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TECHNICAL NOTE

The choice of reference gene set for assessing gene expression in barley (*Hordeum vulgare* L.) under low temperature and drought stress

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Abstract Drought and low temperature are the two most significant causes of abiotic stress in agricultural crops and, therefore, they pose considerable challenges in plant science. Hence, it is crucial to study response mechanisms and to select genes for identification signaling pathways that lead from stimulus to response. The assessment of gene expression is often attempted using real-time RT-PCR (qRT-PCR), a technique which requires a careful choice of reference gene(s) for normalization purpose. Here, we report a comparison of 13 potential reference genes for studying gene expression in the leaf and crown of barley seedlings subjected to low temperature or drought stress. All three currently available software packages designed to identify reference genes from qRT-PCR data (GeNorm, NormFinder and BestKeeper) were used to identify informative sets of up to three reference genes. Interestingly, the data obtained from the separate treatment of leaf and crown have led to the recommendations that *HSP70* and *S-AMD* (and possibly *HSP90*) to be used as the reference genes for low-temperature stressed leaves, *HSP90* and *EF1α* for low-temperature stressed crowns, *cyclophilin* and *ADP-RF* (and possibly *ACT*) for drought-stressed leaves, and *EF1α* and *S-AMD* for drought-stressed crowns. Our results have demonstrated that the gene expression can be highly tissue- or organ-specific in barley and have confirmed that reference gene choice is essential in qRT-PCR. The findings can also serve as guidelines for the selection of reference genes under different stress conditions and lay foundation for more accurate and widespread use of qRT-PCR in barley gene analysis.

Keywords Crowns · Drought · Leaves · Low temperature · Normalization · Real-time RT PCR

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Introduction

Humans have been using barley as food or barley-based alcoholic drink and for animal feed for centuries. To this end, barley production ranks fourth among the cereals following maize, rice and wheat (FAO 2013). Increased interest in nutrition and health together with barley's reputation as a stress-tolerant crop bode well for its present and future usefulness. However, the information on barley genome expression and adaptation capacity is lacking. During a typical life cycle and due to climatic changes, plants undergo environmental stresses, such as low temperatures or long-term water deficiencies. To survive such adverse conditions, plants employ strategies that trigger a cascade of events that alter gene expression and lead to biochemical and physiological changes (Achard et al. 2008). The molecular responses to cold, heat, drought, and salt have been evaluated in different species by monitoring genes with enhanced expression in plants under environmental stress (Nicot et al. 2005; Milella et al. 2006; Cao et al. 2011; Ovesná et al. 2011; Rapacz et al. 2012).

Real-time quantitative PCR (qRT-PCR) represents the current state-of-the-art approach for measuring gene expression; and the method has numerous applications in both biology and biomedicine (Vandesompele et al. 2002; Terzi et al. 2010). In cereal crops, the correct targeting of gene expression with qRT-PCR demonstrated to be crucial (Demidenko et al. 2011; Rapacz et al. 2012).

Although qPCR is a robust technique, results can vary depending on factors such as RNA integrity, reverse transcriptase (RT) efficiencies, sample-to-sample variations in amplification efficiency, and variation in cDNA sample loading. Using equal sample sizes, assessing RNA integrity and equalizing RNA concentrations prior to RT are fundamental normalization steps in qPCR. Still, normalization to some internal control is essential for accurate qPCR to balance sample-to-sample variations within the RT and PCR reactions. Currently, the preferred internal control is achieved using reference genes or better a normalization factor based on several reference genes calculated using e.g. geNorm or other statistic tools (Nicot et al. 2005; Martin et al. 2008; Cao et al. 2011; Sharoni et al. 2012; Ovesna et al. 2012). The sensitivity of the method relies heavily on the choice of reference gene(s) to normalize the expression data (Wong and Medrano 2005) and the general strategy is to choose gene(s) the expression of which is as much as possible independent of the environmental treatments under consideration and the tissue types being assayed (Faccioli et al. 2007; Migocka and Papierniak 2011; Ovesna et al. 2012). This requirement is, at best, only approximately satisfied with the most commonly used reference genes, such as those encoding ubiquitin, actin or tubulin. Previous papers suggested the use of multiple reference genes to overcome this weakness, (Vandesompele et al. 2002; Rapacz et al. 2012). The reason is that in contrast to plant resistance to biotic stress, the response to abiotic stress is a complex and multigene-controlled mechanism (Vinocur and Altman 2005; Milella et al. 2011). Moreover, a strong interaction was observed between barley leaf response to drought and developmental factors (Rapacz et al. 2012). Thus, to improve drought and temperature tolerance, a better understanding of the genetic bases and the mechanism of drought response in different plant tissues is required. To address this issue, our study is focused on the drought- and cold-induced changes in the expression of genes involved in signaling and regulatory pathways or genes encoding proteins related to stress tolerance. Here, we assess the utility of a selection of reference genes to act as internal standards for a qRT-PCR experiment targeting for the first time the different response of barley tissues to low temperature and drought stress.

Materials and methods

Plant material and stress treatments

Low-temperature stress

Grain of the barley cvs. Luxor (very winter hardy), Igri (mildly winter hardy) and Atlas 68 (spring type) were pre-germinated and the seedlings were raised under a 12 h photoperiod (irradiation intensity $\sim$200 µmol m^{-2} s^{-1}) and a day/night temperature of 18/13 °C. When the second leaf was fully expanded, the seedlings were exposed for 3 weeks to +3 °C during the lit hours (irradiation intensity $\sim$120 µmol m^{-2} s^{-1}) and +2 °C during the dark hours. At the end of this acclimation period, the seedlings were subjected to −3 °C for 24 h. Destructive samples of the second leaf and the crown were taken in the middle of the lit period after 0, 1, 3, 7 and 21 days at +3/2 °C and following the −3 °C treatment.

