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Housekeeping gene selection for quantitative real-time PCR assays in the seagrass *Zostera marina* subjected to heat stress

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Abstract

In light of increasing sea surface temperatures, quantifying the expression of stress-inducible genes in coastal organisms is an important topic of marine molecular ecology and evolution. As a prerequisite for quantitative real-time PCR (QPCR) assays, we tested the suitability of 12 candidate housekeeping genes (HKGs) for normalization of messenger RNA abundance and quality in the ecologically important seagrass species *Zostera marina*. In comparing several algorithms used to identify appropriate HKGs, two approaches using reciprocal cross-validation among a larger group of HKG candidates implemented in geNorm or NormFinder yielded largely consistent results. Using these approaches, 3 HKG candidates were selected that are stable alone or in combination in the target tissue (leaf) with respect to the imposed temperature stress treatments (TATA box binding protein, translation elongation factor, eukaryotic initiation factor 4A). Example calculations revealed spurious gene expression changes of a factor of 2 in hypothetical target genes when using less stable HKGs as reference for standardization. These results highlight the need for devoting considerable effort to selecting appropriate HKGs for normalization of QPCR data before performing experiments with the target genes.

Introduction

Rising sea surface temperatures due to global warming are imposing increasing temperature stress on aquatic organisms (Rowan 2004, Stillman 2003). To predict either phenotypic plasticity and acclimation (Stillman 2003) or possible adaptation to rising temperatures (Oleksiak et al. 2002), quantifying the expression of stress-inducible genes in coastal organisms is highly relevant to marine molecular ecology (Hashimoto et al. 2004). Here we present quantitative real-time PCR (QPCR) assays of a suite of housekeeping genes for studying target gene expression in the ecologically important seagrass species *Zostera marina* (eelgrass) (Williams and Heck 2001). Previous work has shown that many eelgrass populations are under chronic temperature stress (Williams 2001) and that, particularly in northern Europe, maximal sea surface temperatures during summer months are at the lethal limit for local populations (Greve et al. 2003; Reusch et al. 2005).

QPCR is a highly specific and sensitive technique that can quantify the amount of template DNA molecules in a polymerase chain reaction (Heid et al. 1996). The advantages of speed, throughput, and excellent reproducibility make this technique a powerful new tool in molecular ecology (Haller et al. 2004). The technique has been most widely used in aquatic ecology to quantify the abundance and composition of small plankton in marine and freshwater plankton samples using taxon-specific probes (e.g., Coyne et al. 2005). In contrast, the potential of this technique to study gene expression by quantifying messenger RNA (mRNA) abundance has only recently been used in marine systems (e.g., Hashimoto et al. 2004). The expression of a target gene can be related to the total RNA input, or alternatively it can be quantified relative to the expression of a reference gene, the housekeeping gene (HKG). The term "housekeeping" reflects the assumption that, due to its housekeeping tasks, the reference gene would always be expressed at the same level. Unfortunately, a universal reference gene that is expressed into mRNA uniformly, at all biological conditions in all tissues, simply does not exist (Haller et al. 2004). Suzuki et al. (2000) pointed out that in 1999, in high-impact journals, nearly 90% of the gene expression studies used glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), or ribosomal (18S or 28S rRNA) genes as a single control without previ-

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ous gene stability studies. Subsequent studies revealed that the expression of these particular housekeeping genes is far from uniform across experimental conditions, making many conclusions based on untested HKGs questionable (Suzuki et al. 2000; Vandesompele et al. 2002). A consensus is therefore emerging that, before performing experiments using QPCR to quantify gene expression, it is necessary to validate the stability of several potential HKG candidates (Nicot et al. 2005; Suzuki et al. 2000; Vandesompele et al. 2002).

