl et al. 2004) evaluates the stability of reference genes calculating their standard deviation (*SD*) using *CP* (crossing point) or *Ct* values as input in different experimental conditions. The genes with a $SD\ [\pm CP]$ value below 1.0 are considered to be stably expressed.

The ΔCt method (Silver et al. 2006) chooses the best reference gene by comparing the variation of the cycle threshold values between different pairs of reference genes. If the ΔCt value between two genes remains constant when analyzed in several experimental samples, this means that both genes are stably expressed or that they are co-regulated.

Table 2 List of primers used in the study for reference/target genes and related information

Gene	Gene description	CONTIG (number)	GenBank accession number	Primer sequences (5′-3′) forward/reverse	Annealing temperature (°C)	Amplicon length (bp)	Efficiency (*E*) (%)	R^2
EF1α	Translation Elongation Factor 1-α	862 3341 4476 5314 5089 17,109	GCIB01000869.1 GCIB01003378.1 GCIB01004529.1 GCIB01005383.1 GCIB01005879.1 GCIB01017381.1	GGCCTAATC ACACCGGTT TC CTGGTTTTG AGGGTGACA ACA	58	205	102.1	0.990
EF1β	Translation Elongation Factor 1-β	4274 5514 13,868	GCIB01004324.1 GCIB01005581.1 GCIB01014076.1	CCATGTCAT CAACAGAAA CCA GATGACGAA ACCGACATG AA	57	143	108.30	0.991
G6PDH	Glucose 6-phosphate Dehydrogenase	3151	GCIB01003184.1	TTGACCCCT CCTCTTGTA TGG ACTAGCTCT CTTTGCAAT GGA	60	105	103.60	0.997
CYP	Cyclophilin	20,377 65,365	GCIB01020685.1 GCIB01066152.1	CTTGAAGCA GCACCAGTT GT CGGACGATG CTAAGCCAA AT	62	213	91.90	0.990
PP2Acs	catalytic subunit of Protein Phosphatase 2A	15,052 46,109 47,270	GCIB01015272.1 GCIB01046738.1 GCIB01047913.1	AGCTGGATC GAACTGAAG GA GCTACACAT TCGGGCAAG AT	61	227	101.3	0.992
PGK	Phosphoglycerate Kinase	454 8304 136,667	GCIB01000465.1 GCIB01008417.1 GCIB01137419.1	AGAGAGGAT CCGACTGCG TA TGTTGCTGG CTACTTGAT GC	59	216	103	0.994
FBX	F-box family protein	13,951 61,416 78,694	GCIB01014160.1 GCIB01062182.1 GCIB01079543.1	CTCCCCACT CAGCAAGGT TA TCACTC AATTTCCGG TCACA	61	166	95	0.998
PLPA2	Phospholipase A2	17,683 19,898 59,935	GCIB01017961.1 GCIB01020197.1 GCIB01060692.1	AAAACCCAG CCTTCCCAG AT GTCCTGGAG AGAAGCCTT GT	58	150	109.4	0.986
PTB	Polypyrimidine Tract-Binding protein	62,834 31,075	GCIB01063605.1 GCIB01031534.1	TGGCTCCAA CATTGCACT TC TCTAGTCAG CCACAGTTT CGT	57	148	95.80	0.995

Table 2 (continued)

Gene	Gene description	CONTIG (number)	GenBank accession number	Primer sequences (5′-3′) forward/reverse	Annealing temperature (°C)	Amplicon length (bp)	Efficiency (*E*) (%)	R^2
TBP	TATA-box Binding Protein	14,864	GCIB01015085.1	CGCCAGTAA CCACCATCT TG AGGTAGCGT CAGTGGCAT TA	59	206	90.2	0.995
PBGD	Porphobilinogen Deaminase	37,630 17,795 16,934	GCIB01038167.1 GCIB01018076.1 GCIB01017200.1	GAAAGCTCG TTCACATGC CA AGCTGAATG AAGGGGTTT GC	57	230	97	0.999
Target gene								
CqCAT	Catalase	11,039	GCIB01011193	GCAACTTTC ATCCCCAAA GA CGTGAGGCT GAGAGATTT CC	59	248	109	0.993

However, if the ΔCt oscillates, the introduction of a third, fourth or fifth gene in the comparison provides information on which pairs show a lower variability and thus which gene has a more stable expression among the tested samples.

Validation of reference genes

For the validation of selected reference genes for qRT-PCR analysis under different abiotic stress in *C. quitensis*, the relative transcript accumulation of catalase gene from *C. quitensis* (*CqCAT*) was normalized using the two most stable (*EF1α* and *PP2Acs*), and the least stable (*PLPA2*) reference genes identified in this study. Sequence information and specific primers for *CqCAT* were obtained as described above. Primers sequence and related information are shown in Table 2. Quantitative analysis for *CqCAT* gene was performed according to the $2^{-\Delta\Delta Ct}$ method by applying the efficiency correction formula (Pfaffl 2001).

Results

Selection of candidate reference genes and their characterization

In this study, eleven reference genes showing stable expressions in *C. quitensis* transcriptome data from both inside (IN) and outside (OUT) OTC samples were selected as candidate for normalization of gene expression analysis. The cut-off value for the stability of gene expression between the two conditions was fixed as $-1 < \text{Log}_2$ Fold Change IN/OUT < 1 (Table 1).