Drought stress

Grain of the barley cvs. Amulet (drought tolerant) and Tadmor (highly drought tolerant) were pre-germinated and the seedlings were raised under a 14 h photoperiod (irradiation intensity $\sim$350 µmol m^{-2} s^{-1}) and a day/night temperature of 25 °C/20 °C. Well-watered 16-day-old seedlings (30 wt%) were taken as a control, while the drought treatment consisted of withholding water for 9 days from 11-day-old seedlings (10 wt% Amulet, 11 wt% Tadmor).

RNA isolation

Plant tissue was snap–frozen and stored at −80 °C for <2 weeks before being used for RNA extraction based on the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The resulting RNA was purified by passage through an RNeasy column in the presence of DNase (Qiagen, Hilden, Germany). RNA quality was assessed both by agarose gel electrophoresis and by analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA). Each biological sample was represented by three replicates, made up from the bulked tissue harvested from four seedlings.

Primer design

Target cDNA sequences were derived from the Affymetrix Barley Genome Array EST. PCR primers were designed using Primer 3 Plus software (Untergasser et al. 2007), and their specificity was verified by a BLAST search of the NetAffx^{TM} Analysis Center and NCBI databases.

Two-step real-time reverse transcription PCR (qRT-PCR)

The RNA was diluted to 150 ng μl^{-1} of which a 2 µl aliquot was used as template in a reverse transcription reaction carried out in a volume of 100 µl using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA), according to manufacturer's protocol. A 2 µl aliquot of the reaction product was then taken as the template for a subsequent 20 µl qRT-PCR containing 7.2 µlH2O, 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and 200 nM of each relevant primer. The amplification regime comprised a 10 min denaturation at 95 °C, followed by 40 cycles of 95 °C/15 s and 60 °C/60 s. The signal was recorded during the annealing phase of each cycle. Melting curves of PCR products were also recorded. The specificity of the amplicon was checked by electrophoresis through a 2 % w/v agarose gel and the melting curves were evaluated (data not shown). Three technical replicates of each biological sample were included.

The qRT-PCR efficiency for each target gene was calculated by the formula 100−[ABS (100−(10^{-1}/slope−1) × 100)]. Only sequences associated with an efficiency of >90 % were taken forward (Table 1). The suitability of each candidate reference gene for use with a particular abiotic stress and organ was evaluated using three programs implemented within Microsoft Excel, namely GeNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004).

Results

Selection of candidate reference genes, amplification specificity and PCR efficiency

The set of 13 candidate reference sequences was based on prior micro-array-based gene expression experiments

Table 1 Primer sequences of the candidate reference genes, amplicon lengths and amplification efficiencies

Gene name	Primer sequences (forward/reverse)	Length of PCR product (bp)	Amplification efficiency cold	Amplification efficiency drought
ACT	tggatcggagggtccatcct/gcacttcctgtggacgatcgctg	105	1.91	1.97
α-TUB	aaggtccagagggctgtgtg/accagtggacaaaggcacgcttg	115	1.89	1.97
GAPDH	gttggcaaggtgctcccaga/gctcataggtggctggcttg	121	1.90	1.93
IF5A	cgtccaagacctaccctatgcagg/tagcatgaccgtgctttcca	123	1.88	1.91
Cyclophilin	Burton et al. (2004)	122	1.84	1.92
S-AMD	TC130707; (Faccioli et al. 2007)	101	1.92	1.98
EF1α	TC146566; (Faccioli et al. 2007)	101	1.94	1.96
GR	TC146685; (Faccioli et al. 2007)	112	1.89	1.95
ADP-RF	TC138681; (Faccioli et al. 2007)	127	1.90	1.95
HSP70	TC138926; (Faccioli et al. 2007)	102	1.95	1.96
HSP90	TC131381; (Faccioli et al. 2007)	101	1.93	1.98
HOGAPDH	TC146536; (Faccioli et al. 2007)	105	1.88	1.91
SIGPRP	TC139176; (Faccioli et al. 2007)	102	1.93	1.95

ACT actin, *α-TUB* α-tubulin, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *IF5A* translation elongation factor 5A, *S-AMD* homologue of S-adenosylmethionine decarboxylase, *EF1α* elongation factor 1α, *GR* homologue of an RNA-binding glycine-rich protein, *ADP-RF* ADP-ribosylation factor 1-like protein, *HSP70* homologue of heat shock protein 70, *HSP90* cytosolic heat shock protein 90, *HOGAPDH* homologue of a putative glyceraldehyde-3-phosphate dehydrogenase, *SIGPRP* similar to *GPRP* (proteins rich in glycine and proline)

carried out in the leaf and crown of cv. Luxor (Janská et al. 2011). Four of the qRT-PCR primer pairs were designed from the Affymetrix barley arrays sequence; the genes targeted were *Actin* (*ACT*, Contig1390_3_s_at), *α-Tubulin* (*α-TUB,* Contig333_3_x_at), *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*, Contig865_3_s_at) and *Translation elongation factor 5A* (*IF5A,* Contig2580_3_s_at). The primer sequences for the remaining genes were obtained from the literature (Table 1, references).

The qRT-PCR conditions for each target were optimized to provide a single amplicon of expected length, and controls were included (genomic DNA, no template, qRT-PCR mastermix). The negative controls proved uniformly negative with respect to amplification, and neither primer-dimers nor non-specific products were detected. The amplification efficiency of each of the candidate reference genes is shown in Table 1.

Expression stability of candidate reference genes

Ct values, representing the mean of the three technical replicates included, varied from 17 to 24 for the low-temperature stress (Online resource 1) and from 18 to 25 for the drought stress (Online resource 2). The Ct values were used either directly (BestKeeper), or transformed using a comparative Ct method (GeNorm and NormFinder).