This can be a circular problem, however, as the expression data of the HKG candidate to be tested also needs to be standardized. One possible solution is to use a sample of more than just one HKG for determining the most stable candidates (Pfaffl et al. 2004). Several rounds of reciprocal cross-validation of a complete matrix of all pairwise HKG comparisons are performed. Different procedures have been suggested that formalize the choice of one or more HKG, but they have rarely been compared. One purpose of this technical note is therefore to assess whether these methods provide identical results. In our case, we sought one or more housekeeping genes that are uniformly expressed during heat stress in leaves of eelgrass (*Zostera marina*) (Vandesompele et al. 2002; Pfaffl et al. 2004). We compared 3 different algorithms, implemented in the programs Bestkeeper (Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002), and NormFinder (Andersen et al. 2004). We discuss differences in the results, taking into account the different approaches that are implemented in each algorithm to identify stable HKGs.

Materials and procedures

Study species—Zostera marina, or eelgrass, is a marine angiosperm or seagrass. This species is widespread among shallow sedimentary shorelines in the northern hemisphere from subtropical to cold-temperate latitudes. As a keystone species and ecosystem engineer, it provides the structural basis for a rich community of associated microalgae, invertebrates, and fish species (den Hartog 1970).

Plant material—Entire shoots (with attached roots) of *Zostera marina* were collected in an extended Baltic Sea meadow at Schilksee (10°10’22”E, 54°25’39”N), Schleswig-Holstein, Germany, while snorkeling. Sampling was conducted at ambient water temperatures on both 24 January 2005 (3-4°C) and 4 August 2005 (~18°C) in a water depth of 2.5 to 3 m. In untreated (control) plants (24 January 2005), leaf samples were cleaned with a tissue, frozen in liquid nitrogen within 15 min of collection, and maintained at –80°C until later RNA extraction. Plant materials from the 4 August 2005 were replanted within 2 h into aquaria to be exposed to defined water temperature treatments (see below).

Plant treatments—Shoots exposed to temperature treatments (ambient samples collected in August only) were planted into ambient sediment into aerated 120-L aquaria with illumination, filled with Baltic seawater (salinity ~15 g/kg). Within 4 days, the temperature in half of the aquaria was increased at a rate of 1 to 2°C per day to a final temperature of 25°C. The temperature of the other aquaria was maintained at 18 to 20°C. After treatment, leaf samples were collected, picking the third (youngest) leaf of each shoot. The leaves were cleaned, and the first step of the extraction was performed immediately. The disrupted leaves were frozen in the lysis buffer and stored at –80°C until the rest of the extraction was performed.

RNA extraction and cDNA preparation—We isolated total RNA from leaf samples using RNeasy-96 (Qiagen) or Insorb Plant RNA Mini Kit for total RNA extractions (Invitek). RNA quality was confirmed by gel electrophoresis. In general, the 28S band was stronger than the 18S rDNA band, indicating high RNA quality. Total RNA concentration was quantified using a Nano-Drop spectrophotometer. First-strand cDNA synthesis was carried out using the Omniscript kit (Qiagen) using 0.1 µg total RNA diluted according to the initial concentration with RNAse-free water. For reverse transcription, a total reaction volume of 20 µL contained 1× reaction buffer, 200 mM of each dNTP, 1 µM oligo-dT primer, and 4 units of reverse transcriptase (Qiagen, 4 unit/µL). The reaction was heated for 5 min at 65°C, chilled on ice, incubated for 3 h at 37°C, and finally heated for 5 min at 95°C. The resulting cDNA was diluted 1:10 before use in QPCR assays.

Gene identification and primer design—Zostera marina genes were obtained from reverse-transcribed cDNA in an expressed sequence tag (EST) library prepared from a combination of meristematic and leaf tissue using the Creator SMART library construction kit (BD Clontech), according to the manufacturer’s protocol (TBH Reusch, unpublished data). In total, 1103 unique genes were identified, from which 12 widely used housekeeping gene (HKG) candidates were selected (GenBank accession numbers AM268883-AM268894). Primers were designed using the primer analysis software Primer Express v. 2.0 (Applied Biosystems) with the default parameters of the TaqMan MGB Probe and Primer design procedure. We selected PCR amplicons to range from 101 to 157 bp in size, facilitating cross-comparison of assays and assuring equal PCR efficiencies. Whenever possible, the forward and reverse primers were placed across an exon-exon boundary to avoid any genomic amplification; however, we were unable to achieve this for cyclophilin genes and for the different ribosomal proteins of large subunit gene. To locate exon-exon boundaries, target sequences were aligned with putatively homologous sequences after BLAST search using BioEdit. Only BLAST hits with a P value $< 1 \times 10^{-3}$ were considered. Primer sequences are given in Table 1.