The selected genes could be grouped into three categories: (i) genes involved in transcription, i.e. *TBP* (TATA-box Binding Protein) and *PTB* (Polypyrimidine Tract-Binding protein); (ii) translation, i.e. *EF1α* and *EF1β* (Translation Elongation Factor 1-α and 1-β); (iii) cellular metabolism, i.e. *PBGD* (Porphobilinogen Deaminase), *G6PDH* (Glucose-6-Phosphate Dehydrogenase), *PLPA2* (Phospholipase A2) and *PGK* (Phosphoglycerate Kinase). In addition, three traditional housekeeping genes were also selected: *CYP* (Cyclophylin), *PP2Acs* (Protein Phosphatase 2A catalytic subunit) and *FBX* (F-box family protein). Gene names and description, accession ID, primer sequences, annealing temperatures and amplicon length as well as amplification efficiency and regression coefficient (*R-squared*) are listed in Table 2. Gene-specific amplification was confirmed by the presence of a single peak in the melting curves obtained by qRT-PCR (Online Resource 4). Moreover, no primer dimers were detected during the amplification reactions neither non-specific products were detected in negative controls. Efficiency values were accepted within a range of $100 \pm 10\%$, accompanied by a linear regression value as close to 1.

Expression profiles of the candidate reference genes

In order to detect the expression profiles of the eleven candidate reference genes of *C. quitensis* and to study their stability in qRT-PCR, we analyzed several experimental samples subjected to different abiotic stresses (i.e. nitrogen supply, salinity and OTC).

The cycle threshold (*Ct*) values were determined for each gene through all the experimental conditions and the mean *Ct* values along with the standard deviation (*SD*) were determined (Online Resource 5). The *Ct* values measured in each run allowed us to assess the transcript abundance of the candidate genes. The mean *Ct* value ranged from 19.3 to 25.96, which is considered a moderate to high level of expression. In particular, *EF1α* showed the highest expression level with a mean *Ct* value of 19.3 ± 2.62, whereas *G6PDH* showed the lowest transcript abundance with a mean *Ct* value of 25.96 ± 1.30. Moreover, *SD* of *Ct* values, which can reveal the expression stability of candidate reference genes, ranged from 1.30 to 2.98 for six genes (*G6PDH*, *FBX*, *PP2Acs*, *EF1α*, *CYP* and *EF1β*), indicating a good stability (Online Resource 5). On the other hand, *PGK* and *PLPA2* showed *SD* values of 3.25 and 4.24, respectively, indicating a lower level of stability. Furthermore, it is worthwhile mentioning that *PBGD*, *TBP* and *PTB* displayed a different behavior in relation to the stress conditions. As far as *PTB* is concerned, its expression level was undetectable in samples subjected to nitrogen supply ($Ct > 40$) at any concentration (N0 through N2), whereas *TBP* expression was undetectable during both nitrogen supply (N0 through N2) and salinity stress (S0 through S3); finally, *PBGD* expression was undetectable only at the highest concentration of NaCl (S2 and S3) (Online Resource 5). The different behavior of the last three genes indicates a different impact of the experimental conditions on their expression as already reported in similar studies in other plant species (Chen et al. 2011; Tillett et al. 2011). Since a good reference gene should be expressed at good level and with high stability independently from experimental conditions, we decided to continue our analysis only with eight genes, namely *CYP*, *EF1α*, *EF1β*, *G6PDH*, *PP2Acs*, *FBX*, *PLPA2* and *PGK*. The box plot graph of the *Ct* values for all the selected candidate reference genes is showed in Fig. 1.

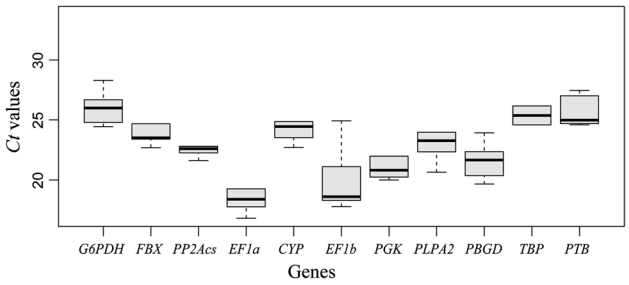


Fig. 1 Box-plot graph of the cycle threshold (*Ct*) values referred to the eleven candidate reference genes across the experimental samples ($n = 9$). The line across the box shows the median value, lower and upper boxes indicate the 25th percentile to the 75th percentile, the whiskers represent the 95% confidence intervals. *Ct* values for all the reference genes are given in Online Resource 5

Expression stability of the candidate reference genes

Since the eight candidate reference genes did not show a completely constant expression level across all samples, *geNorm* and *RefFinder* tools were used in order to evaluate and rank the stability of the selected genes for qRT-PCR studies.

The *geNorm* algorithm calculates the stability *M* value that is used to arrange the gene ranking order from the smallest to the largest value, i.e. from the highest to the lowest stability, with a suggested cut-off range of $M \leq 1.5$ (Vandesompele et al. 2002). During nitrogen supply stress, the lowest *M* values ($M < 0.15$) were attributed to *PGK*, *FBX*, *PP2Acs* and *CYP* even though all other genes displayed very low *M* values (< 0.6), indicating that they can be all considered reliable candidates for normalization studies under this stress condition (Fig. 2 and Online Resource 6).