GeNorm analysis

GeNorm software generates a measure of gene stability *M* for each gene, and calculates the measure of pairwise variation *V* which reflects inherent machine, enzymatic and operator variation (Vandesompele et al. 2002). The most stable genes for the cold treatment (the lowest *M* value)

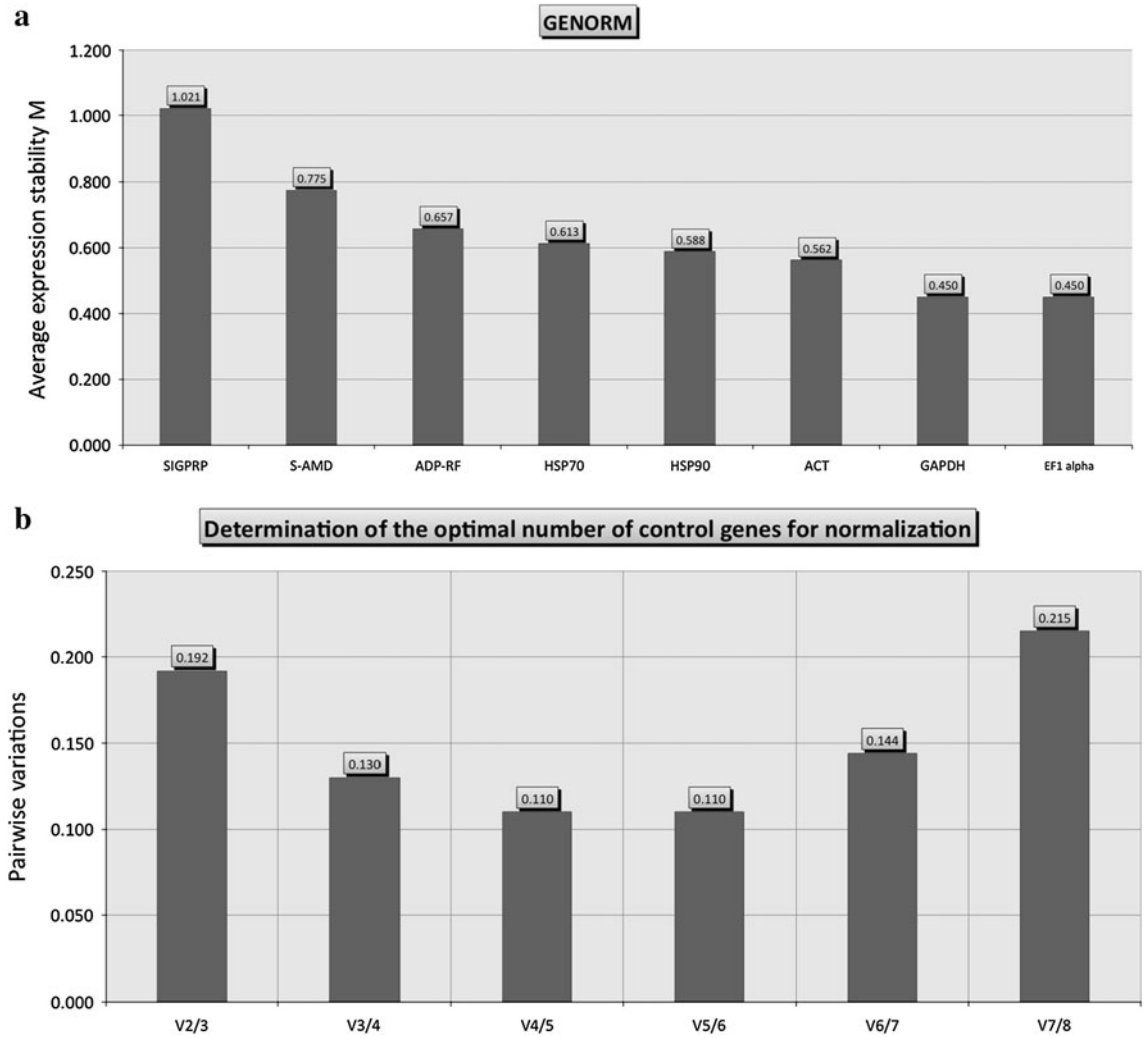

Fig. 1 **a** Determination of the most stable reference genes in the low-temperature stress treatment according to GeNorm. For GeNorm, the most stable genes are those with the lowest values. **b** Determination of the optimal number of reference genes. A pairwise variation <0.15 indicates no significant contribution made by the inclusion of an additional reference gene. The optimal number of reference genes was three