Quantitative real-time PCR—QPCR permits the quantification of the amount of template DNA (or cDNA) in the sample by following the accumulation of product during PCR (in real-time). Fluorescent dyes such as SYBR green show maximal fluorescence only with double-stranded DNA. Fluorescence is monitored once per cycle after product extension, and increases above background fluoresce at a cycle number that depends on the initial template concentration. QPCR was performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the SYBR Green QPCR Master Mix (ABgene). The PCR

Table 1. Candidate housekeeping genes for normalization in eelgrass *Zostera marina* quantitative real-time PCR assays.

Gene name	Symbol	Acc. no.	Function	Primer sequence 5′–3′ (forward and reverse)	Amplicon Size	T_M
Ubiquitin 01	Ub01	AM268883	Protein degradation	GGAGGTACAGACGACTGGAAA GGGGGTATCCCTTCTTTGTC	136	—
Ubiquitin 10	Ub10	AM268884	Protein degradation	TCGCAGCAAGCGTTCTTA TGCTGGTCAGGAGGGATT	147	—
Elongation factor	Elo_f	AM268885	Translational elongation factor	CCAGCAATGGCAGTTTCGT CAGATGGAACCGATGAGATTGA	151	77
Tubulin alpha	Tub_a	AM268886	Microtubule based process	TGGTGGAGGAGATGATGCTTT CGATCACAGTCGGTTCAAGATC	101	81
Tubulin beta	Tub_b	AM268887	Microtubule based process	GCTGGTAACAACTGGGCTAAGG CCAAGGGAGTGGCAAACTTG	131	78
Cyclophilin 1*	Cyp1	AM268888	Protein folding	AAAGCGGTGACACACAAGGTT GCAGTTTTAGGCACAGCTTTCC	101	79
Cyclophilin 2*	Cyp2	AM268889	Protein folding	CACTCCACTACAAGGGATCGAAA GGACCTGTATGCTTCTTAACGAAGT	151	80
Eukaryotic initiation factor4A	eIF4A	AM268890	Translation initiation factor	TCTTTCTGCGATGCGAACAG TGGATGTATCGGCAGAAACG	125	80
TATA box binding protein	TBP	AM268891	General RNA polymerase II transcription factor	CGGAGAGCTCATTGAAACAGCTA GGAACTTTTCCTTCCAACTTCAGA	117	76
Ribosome structural protein L28*	RPL28	AM268892	Structural component large ribosomal submit	TTCCGCACCTAGGGTTTCG ATATTGGCGCAGCGATTTTG	101	79
Ribosome structural protein L17*	RPL17	AM268893	Structural component large ribosomal submit	AACAAATTTCGCATGTCATTGG ATCCCCTTGACGGAAATGATG	101	77
Ribosome structural protein L18*	RPL18	AM268894	Structural component large ribosomal submit	GGTCAATTTCGCTTTCACCAGTA TTGGTTGCCCACAGCTTCA	101	81

T_M, melting temperature of PCR amplicon. Gene sequences were identified using an EST (expressed sequence tag) library, using homology search via BLAST_N (cutoff P value $< 1 \times 10^{-3}$). PCR primers were designed with software Primer Express (Applied Biosystems), preferentially across exon-exon boundaries.
*PCR amplicon not designable across an exon-exon boundary.

reaction consisted of 10 µL SYBR Green PCR Master Mix, 100 nM forward and reverse primers, and 4.0 µL 1:10-diluted template cDNA in a total reaction volume of 20 µL. Thermocycling was performed using the following conditions: 2 min at 50°C, 15 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. To verify that the reaction yielded only a single product, a dissociation protocol after thermocycling revealed the melting curve of the PCR amplicon from 65 to 95°C.