During salinity stress, *EF1β*, *EF1α* and *PGK* were found to be the most stable genes ($M < 0.6$). However, *M* values < 1.5 were also found for *PP2Acs*, *CYP* and *FBX*, indicating that also these genes can be suitable for normalization studies under this stress condition. On the contrary, *G6PDH* and *PLPA2* with *M* values > 1.5 should be considered the least stable (Fig. 2 and Online Resource 6). As far as OTC stress is concerned, *PP2Acs*, *G6PDH* and *PGK* were the most stable genes showing *M* value ≤ 0.1. The other genes displayed an *M* value slightly higher but, in any case < 1.5, so they all can be considered reliable and stable reference genes under this stress condition (Fig. 2 and Online Resource 6). When all stress conditions were taken into account together, the best reference genes were *EF1β*, *PGK* and *EF1α* with *M* values < 0.9. In addition, *PP2Acs*, *CYP*, *FBX* and *G6PDH* displayed *M* value < 1.5, indicating that they are all suitable reference genes, whereas *PLPA2* with an *M* value slightly greater than 1.5 is borderline (Fig. 2 and Online Resource 6).

The *geNorm* program was also used to assess the optimal number of reference genes required for accurate normalization across the experimental conditions by calculating the pairwise variation (V_n/V_{n+1}) between two sequential normalization factors *NF* (NF_n and NF_{n+1}). According to this software, a cut-off value of 0.15 is the recommended threshold to determine whether additional reference genes are required for better normalization (Vandesompele et al. 2002). A little variation between V_n/V_{n+1} indicates that the number of reference genes identified is sufficient for normalization. The *V2/3* values in nitrogen supply and OTC stresses were lower than 0.15 (Fig. 3) suggesting that the top two reference genes were sufficient for accurate normalization. Nevertheless, when salinity and all stress conditions were considered, the lowest pairwise variation value was above 0.15. As recommended in this case, the lowest V_n/V_{n+1} value could be considered to determine the number of