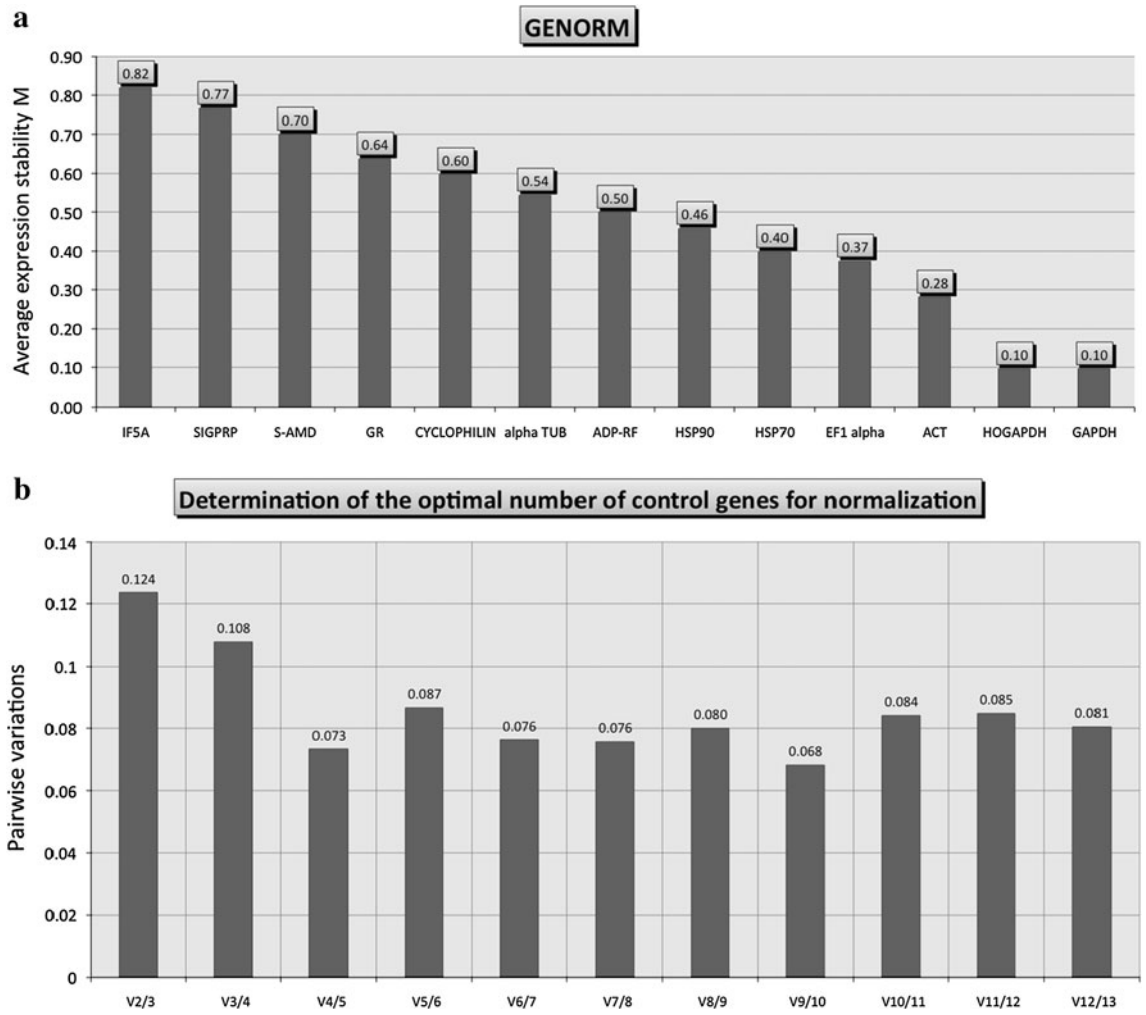

Fig. 2 **a** Determination of the most stable reference genes in the drought stress treatment according GeNorm. For GeNorm, the most stable genes are those with the lowest value. **b** The determination of the optimal number of reference genes. A pairwise variation <0.15 indicates no significant contribution made by the inclusion of an additional reference gene. The optimal number of reference genes was two

were *EF1α* (0.45), *GAPDH* (0.45) and *ACT* (0.56), and the least stable was *SIGPRP* (1.021) (Fig. 1a). The pairwise variation *V*, based on the comparison between NFn (normalization factor) and NF_{n+1}, resulted in a suggested number of reference genes for the cold treatment of three, because the *V* value of the third best-performing gene was 0.13 (Fig. 1b), which is below the recommended threshold of 0.15 (Vandesompele et al. 2002). The best-performing set of three reference genes for the drought treatment was *GAPDH* (0.096), *HOGAPDH* (0.096) and *ACT* (0.281), and the least stable gene was *IF5A* (0.819) (Fig. 2a). In this case, the recommended number of reference genes was two (the *V* value for the second best gene was 0.124) (Fig. 2b).

NormFinder analysis

NormFinder estimates the overall expression variation of the candidate reference genes and assesses the variation between sample subgroups. Its output is a stability value for each candidate reference gene, with the lowest value indicating the most stable expressed gene (Andersen et al. 2004). The best-performing genes for the cold treatment were *HSP70* (0.149), *ADP-RF* (0.169) and *ACT* (0.215), while for the drought stress, the genes were *ADP-RF* (0.128), *EF1α* (0.162) and *GAPDH* (0.222), and the least stable genes for the two stress treatments were *SIGPRP* (1.181) and *IF5A* (0.717), respectively.

BestKeeper analysis

The BestKeeper calculation is based on the calculation of a Pearson correlation coefficient between the "BestKeeper index" (a geometric mean of candidate reference genes' Ct values) and the pairwise correlation of all possible gene pairs (Pfaffl et al. 2004). In contrast to the other two methods, BestKeeper operates on raw Ct values. Genes