The expression of the remaining 8 HKG candidates was tested in 4 individuals in triplicate at 3 different temperatures (4, 20, and 25°C), resulting in 36 QPCR assays per gene. Data were recorded as fractional cycle at an arbitrary C_T-value during the exponential phase of the reaction. All the data from one gene were recorded at the same time and have the same threshold. In only one case was one of the triplicate values omitted because it differed by more than 0.5 C_T (Neuvians et al. 2005). The average of each triplicate was calculated and then served as input for comparing the different HKG candidates, as validated by 3 different algorithms. Two different sets of analyses were performed with the data. First, all 3 temperature conditions across a deliberately unrealistic biological range (4°C ambient winter, 20°C, and 25°C) were considered. A second analysis was performed with the 2 temperatures used in our later bioassays (20 and 25°C), the treatment most relevant for our question, namely the effects of summer temperature stress. These analyses were done to investigate possible differences between the experimental conditions.

PCR efficiency—To determine the amplification efficiency of each PCR assay, a standard curve of 4 serial dilution points (in steps of 10-fold) of a cDNA mixture was analyzed using linear regression. A standard curve (E_S) was calculated from the slopes of the standard curve by using the equation $E_S = 10^{(-1/\text{slope})}$ (Pfaffl 2001). The amplification efficiency of an individual reaction (E_R) was determined with the program LinReg PCR (Ramakers et al. 2003). PCR efficiencies of single genes ranged from 1.84 to 1.96. Slopes of regressions were not significantly different (data not shown).

Bestkeeper—A first procedure for addressing HKG candidates is implemented in the software Bestkeeper (a Microsoft Excel sheet is available at http://bioinformatics.gene-quantification.info/) and simply uses the standard deviation between the whole data set (all treatments random). According to the observed variabil-

ity, housekeeping genes can be ordered from the most stable expressed, exhibiting the lowest variation, to the least stable gene, exhibiting the highest variation. Any studied gene with the SD higher than 1, corresponding to a variation in template amount by a factor of 2, can be considered inconsistent and should be excluded. With the remaining genes, a Bestkeeper index is then calculated based on individual C_T values as the geometric mean of a number of Z candidate HKGs (Eq. 1), that can be use to normalized the targets genes (Pfaffl et al. 2004).

$$\sqrt[Z]{C_{T_1} * C_{T_2} * C_{T_3} * \ldots * C_{T_Z}} \tag{1}$$

geNorm—The second approach for evaluating gene expression stability was the procedure implemented in geNorm (a Microsoft Excel program available at http://medgen.ugent.be/~jvdesomp/genorm/). This approach needs several candidate genes that reciprocally serve as cross-references to single out the most stable ones. The underlying assumption of this procedure is that ratios between a sample of uniformly expressed, nonnormalized housekeeping genes should remain relatively constant among a suitable selection of HKGs. To show this, M values are calculated, corresponding to the average pairwise variation of a single HKG candidate to all other genes. Low M values indicate stable expression. In iterative steps, genes with the lowest stability (i.e., the highest M value) are removed. A new M value for each remaining gene is calculated until only 2 genes remain. Because these calculations are based on ratios, the final 2 genes cannot be resolved from each other. For the analyses, C_T values were converted into relative quantities for analysis with geNorm, considering the PCR efficiencies of the genes. The program selects from a panel of candidate reference genes the 2 most stable genes or a combination of multiple stable genes for normalization (Vandesompele et al. 2002; Meller et al. 2005).

NormFinder—The third approach tested for the selection of housekeeping gene candidates is implemented into NormFinder, a Microsoft Excel add-in (available at http://www.mdl.dk/publicationsnormfinder.htm). NormFinder assesses the expressional stability of a gene by evaluating its expression variability within treatments ("groups," in NormFinder terminology) compared to variation among treatments. Low variation between treatments corresponds to a small stability value, and thus, to high expressional stability. In our analyses, each temperature treatment is considered an experimental group, so we had 3, respectively, 2-treatment groups, each composed of 4 individuals. It is recommended that more than 5 potential housekeeping gene candidates be evaluated for the lowest stability value, i.e., suitability as HKG. In addition, a combination of 2 genes is recommended that need not necessarily be identical (Andersen et al. 2004; Ohl et al. 2005).