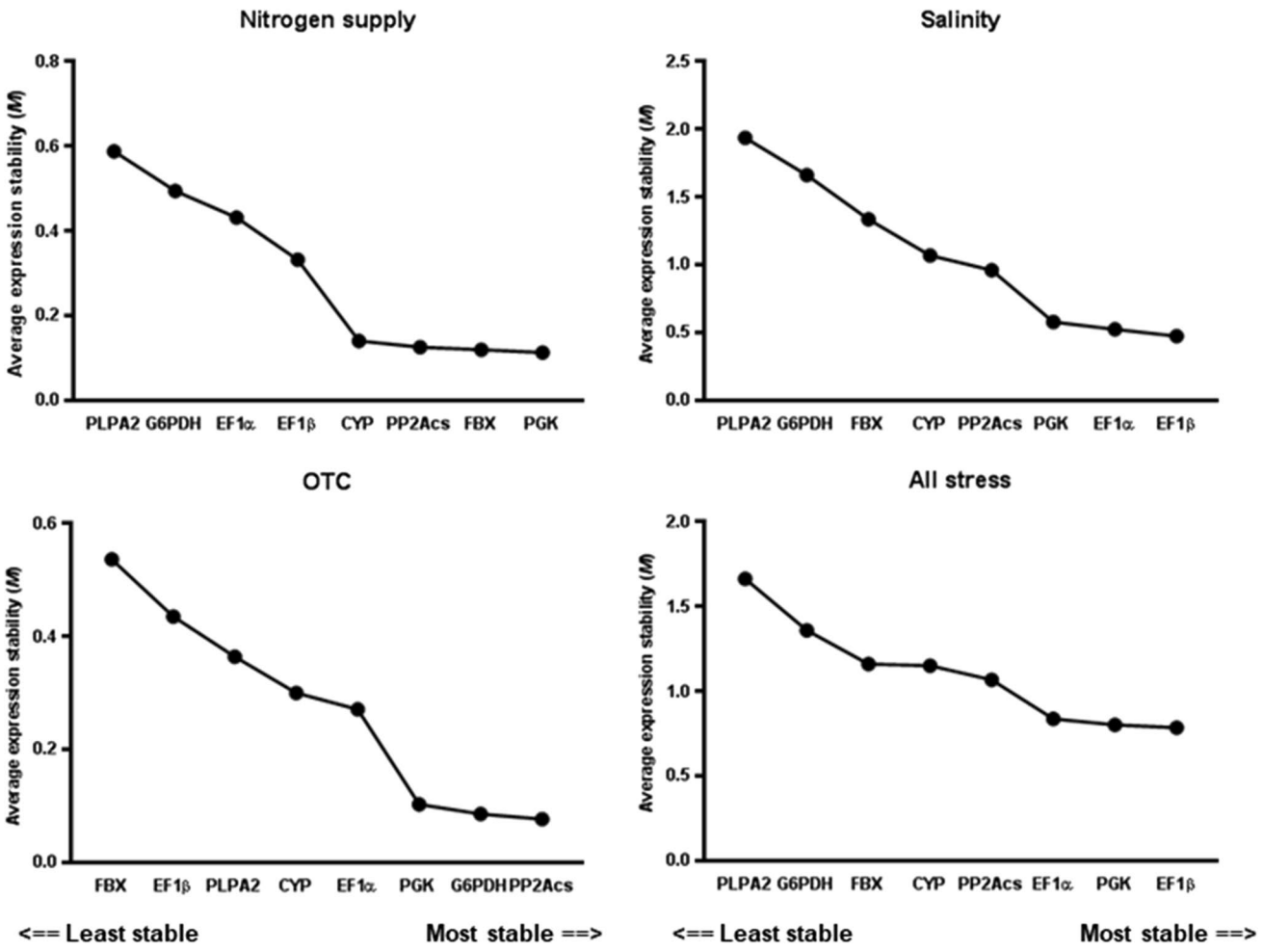


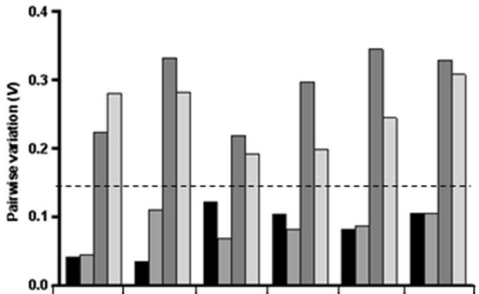
Fig. 2 Expression stability values (*M*) of eight candidate reference genes calculated by *geNorm* software in different sample groups. The genes with $M \leq 1.5$ are considered significant with stable expression

reference genes adequate for normalization (Vandesompele et al. 2002). Thus, the use of the four most stable genes could be considered a valid normalization strategy for both conditions and the addition of two more genes has a significant effect on the results compared to the other conditions.

In Fig. 4 the expression stabilities of the selected reference genes according to *NormFinder*, *BestKeeper* and ΔCt algorithms, as derived by *RefFinder*, are reported. In particular, the results of *NormFinder* analysis highlighted that all genes have a good stability during OTC and nitrogen supply stresses displaying expression stability values ranging from 0.289 (*PP2Acs*, *PGK*, *CYP*) to 1.0 (*PLPA2*) for nitrogen stress and from 0.354 (*G6PDH*, *PP2Acs*, *CYP*, *PLPA2*, *EF1α*) to 0.9 (*EF1β*) for OTC stress (Fig. 4a and Online Resource 7). On the contrary, great variability among the reference genes was found during salinity stress, ranging from 0.126 (*EF1α*) to 2.918 (*G6PDH*). When all stress conditions were taken into account together, *PP2Acs* (0.554) ranked first as the most stable expressed gene, followed by *EF1α* (0.566) and *EF1β* (0.633), whereas *PLPA2* was the least stable with a stability value of 2.184.

BestKeeper analysis (Fig. 4b and Online Resource 8) showed a good stability for all reference genes when considering OTC and nitrogen supply stresses, as found with *NormFinder* analysis. In fact, *SD* [$\pm$ *CP*] values ranged from 0.0 (*PP2Acs*, *PGK*, *CYP*) to 0.67 (*PLPA2*) during nitrogen stress and from 0.0 (*FBX*, *PGK*) to 1 (*EF1α*) under OTC stress. As regards to salinity stress, *G6PDH* was the only gene with a *SD* [$\pm$ *CP*] close to 1 (1.11), indicating a good stability under these conditions, whereas all other reference genes displayed values > 1. When considering all stress conditions together, *G6PDH* (1.03) ranked first while *PLPA2* (3.08) was the least stable reference gene as also found using the *NormFinder* and *geNorm* algorithms.

According to the previous results, the analysis with ΔCt method showed that all genes have a good stability during OTC and nitrogen supply stresses having average of *SD* values below 1 (Fig. 4c and Online Resource 9). In particular,



	V2/V3	V3/V4	V4/V5	V5/V6	V6/V7	V7/V8
Nitrogen supply	0.043	0.035	0.123	0.105	0.083	0.106
OTC	0.045	0.11	0.069	0.082	0.087	0.105
Salinity	0.224	0.332	0.219	0.297	0.345	0.329
All stress	0.28	0.282	0.192	0.199	0.245	0.308

Fig. 3 Pairwise variation (*V*) analysis of eight candidate reference genes in different sample groups, calculated by *geNorm* software. The optimal number of the reference genes required for accurate normalization was determined by pairwise variation (V_n/V_{n+1}). The dotted line indicates the recommended threshold value of 0.15 below which the inclusion of an additional reference gene is not necessary

values ranged from 0.47 (*PP2Acs*, *PGK*, *CYP*) to 1.06 (*PLPA2*) during nitrogen stress and from 0.30 (*G6PDH*, *PP2Acs*, *CYP*, *PLPA2*, *EF1α*) to 0.91 (*EF1β*) for OTC stress. As far as salinity stress is concerned, values of *SD* average varied from 1.33 (*CYP*) to 2.98 (*G6PDH*), indicating a moderate stability as reference genes. When all stress conditions were considered together, the stability values ranged from 1.28 (*EF1α*) and 2.34 (*PLPA2*). Once again, *PLPA2* displayed the highest value correlated to the lowest stability, as also found using the other algorithms.

On the basis of the above results and taking into account all software (Figs. 2 and 4), we can infer that under nitrogen stress, *PP2Acs*, *PGK* and *CYP* could be considered as the most stable genes, while the least stable should be *PLPA2*. Under salinity stress, the gene stability order was different among the four software, but in general, *EF1α*, *CYP* and *PP2Acs* were the most stable, while *G6PDH* and *PLPA2* were the least stable. Finally, as far as the OTC stress is concerned, the most stable genes were *G6PDH* and *PP2Acs*, being *EF1β* the least stable one. Although the stability order of the candidate reference genes varied as a function of the software used and the experimental conditions, a general consensus can be inferred when the stress conditions were considered all together. Indeed, *PP2Acs* and *EF1α* were often among the most stable genes, while *PLPA2* and *G6PDH* were frequently the least stable in all conditions.