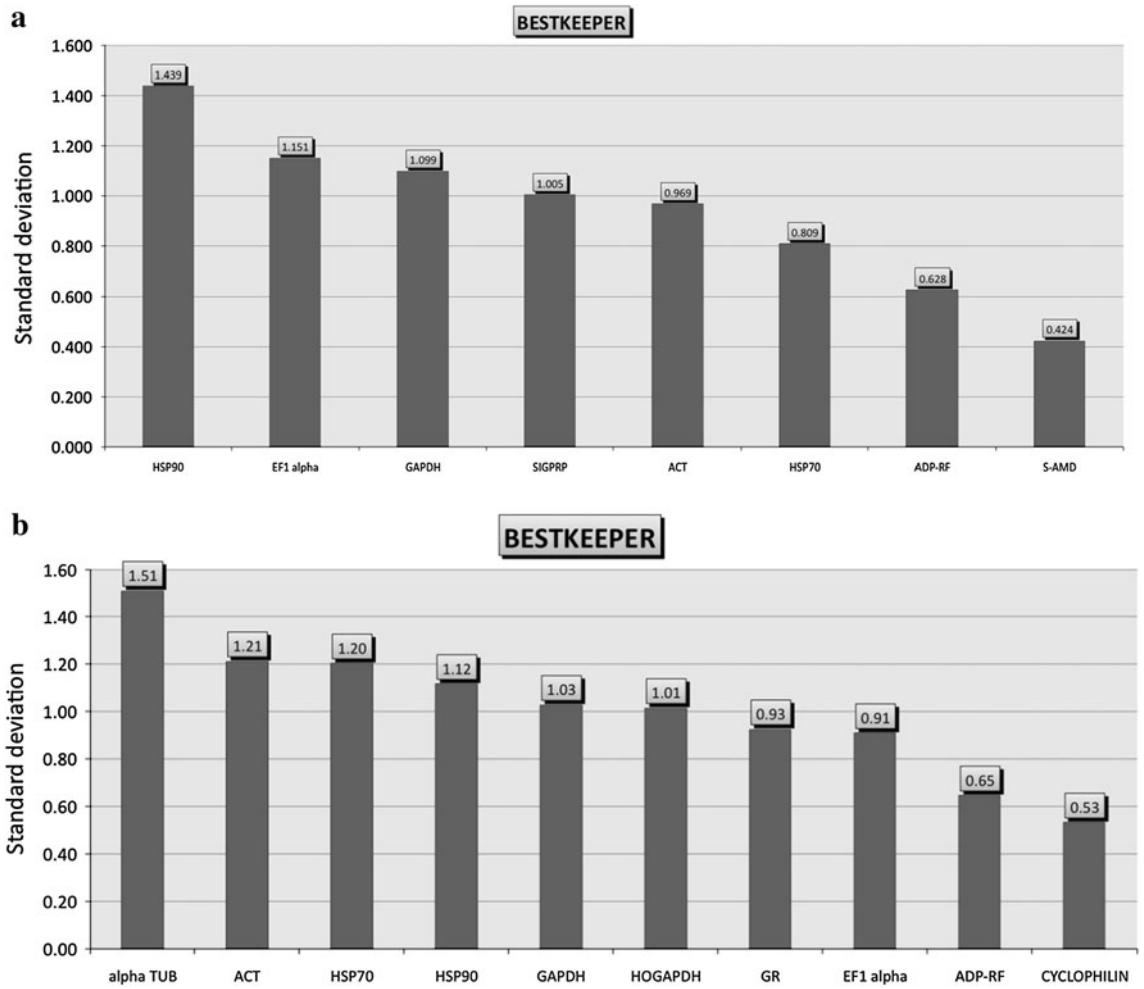

Fig. 3 Determination of the most stable reference genes in the low temperature (**a**) and in the drought stress (**b**) treatments according to BestKeeper, the higher the correlation coefficient, the more stable the gene's expression, the lower the standard deviation (SD), the more stable the gene's expression

associated with a Ct value standard deviation (SD) of >1 are considered inconsistent and are discarded. The best correlations were obtained for *ACT* (0.968), *HSP70* (0.937) and *EF1α* (0.927) in the low-temperature treatment, and for *α-TUB* (0.989), *HSP70* (0.985) and *GAPDH* (0.979) in the drought treatment. The least stable genes were *SIGPRP* (−0.2) and *GR* (0.843), respectively. The full list of genes showing an SD < 1 were *S-AMD*, *ADP-RF*, *HSP70* and *ACT* in the low-temperature treatment (Fig. 3a) and *cyclophilin*, *ADP-RF*, *EF1α* and *GR* in the drought treatment (Fig. 3b). The overall SDs for the two methods were 0.64 and 0.99, respectively, and, therefore, was considered for further work.

Sets of reference genes dependent on organ type and nature of the abiotic stress treatment

The conclusions were based on the full data set, so an analysis was also carried out separately for the leaf and crown. The *V* parameter produced by GeNorm was used to determine the optimal number of reference genes. Across the full data sets, two reference genes appeared to be sufficient for normalization in the leaf (Fig. 4a, b), but not in the crown (Fig. 5a, b) and, therefore, had to be extended to three reference genes. In the leaves exposed to low-temperature stress, GeNorm identified the three reference gene sets as *S-AMD* (0.325), *HSP70* (0.325) and *ADP-RF* (0.362), while the NormFinder identified *HSP70* (0.143), *S-AMD* (0.178) and *ADP-RF* (0.202). The BestKeeper correlation coefficient was highest for *SIGPRP* (0.915), *HSP90* (0.904) and *S-AMD* (0.903). The BestKeeper SD for all of the candidate genes was <1 (data not shown). In the crowns exposed to low temperature, the gene sets selected by GeNorm, NormFinder and BestKeeper were *ACT* (0.192), *HSP90* (0.192) and *HSP90* (0.039), and *ACT* (0.131), *HSP70* (0.963) and *S-AMD* (0.957), respectively. A similar analysis focused on the drought-stressed leaf resulted in the choice of *HOGAPDH* (0.101), *GAPDH*