Assessment

Twelve candidate genes from different functional classes and gene families that have been widely used as HKGs (Andersen et al. 2004; Brunner et al. 2004; Pfaffl et al. 2004; Stürzenbaum and Kille 2001; Vandesompele et al. 2002) were initially chosen for the present study (Table 1). These include genes encoding for ubiquitin variants, translation elongation factor, tubulins, cyclophilins, eukaryotic initiation factor, TATA box binding protein, and 3 different ribosomal large subunit proteins. A priori, we decided not to include ribosomal RNA genes as potential HKG candidates because these are not polyadenylated upon transcription and cannot be used when using poly-dT priming in the reverse-transcription reaction (Stürzenbaum and Kille 2001).

After an initial PCR amplification, both ubiquitin candidates were discarded because UB01 gave a double amplification and UB10 gave no amplification. The tubulin β and tubulin α candidate genes yielded inconsistent amplification and were thus not further examined.

In the melting point analysis, the amplicons of all 8 genes revealed only a single product with a melting temperature (T_M) consistent with the expected value (Table 1).

The method implemented in Bestkeeper uses the standard deviation (SD) of C_T values across the entire data set (Table 2) with a useful reference gene revealing a low overall SD. The least stable candidate genes with the highest SD in C_T values were Cyp2 and RPL18, respectively, for the two different temperature treatment combinations (Table 2), indicating that these genes are clearly not reliable reference genes for temperature-related expression studies. In contrast, RPL17 displayed high expressional stability in both analyses (4/20/25°C or 20/25°C).

When using geNorm (Table 3), any 1 gene with a high M value has a high variance in gene expression. These were Cyp2 and RPL18, in accordance with results obtained from Bestkeeper. The program uses an iterative procedure. In each step, the least stable candidate is removed and a new M value is calculated. At the end of iterations the two most stable candidates were eIF4B and TBP or Elo_F and TBP, for the temperature combinations 4/20/25°C or 20/25°C, respectively (Table 3).

The NormFinder approach indicated eIF4B as the single best HKG and Elo_F and Cyp1 as the best pair of HKGs for 4/20/25°C treatments (Table 4). Thus, in this case, the most stable combination of 2 HKGs does not include the single most stable candidate gene (Table 4). For the realistic summer temperature treatments 20/25°C, however, the program identified TBP as the best gene, and eIF4B together with TBP as the best pair of 2 genes (Table 4). In accordance with previous results considering all 3 temperatures, the least stable genes were Cyp2 and RPL18.

Discussion

In this study, we sought suitable housekeeping genes to set up a QPCR assay to study the effects of temperature stress for expression of target genes in an ecologically important coastal plant, eelgrass (*Zostera marina*). To this end, we have compared 3 different approaches, implemented in the software programs NormFinder, geNorm, and Bestkeeper. Not surprisingly in light of their diverging mathematical approaches, these programs

Table 2. Selection of stable housekeeping genes in eelgrass (*Zostera marina*) based on the Bestkeeper approach (Pfaffl et al. 2004).