These evidences were confirmed by the comprehensive expression stability ranking of the candidate reference genes obtained by *RefFinder* (Table 3). Results confirmed *PP2Acs* and *EF1α* as the most stable genes due to the lowest *Geomean* of the ranking values. Furthermore, regardless of the order, the software substantiates the lower stability for *PLPA2* and *G6PDH* ranking them as the most unstable gene in almost all sample sets, with the exception of the OTC stress. It is worth highlighting that small discrepancies were obtained between the original *geNorm* software package and the *geNorm* output of the *RefFinder* tool, probably because *RefFinder* uses the raw *Ct* values as input data, while the original software makes use of PCR efficiencies (Online Resources 6 and 10).

Catalase gene expression analysis under different abiotic stresses for reference genes validation

In order to validate the reliability of the best reference genes identified, the expression profiling of the *C. quitensis* catalase *(CqCAT*) gene was evaluated using the most stable reference gene (*EF1α*), the combination of the two most stable genes (*EF1α* + *PP2Acs*) and the least stable gene (*PLPA2*) as normalizers. The expression levels of *CqCAT* were determined by qRT-PCR on *C. quitensis* leaves harvested from plants subjected to different abiotic stresses, i.e. nitrogen supply, salinity and OTC (Fig. 5).

In this study, when the most stable reference genes (*EF1α* and *EF1α* + *PP2Acs*) were used for accurate normalization of the samples under nitrogen supply stress, *CqCAT* transcript was found to increase along with the rise of ammonium concentration, whereas using the least stable reference gene (*PLPA2*), a decrease on *CqCAT* expression was observed at the highest ammonium concentration (Fig. 5a). A different expression pattern of *CqCAT* was also highlighted under salinity stress when the best or the least stable reference genes were used for data normalization. In particular, EF1α and *EF1α* + *PP2Acs* allowed to detect an increasing *CqCAT* expression between 0 and 250 mM NaCl and a strong decrease when 500 and 750 mM NaCl concentrations were reached. The expression pattern of *CqCAT* gene did not significantly change when the four top genes (*EF1α*, *PP2Acs*, *PGK* and *EF1β*) were used for normalization as suggested by the *geNorm* pairwise variation results (data not shown). On the other hand, using *PLPA2* a very strong increase in *CqCAT* expression was registered until 500 mM NaCl (Fig. 5b). Finally, when OTC stress was considered, the *CqCAT* expression profile followed the same trend with all the reference genes (Fig. 5c). This result could be related to the good performance of *PLPA2* gene, when the OTC stress was considered per se (Figs. 2 and 4).

Discussion

The recent climate changes affecting the entire planet have a strong impact on the Antarctic environment, with clear signs both on land and sea (Schofield et al. 2010). It has been