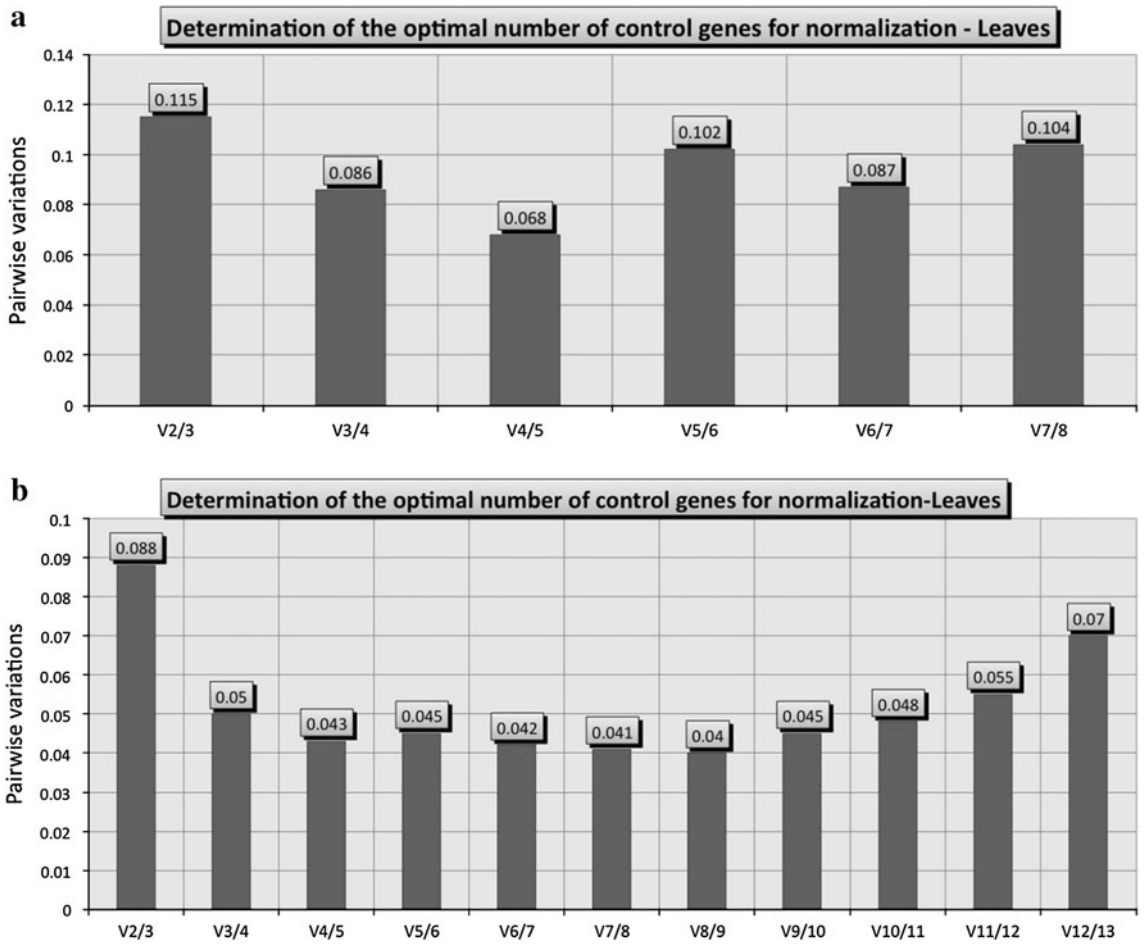

Fig. 4 Determination of the most stable reference genes in the leaf of low temperature (**a**) and drought (**b**) stressed plants. The determination of the optimal number of reference genes. A pairwise variation <0.15 indicates no significant contribution made by the inclusion of an additional reference gene. The optimal number of reference genes was two in both cases

(0.101) and *cyclophilin* (0.214) using GeNorm; *ADP-RF* (0.052), *cyclophilin* (0.081) and *HOGAPDH* (0.127) using NormFinder; and *ACT* (0.994), *cyclophilin* (0.987) and *ADP-RF* (0.987) through BestKeeper software. In the drought-stressed crown, the two optimal reference genes were *S-AMD* (0.077) and *EF1α* (0.077) using GeNorm, *EF1α* (0.088) and *IF5A* (0.113) using NormFinder, while the BestKeeper showed *GAPDH* (1.00) and *HOGAPDH* (0.999). The SD for all these genes was <1 (data not shown).

Expression of a non-reference gene based on different sets of reference genes

As an example of how the estimation of the expression level of a non-reference gene can depend on the choice of reference gene(s), the leaf and crown expression of *RS*, a gene encoding raffinose synthase, during the low-temperature stressed treatment was monitored. The reference gene sets *ACT*, *HSP70*, *ADP-RF*, *GAPDH* and *SIGPRP* and their combinations (*ACT* + *HSP70* + *ADP-RF*, *GAPDH* + *ACT* + *HSP70*, *ACT* + *HSP70*) were chosen. However, the single reference genes were considered as the least stable of all the potential reference genes. In all three varieties, the expression of *RS* in the leaf increased during the acclimation period peaking after day one at +3 °C. The *RS* gene expression was further enhanced when the plants were exposed to −3 °C. This general temporal expression profile was consistent with whichever set of reference gene(s) was used but with the exception of *SIGPRP*. However, the estimated level of expression depended on the choice of reference gene(s) (Fig. 6a). *RS* expression in the crown was rather different than in the leaf, showing a mild increase over the first day at +3 °C, and then a very marked increase when the plants were exposed to −3 °C, irrespective of the choice of reference gene(s). When the expression was normalized based on *SIGPRP* as a reference, the estimated *RS* expression level was very different from all the other estimates. Similar to leaf tissue, the combinations *ACT* + *HSP70* + *ADP-RF*, *GAPDH* +