Three temperature treatments (4/20/25°C)	Gene							
	eIF4B	Cyp1	Cyp2	RPL28	TBP	Elo_F	RPL17	RPL18
n	12	12	12	12	12	12	12	12
GM [C_T]	26.06	27.12	24.83	26.57	28.53	26.01	23.93	27.96
AM [C_T]	26.09	27.20	24.94	26.63	28.56	26.04	23.96	28.02
min [C_T]	24.49	24.31	20.79	24.28	26.63	23.77	21.78	25.72
max [C_T]	29.66	31.31	28.89	30.08	32.27	29.16	25.81	31.68
SD [± C_T]	1.09	1.84	2.10	1.51	1.18	1.02	0.74	1.36
CV [%C_T]	4.17	6.77	8.44	5.66	4.13	3.91	3.10	4.84
Two temperature treatments (20/25°C)								
n	8	8	8	8	8	8	8	8
GM [C_T]	26.53	28.32	26.23	27.40	28.96	26.14	23.83	28.78
AM [C_T]	26.56	28.36	26.27	27.44	29.00	26.19	23.87	28.82
min [C_T]	24.49	25.76	23.49	25.09	26.63	23.77	21.78	27.34
max [C_T]	29.66	31.31	28.89	30.08	32.27	29.16	25.81	31.68
SD [± C_T]	1.15	1.13	1.22	1.15	1.27	1.21	0.99	1.35
CV [%C_T]	4.31	3.97	4.63	4.18	4.38	4.61	4.17	4.68

n, number of plant samples over all treatments; C_T, fractional cycle threshold; GM [C_T], geometric mean of C_T value; AM, arithmetic mean; Min and Max [C_T], the extreme values of C_T; SD, the standard deviation of the C_T value; CV, coefficient of variance as percentage.

gave different results. All of them agreed in qualifying RPL18 as the least stable HKG candidate. In contrast, each program differed in the composition and rank of the most stably expressed HKG candidates (Table 5). These discrepancies may be caused by the differences between the approaches.

A priori, one would suggest that the approach in Bestkeeper is less reliable than the other 2 procedures because it inherently suffers from the circular problem of finding any standardization in the first place, as outlined in the introduction (see also Radonic et al. 2005). Also, any approach using the standard deviation of C_T values across experimental treatments cannot take difference in RNA input or of reverse transcription efficiency into account, while all of these variables will inflate the unexplainable variance. In contrast, NormFinder and geNorm correct for intersample variation. Here, all genes are compared pairwise within a single sample to produce ratios that are then further examined across treatment groups. That the approaches implemented in geNorm and NormFinder largely overlapped in their suggestions of suitable HKGs (Table 5) indicates that their principal approach, namely the serial cross-validation comparing all pairwise combinations of genes within single samples, is robust.

It is important to test the stability of the potential candidates under the relevant experimental condition. To have an idea how different temperature contrasts alone can affect the choice, we have compared the candidates selected when the analyses are done with 3 widely ranging temperature conditions (4, 20, and 25°C) or only 2 conditions (20 and 25°C). Obviously, only the latter range represents a realistic simulation of summer sea surface temperatures. One interesting outcome of our experimental assays is that different HKGs are selected under the more realistic temperature stress conditions than when using a wide range of (unrealistic) temperatures.

It is difficult from our data set to evaluate the absolute accu-

Table 3. Stability of different housekeeping gene candidates according to the approach implemented in geNorm (as *M* value) (Vandesompele et al. 2002).

Three temperature treatments (4/20/25°C)	Gene							
	eIF4B	Cyp1	Cyp2	RPL28	TBP	Elo_F	RPL17	RPL18
Stability value (*M*)	0.817	1.067	1.333	0.911	0.816	1.002	1.280	1.191
Final result	0.429				0.359	0.536		
Two temperature treatments (20/25°C)								
Stability value (*M*)	0.520	0.508	0.542	0.540	0.464	0.465	0.575	1.189
Final result	0.325				0.251	0.307		

Low *M* values indicate high expressional stability. Final result gives the novel *M* values for the group of 3 genes that are considered to be the most stable, after elimination of the 5 less stable genes.

Table 4. Expression stability of 8 candidates of housekeeping genes in eelgrass (*Zostera marina*) using the approach implemented in NormFinder (Andersen et al. 2004).