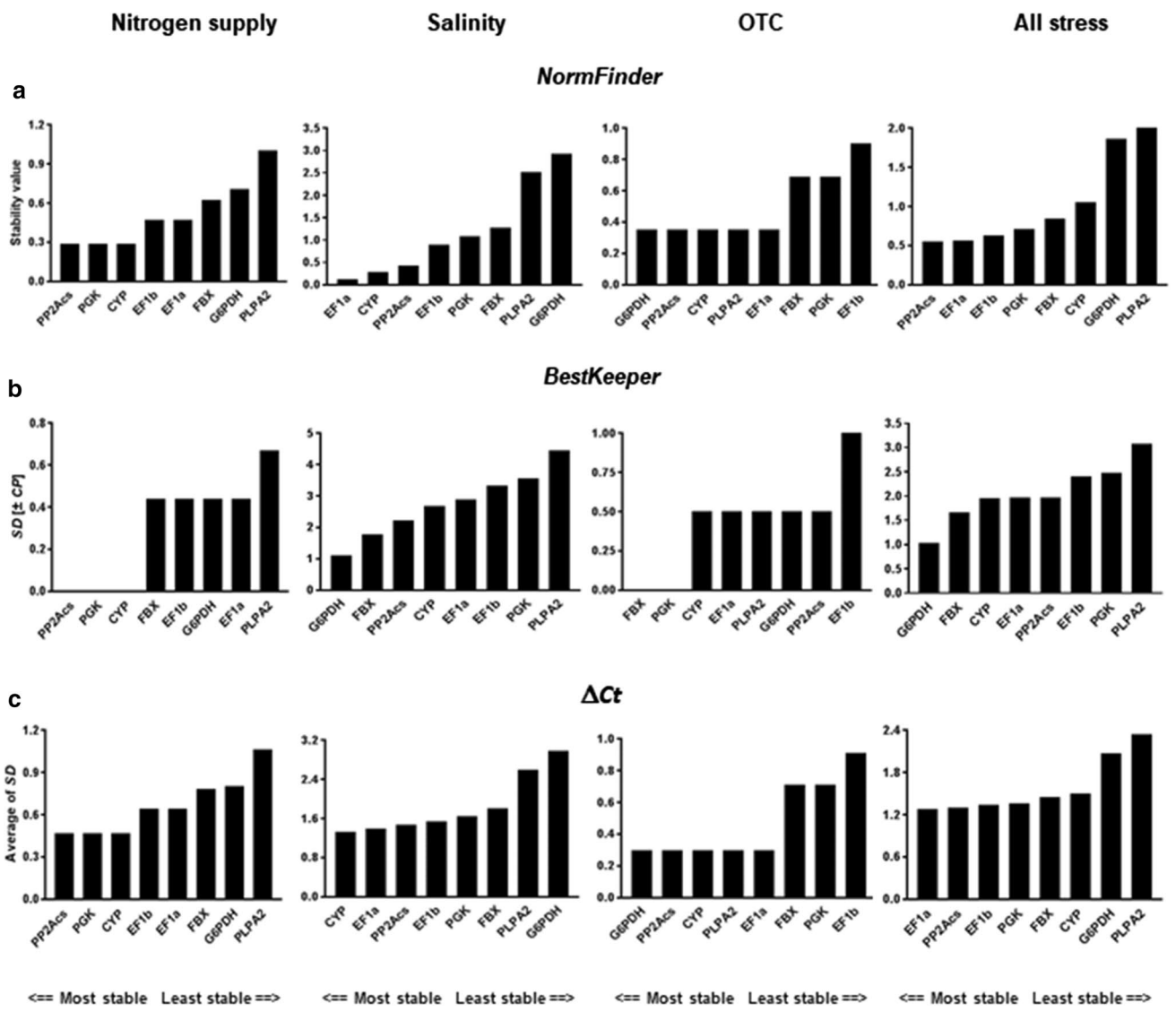


Fig. 4 Expression stability analysis of eight candidate reference genes assayed by the software: *NormFinder* (**a**), based on the stability values calculated by the software; *BestKeeper* (**b**), based on the standard deviation values ± crossing point (*SD* [± *CP*]); Δ*Ct* (**c**), based on the average of standard deviation (*SD*) values

reported an increase of the Antarctic Peninsula air temperature of about 2.6 °C in the last 50 years that influences and shapes the life and expansion of endemic organisms (Turner et al. 2014). In the Maritime Antarctic, only two endemic flowering plants found the way to survive and propagate in this harsh environment and, nowadays, they must overcome a further challenge represented by the climate change. *Colobanthus quitensis* represents the only native dicotyledonous plant able to grow in this region withstanding the hostile climate (Smith 2003) and a recent study assessed its very close origin dating its arrival after the last glacial event (Biersma et al. 2020). Due to the great impact that climate change could have all over the world, we wondered whether *C. quitensis* could be used as bioindicator of regional warming in the Antarctic Peninsula helping discover new molecular biomarkers useful to evaluate biological effects of climate change in Antarctica. The transcriptome of this plant has been recently unveiled (Arthofer et al. 2015; Cho et al. 2018) allowing design specific PCR primers to be used in gene expression profiling studies aimed at disclosing the differentially expressed genes in plants subjected to different stressors. Moreover, studies on the molecular adaptation of different *C. quitensis* haplotypes coming from multiple colonization events of the Maritime Antarctic might support genetic population studies aimed at dating its origin and age in Antarctica. However, although quantitative Reverse Transcriptase-PCR (qRT-PCR) is the technique of choice for gene expression studies, unfortunately it also has some

Table 3 Recommended comprehensive ranking calculated by *RefFinder* in different sample groups

Ranking	Nitrogen supply		Salinity		OTC		All stress	
	Genes	*Geomean* of ranking value	Genes	*Geomean* of ranking value	Genes	*Geomean* of ranking value	Genes	*Geomean* of ranking value
1	*PP2Acs*| *PGK*	0	*EF1α*	2.34	*G6PDH*| *PP2Acs*	0	*EF1α*	2.21
2	*PP2Acs*	1	*CYP*	2.38	*G6PDH*	1.59	*PP2Acs*	2.66
3	*PGK*	2	*EF1β*	3.13	*PP2Acs*	2.29	*EF1β*	2.71
4	*CYP*	2.28	*PP2Acs*	3.41	*PLPA2*	2.99	*PGK*	3.25
5	*EF1β*	4.47	*PGK*	3.64	*FBX*	3.98	*FBX*	4.16
6	*EF1α*	5.14	*FBX*	4.56	*CYP*	4.05	*G6PDH*	4.3
7	*FBX*	5.63	*G6PDH*	4.76	*PGK*	4.92	*CYP*	4.56
8	*G6PDH*	6.48	*PLPA2*	7.24	*EF1α*	5.14	*PLPA2*	8
9	*PLPA2*	8			*EF1β*	8		