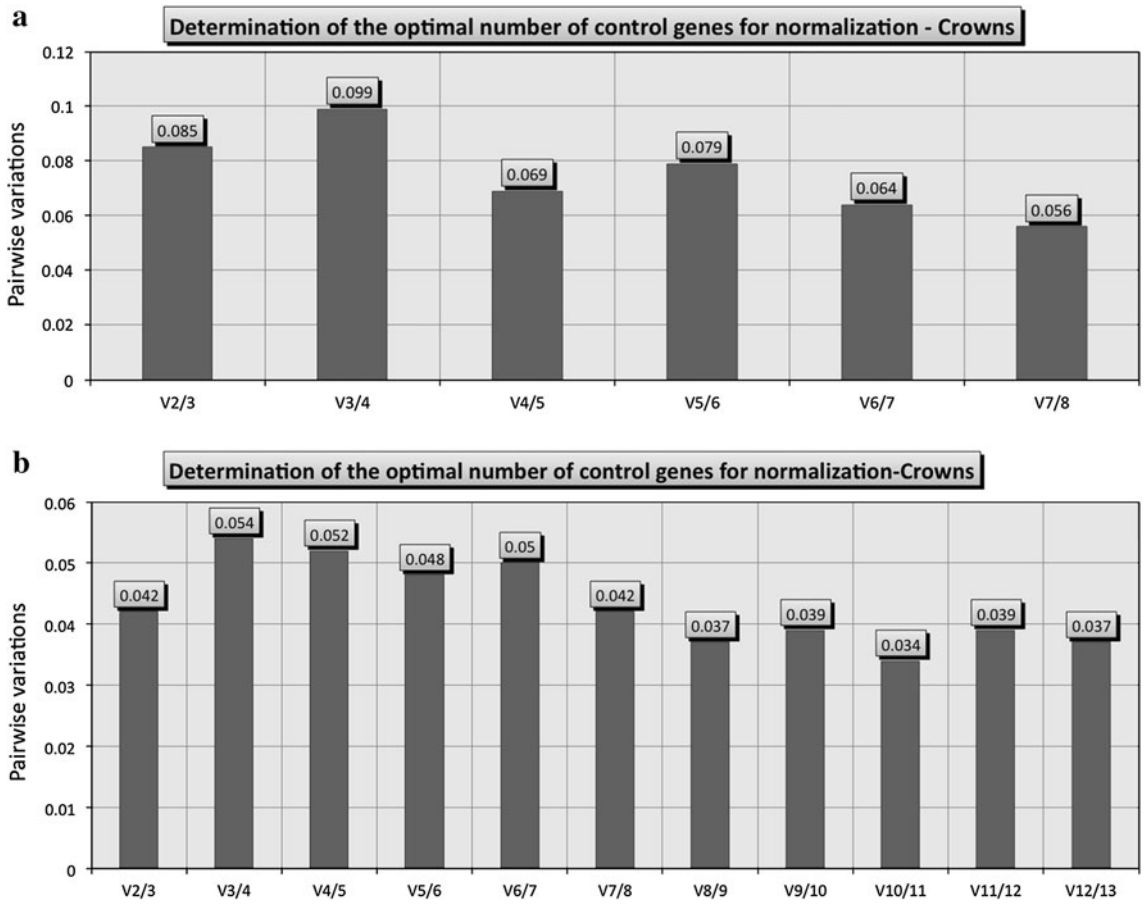

Fig. 5 Determination of the most stable reference genes in the crown of low temperature (**a**) and drought (**b**) stressed plants. The determination of the optimal number of reference genes. A pairwise variation <0.15 indicates no significant contribution made by the inclusion of an additional reference gene. The optimal number of reference genes in this case was three

ACT + *HSP70*, *ACT* + *HSP70* can be expected to give a better level of precision than the reliance on just a single reference gene (Fig. 6b).

Discussion

qRT-PCR has been widely exploited to assess gene expression (Bustin and Dorudi 1998; Czechowski et al. 2004; Maciá-Vicente et al. 2009; Terzi et al. 2010; Zampieri et al. 2010; Takahashi et al. 2010; Chi et al. 2012). It has been recognized that the quality of the data can be affected by a number of experimental factors (Faccioli et al. 2007; Zhong et al. 2011), but many of these problems can be addressed by the appropriate choice of internal controls and the application of appropriate statistical analysis (Faccioli et al. 2007). The assumption is that the reference gene(s) employed should be involved in cellular processes which are as much as possible independent of any exogenous influence (Schmittgen and Zakrajsek 2000; Cappelli et al. 2008), while genes encoding certain specific cytoskeletal proteins, GAPDH, EF1α, 18S or 25S rRNA etc. (Silveira et al. 2009; Zhong et al. 2011) have been recommended, these have not proven to be universally appropriate. Hong et al. (2008) have suggested that any reference gene(s) needs to be validated prior to its use for normalization, while Vandesompele et al. (2002) suggested that a single reference gene cannot provide a sufficient degree of control. A set of potentially informative reference genes were recommended for use in barley by Faccioli et al. (2007), and Rapacz et al. (2012) in relation to barley leaf response to drought stress only and in comparison to developmental factors. Thus, to improve drought and temperature tolerance, a better understanding of the genetic bases and the mechanism of drought response in different plant tissues is required. To address this issue, we describe here a comparison of various candidate reference genes in the context of the response of barley to drought or low-temperature stress, and for the first time targeting the different response of barley leaf and crown to both low temperature and drought stresses It was possible to show that the use of a single reference gene was