Three temperature treatments (4/20/25°C)	Gene							
	eIF4B	Cyp1	Cyp2	RPL28	TBP	Elo_F	RPL17	RPL18
Stability value	0.23	0.53	0.77	0.33	0.26	0.52	0.76	0.52
Two temperature treatments (20/25°C)								
Stability value	0.05	0.07	0.13	0.11	0.04	0.08	0.14	0.42

Stability values compare the variability in gene expression between and within experimental treatments (groups). Best combination (combined stability value) for 3 temperature treatments: Cyp1 and Elo_F, 0.101; for 2 temperature treatments: eIF4B and TBP, 0.033.

racy of the approaches investigated here because we lack an independent standardization. In comparing the NormFinder and geNorm approach, Andersen et al. (2004) showed that NormFinder may be more sensible than geNorm in detecting unstable gene expression (see also Huggett et al. 2005). When the targets were normalized by NormFinder's candidate genes, correct expression levels were obtained, whereas in the reciprocal analysis, the true expression was overestimated. As a cautionary measure, we therefore recommend using the best pair of housekeeping genes given by NormFinder, translation initiation factor and TATA box binding protein. Note, however, that in case of the use of a single housekeeping gene, the recommendations based on NormFinder and geNorm are identical (TATA box binding protein).

We also note in our data set that NormFinder and geNorm are more suited than Bestkeeper given that their suggestion of the best HKG candidate is less affected by addition of another experimental condition (Table 5). In both sets of temperature conditions, the 3 most stable candidates selected were always the same in geNorm and NormFinder (Table 5). In contrast, the ranks of candidate genes changed considerably with temperature treatment range according to Bestkeeper.

For each experimental setup using QPCR, the choice of reference (housekeeping) genes must reflect the tissue used and the experimental treatment. If this is not the case, unsuitable housekeeping genes may be selected, biasing the final results obtained from any target gene experiment (Brunner et al. 2004; Nicot et al. 2005). Devoting considerable effort into the quest for stably expressed housekeeping genes cannot be overemphasized. For example, we performed a simple calculation of the expression data in hypothetical target genes. Had we used the least stable HKG candidates (RPL18 and Cyp2), we would have erroneously inferred a 2-fold downregulation of a hypothetical target gene with constant expression across the imposed temperature treatments. This highlights how results from real-time PCR–based gene expression assays are influenced by the choice of housekeeping genes if they themselves are differentially expressed across the experimental treatments.

Our case study revealed the importance of pilot studies for selecting the best reference genes in gene expression analysis for the first time in a marine plant. As first genomic resources in the cosmopolitan coastal plant *Zostera marina*, EST libraries are currently being developed and sequenced (TBH Reusch unpublished), with several additional marine species where genomic resources are available or will soon become available (Hofmann et al. 2005). With the present work, we provide different potential housekeeping gene primer sequences for QPCR assays in *Zostera marina* and suggest 2 programs, NormFinder and geNorm, to validate their expression stability. Obviously there is no "one good gene for all experiments"

Table 5. Stability of housekeeping gene expression in *Zostera marina*.

	Bestkeeper		geNorm		NormFinder	
Rank*	4/20/25°C	20/25°C	4/20/25°C	20/25°C	4/20/25°C	20/25°C
1	RPL17*	RPL17	eIF4B	TBP	eIF4B	TBP
2	Elo_F	CYP1	TBP	Elo_F	TBP	eIF4B
3	eIF4B	RPL28	Elo_F	eIF4B	RPL28	CYP1
4	TBP	eIF4B	RPL28	CYP1	Elo_F†	Elo_F
5	RPL18	Elo_F	CYP1	CYP2	RPL18	RPL28
6	RPL28	CYP2	RPL18	RPL28	CYP1†	CYP2
7	CYP1	TBP	RPL17	RPL17	RPL17	RPL17
8	CYP2	RPL18	CYP2	RPL18	CYP2	RPL18

Ranks of housekeeping gene candidates from most until least stable ones obtained from three approaches, for two ranges of experimentally imposed temperature treatments.

*For normalization using single candidate genes only.

†Combinations of most stable genes when differing from most stable single genes.

recommendation. For each experimental treatment, a novel housekeeping gene validation will become necessary. Some of the nonsuited candidate genes with respect to temperature as a treatment (Table 1) may be good HKGs in other experiments or using other tissue types. Our shortlist may be helpful to find putative candidate genes for future experiments that address other environmental variables as treatment factors.

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