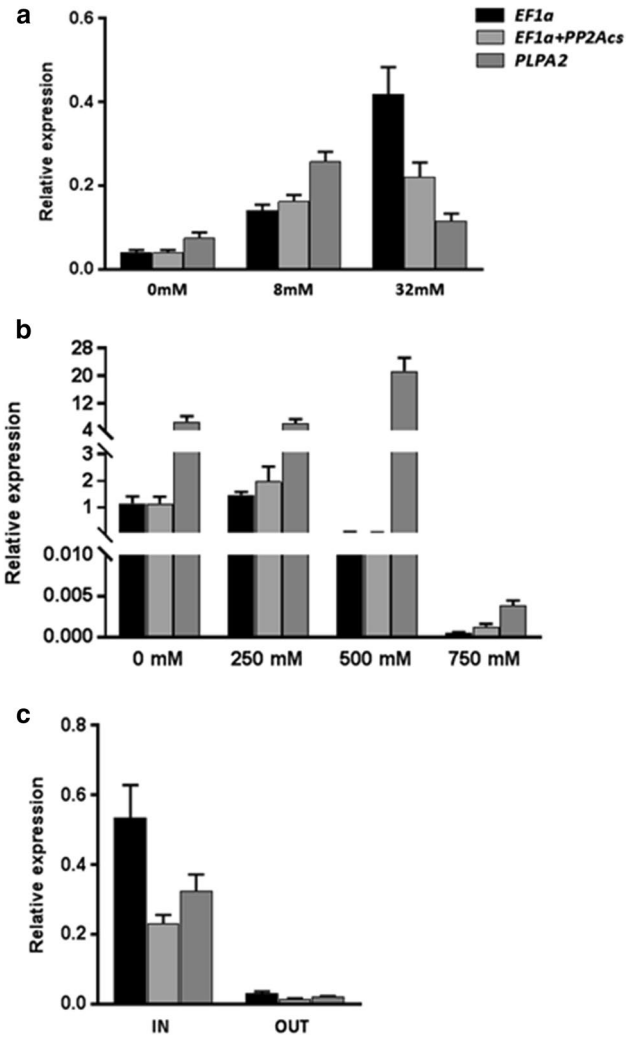


Fig. 5 Relative expression of *Colobanthus quitensis* catalase gene (*CqCAT*) using the selected reference genes for transcript normalization following different stress treatments. (a) Nitrogen supply; (b) Salinity; (c) OTC. Bar indicates the standard error (±*SE*) evaluated from three biological replicates

weaknesses as the need to use reliable normalizing genes to obtain accurate and meaningful expression values (Gutierrez et al. 2008). Furthermore, it should also be taken into account that the transcription levels of reference genes can vary greatly in response to different physiological conditions (plant tissues, organs or developmental stages) as well as under different stress conditions or even depending on the experimental design (Czechowski et al. 2005; Le et al. 2012). Thus, the simply use of housekeeping genes commonly thought to be good reference genes could affect the reliability of results. Therefore, there are no universal reference genes for all seasons, but it is mandatory to select a panel of reliable genes within specific experimental treatments or tissues from the same individual. In a previous work, Cho et al. (2018) selected *18S rRNA* and *TIM* (chloroplast-like Triosephosphate Isomerase, also known as *TPI*) as reference genes for gene expression analysis in *C. quitensis* plants challenged with abiotic stress. We searched for these genes in our experimental system, but could not find any contig coding for *18S rRNA*, since our transcriptome analysis was performed after ribosomal RNA (rRNA) depletion. Moreover, we found three contigs coding for *TIM*, but with very unstable Log_2 Fold Change (viz. -0.61, -0.82, -2.2). Indeed, genes that are good normalizers for some stresses may not be reliable for others, thus we searched for new reference genes and in this work we tested the reliability of eleven different candidates under diverse experimental conditions. Between them are some traditional housekeeping genes, such as *EF1α* and *EF1β*, widely used in several plant species as conventionally considered stably expressed under different treatments and various development stages (Zhou et al. 2016; Wan et al. 2017), and some new genes reported to be highly stable in diverse conditions, such as *CYP*, *PP2Acs*, *PGK, TBP*, *PTB* and *FBX* (Expósito-Rodríguez et al. 2008; Hao et al. 2014; Li et al. 2015; Wang et al. 2015; Taylor et al. 2016; Zhou et al. 2016). Moreover, three more

genes, i.e. *PLPA2, G6PDH* and *PBGD,* which have proved to be suitable reference genes in insects (Meunier et al. 2006), fungi (Yin et al. 2013) and humans (Dundas and Ling 2012), respectively, were added to this study with the aim of testing their reliability in plants as well. At first, the expression profile of the candidate reference genes under various abiotic stress conditions (i.e., nitrogen supply, salinity and OTC) was evaluated by qRT-PCR. This preliminary analysis revealed that the expression of three out of eleven genes (i.e. *TBP, PTB* and *PBGD*) varied strongly as a function of the stress condition (Table 3 and Fig. 1), thus they were excluded from the following computational analysis. Although *TBP* and *PTB* were reported to rank between the first top reference genes in some plant systems (Hao et al. 2014; Taylor et al. 2016; Expósito-Rodríguez et al. 2017), they were also reported to be strongly unstable in tea plants under metal stress, in *Oenanthe javanica* under abiotic stress, in *Olea europea* between different cultivars, in cotton between different tissues (Jiang et al. 2014; Ray and Johnson 2014; Sun et al. 2015; Wang et al. 2017), corroborating our results. As far as *PBGD* is concerned, its reliability was well known in animal systems (Dundas et al. 2012; Yu et al. 2015), but its use as reference gene was unsuccessful in our model system. The stability ranking of the remaining eight candidate reference genes was evaluated using *geNorm* software package and *RefFinder*, a comprehensive software platform which integrates four different statistical algorithms, i.e. *geNorm*, *NormFinder, BestKeeper* and Δ*Ct*. All software generally ranked the same genes as the least stable, but showed discrepancy in the ranking of the most stable candidate reference genes (Figs. 2 and 4). In particular, the least stable genes were identified as *PLPA2* and *G6PDH* considering both individual stress, such as nitrogen supply or salinity, and all stress conditions jointly. These genes, which are not frequently used in plants, were selected with the aim of identifying new reference genes suitable for plant systems, but they turned out to be unreliable in our study. However, our findings are in agreement with some previous studies where these genes ranked between the last positions. In particular, *PLPA2* was found to be unsuitable as internal control in peach under different treatments and development stages (Tong et al. 2009), and *G6PDH* was estimated as the least stable gene in soybean exposed to cadmium (Wang et al. 2012). Nevertheless, if OTC stress was considered per se, *G6PDH* and *PLPA2* ranked between the first top genes according to all software, proving to be reliable reference genes in *C. quitensis* under this condition. As regards the best reference genes, only under nitrogen stress the four algorithms agreed identifying the same ranking positions for all the genes, classifying *PP2Acs* and *PGK* as the best reference genes. For salinity and OTC stresses, the four algorithms differed in the choice of the best reference genes, being Δ*Ct* and *Normfinder* the algorithms with a more similar output. According to *RefFinder* analysis (Table 3), the comprehensive ranking recommended *G6PDH* and *PP2Acs* as the best reference genes under OTC stress and *EF1α* and *CYP* under salinity stress. Interestingly, *EF1α* and *CYP* were also reported to be the most stable genes in *Deschampsia antarctica* under salt stress (Lee et al. 2010). When all stress conditions were considered together, the comprehensive ranking indicated *EF1α* and *PP2Acs* as the most stable reference genes. Our findings were corroborated by several previous studies that reported the above genes as the most stable under different abiotic stresses. For example, they were recommended as internal control genes in *Agrostis stolonifera* (bentgrass) and *Cenchrus americanus* (pearl millet) under multiple abiotic stresses (Chen et al. 2014; Shivhare and Lata 2016). Moreover, *EF1α* was selected as the best reference gene in tomato under nitrogen and cold stresses (Løvdal and Lillo 2009), in cucumber under osmotic and salt stresses (Migocka and Papierniak 2011), in sugarcane (Guo et al. 2014) and *Glycine max* (soybean) (Ma et al. 2013) under salinity and drought stresses. Likewise, *PP2Acs* was found to be the most stable reference gene in pearl millet under salinity and drought stresses (Saha and Blumwald 2014), in *Pisum sativum* under salinity stress (Die et al. 2010), in soybean exposed to cadmium (Wang et al. 2012).