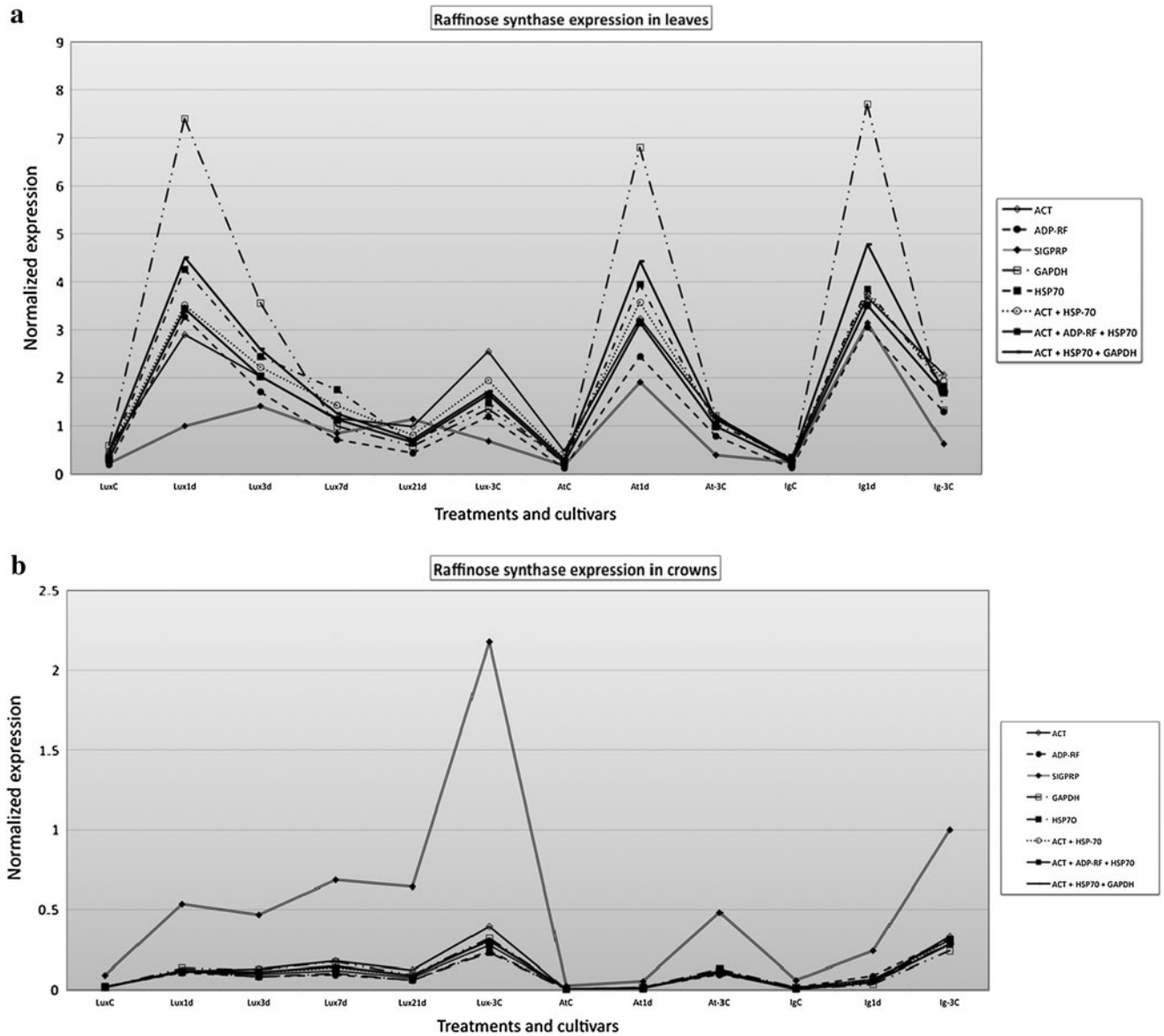

Fig. 6 Normalized expression of RS (encoding raffinose synthase) during low-temperature stress in the leaf (**a**) and the crown (**b**) of the barley cultivars Luxor (Lux), Igri (Ig) and Atlas 68 (At). The expression of RS was normalized using as reference gene(s) either *ACT* + *HSP70* + *ADP-RF*, *GAPDH* + *ACT* + *HSP70*, *ACT* + *HSP70*, *ACT* alone, *HSP70* alone, *ADP-RF* alone, *GAPDH* alone or *SIGPRP* alone

insufficient for normalization purposes. Specifically, raw Ct plots (Online resources 1 and 2) did not allow a clear choice of reference gene.

Three software packages (GeNorm, NormFinder and BestKeeper) have been developed to identify sets of reference genes. As reported by Demidenko et al. (2011), they do not necessarily yield exactly the same set of recommended reference genes from a given data set, but their predictions did not greatly diverge from one another. In barley seedlings exposed to low-temperature stress, the optimal reference genes for estimating gene expression in the leaf were *ACT*, *HSP70* and *ADP-RF*. All three software packages identified the expression of *ACT* as being quite stable, while that of *HSP70* was only classed as very stable by NormFinder and BestKeeper, and that of *ADP-RF* only by NormFinder. Neither *GAPDH* nor *EF1α* featured in the set, because BestKeeper analysis indicated an SD of >1 for both. With respect to the drought treatment, the optimal reference gene set consisted of *GAPDH*, *HOGAPDH* and *EF1α*. *GAPDH* expression was classified as very stable by all three packages, *EF1α* by NormFinder and BestKeeper, and *HOGAPDH* by GeNorm. Although, GeNorm predicted that two reference genes would be sufficient for normalization, a third was included for greater precision. Note that Vandesompele et al. (2002) have made a general recommendation that the reference gene set should include three

members. Given that gene expression can be highly tissue- or organ-specific, there is an argument for optimizing the reference gene set for each tissue/organ under study. A separate treatment of the leaf and crown expression data led to the recommendations that *HSP70* and *S-AMD* (and possibly *HSP90*) be used as the reference genes for low-temperature stressed leaves, *HSP90* and *EF1α* for low-temperature stressed crowns, *cyclophilin* and *ADP-RF* (and possibly *ACT*) for drought-stressed leaves, and *EF1α* and *S-AMD* for drought-stressed crowns.

The results confirmed that reference gene expression is a primary need in qRT-PCR in barley and demonstrated that the correct choice of appropriate genes is essential and strictly related to stress and plant tissues. These results provide for the first time clear guidelines for the selection of reference genes in barley leaf and crowns under temperature and drought-stress conditions. This information would contribute toward more accurate and widespread use of qRT-PCR in barley gene analysis which is crucial to understand response mechanisms and to select genes for a transgenic approach.

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