To further validate the selected reference genes in *C. quitensis*, the expression of catalase gene (*CqCAT*) under nitrogen supply, salinity and OTC stresses was evaluated. The choice of *CqCAT* as a target gene was due to its important role in scavenging the ROS notoriously produced at high levels under the considered abiotic stresses. Moreover, this gene is often exploited as an abiotic stress-responsive marker in many plant species. Indeed, many papers report the behaviour of *CAT* expression in different model systems under the same abiotic stresses studied in this paper and they can be taken into account to compare its expression trend in our model plant. The qRT-PCR analysis was carried out using the most stable gene (*EF1α),* the combination of the two most stable genes (*EF1α* + *PP2Acs*) and the least stable gene (*PLPA2*), according with the *RefFinder* comprehensive ranking as normalizers (Table 3). Catalase is a very important enzyme in protecting cells against ROS, catalyzing the breakdown of hydrogen peroxide to water and oxygen. Its expression was found to be greatly affected in Arabidopsis and rice plants after hormone treatment (Proietti et al. 2013; Bertini et al. 2019a) as well as in *Posidonia oceanica* under heavy metal stress (Bertini et al. 2019b). A significant increase in CAT activity was also reported in wheat under drought, salinity, nitrogen or heat stresses (Sairam et al. 2002; Polesskaya et al. 2004; Luna et al. 2005; Almeselmani et al. 2006; Esfandiari et al. 2007; Heidari 2009; Gupta et al. 2013) as well as in *Cicer arietinum* (chickpea) seedlings, in the unicellular alga *Chlorella* sp., in

the aquatic macrophyte *Najas graminea*, in the mangrove plant *Suaeda maritime* under salt stress (Eyidogan and Öz 2007; Mallik et al. 2011). Nevertheless, high saline concentrations (200 to 500 mM NaCl) were found to induce a strong decrease in CAT activity in *Triticum aestivum* (wheat) leaves (Heidari 2009) as well as in leaf tissue of chickpea seedlings (Eyidogan and Öz 2007). According to these findings, we found *CqCAT* overexpressed following nitrogen supply, OTC and salinity ($\leq$ 250 mM NaCl) treatments; a strong decrease in its expression pattern was registered only under $\geq$ 500 mM NaCl (Fig. 5). When the most stable reference genes (*EF1α* and *EF1α* + *PP2Acs*) were used for normalization, *CqCAT* expression trend was found similar along all the treatments. On the other hand, when using the least stable reference gene, *PLPA2*, the expression pattern of *CqCAT* was different and generally overestimated from that obtained using the other reference genes, in particular for nitrogen supply and salinity stresses. Under OTC stress, *PLPA2* showed a greater stability, as confirmed by the stability ranking derived from all four software kinds when single stress was considered (Figs. 2 and 4).

In summary, we identified two stable reference genes, namely *EF1α* and *PP2Acs*, which can be used for accurate transcript normalization in gene expression studies using qRT-PCR in *C. quitensis* under different individual abiotic stresses. Nevertheless, it is well known that the expression level of genes identified as good reference genes in specific experimental conditions may vary when diverse experimental conditions are considered (Huggett et al. 2005), thus could be necessary to test the reliability of other candidate reference genes in order to identify the most appropriate ones for any specific circumstance.

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Author contributions CC and LB conceived and designed research; CC collected the field samples; LB, SP, FC, FF and CR conducted experiments; LB, SP, FC and FF analyzed data; CC and LAB retrieved funds; CC and LB wrote the manuscript; CC, LB, SP, CR and LAB reviewed the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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