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Evaluation of reference genes for quantitative RT-PCR in *Lolium temulentum* under abiotic stress

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ABSTRACT

Lolium temulentum L. (Darnel ryegrass) is a valuable model grass species for the study of stress in forage and turf grasses. Gene expression analysis by quantitative real-time RT-PCR relies on the use of proper internal standards. The aim of this study was to identify and evaluate reference genes for use in real-time quantitative RT-PCR for abiotic stress studies in *L. temulentum*. Partial sequences of nine *L. temulentum* housekeeping genes were obtained by RT-PCR using degenerate primers designed from the corresponding genes in closely related species. Primers for quantitative RT-PCR were designed based on these partial sequences. The housekeeping genes were evaluated for their expression stability in samples from plants subjected to different forms of abiotic stress. The analysis found that *eEF-1*α and *UBQ5* were the most stable and *ACT11* was the least stable of the genes tested. Analysis by geNorm indicated that the two most stably expressed housekeeping genes (*eEF-1*α and *UBQ5*) should be utilized and are sufficient for normalization of gene expression during stress-related studies in *L. temulentum*.

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1. Introduction

Significant progress in understanding various aspects of plant biology has been made through the use of model systems. The genetic intractability associated with many grass species is primarily due to the fact that most grasses are generally polyploid, obligate out-crossers that are self-incompatible [1–4]. Despite the phenotypic homogeneity displayed by many of these grasses, their populations possess a high degree of genetic heterogeneity. There can be as much genetic diversity among individual plants of the same variety as there is between varieties of the same species. To further complicate matters, many forage grass species require a vernalization period to induce flowering. Because of their high genetic diversity as well as their growth characteristics, the use of molecular genetic approaches to study stress and tolerance can be impractical. Fortunately, *Lolium temulentum* L. (Darnel ryegrass) lacks many of these negative genetic attributes and is therefore better suited for genetic and molecular analyses than related grasses. *L. temulentum* is a diploid self-fertile species with a short life cycle (2–3 months) [5,6]. Furthermore, *L. temulentum* is able to be out-crossed with other *Lolium* species making it an ideal model system for forage and turf grass research [7–10].

L. temulentum has been used extensively for studying flowering in grasses [11–14]. The use of *L. temulentum* as a model grass system has gained momentum in recent years. The establishment of a responsive anther tissue culture line that can be transformed and subsequently regenerated into fertile plants has recently been reported [10,15]. Additional tools such as molecular markers for the study of diversity in *L. temulentum* germplasm accessions are also available [16]. Recently, suppression subtractive hybridization (SSH) has been used to examine genes expressed during salt stress [17,18] and post-harvest stress [19] in *L. temulentum*. These analyses demonstrate the utility of *L. temulentum* as a model for the identification and isolation of genes associated with stress responses in grasses.

Gene expression analysis of selective genes identified in these SSH screens was performed using Northern analysis. Generally, Northern analysis provides an accurate assessment of gene expression. While Northern analysis is not affected by the presence of inhibitors and small amounts of genomic DNA, only a limited number of samples can be analyzed at once and genes that are expressed at low levels are difficult to detect. Another drawback to Northern blot analysis is its limited ability to quantify expression levels of genes due to the non-linear response or limited dynamic range of signal detection associated with film and imaging systems. Additionally, problems of cross-hybridization can occur when dealing with closely related members of a gene family. To

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Abbreviations: NaCl, sodium chloride; PEG, polyethylene glycol; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; SAGE, serial analysis of gene expression; SDS, sodium dodecyl sulfate; SSH, suppression subtractive hybridization; UV, ultraviolet.

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avoid the resulting false positives, probes can be made to the less conserved 3′ or 5′ non-coding regions of closely related genes and blots can be reprobed. However, these additional steps are time consuming and tedious. Furthermore, as genomic analysis progresses, methods for analyzing gene expression at the level of individual tissues or cell types will become more essential.

Quantitative RT-PCR offers advantages in sensitivity and specificity and has a broad range of detection. It is also valuable when studying rare transcripts or working with members of a multigene family or with small sample sizes [20]. While quantitative RT-PCR has the added advantage of allowing the utilization of high throughput methods, this method is only valid if proper internal controls are included. Proper internal control genes should be expressed consistently in the tissues being examined under different experimental treatments. These internal control genes are used to normalize RT-PCR expression analysis by minimizing differences caused by sampling techniques, the quality and quantity of RNA, the presence of inhibitors in certain tissues and variations in the reverse transcription reaction. Several reports describing proper selection and evaluation of multiple housekeeping genes as internal control genes for accurate normalization of real-time quantitative RT-PCR have been published [21–26]. These studies have validated the necessity of utilizing multiple reference genes when evaluating quantitative RT-PCR data. In many model organisms, most housekeeping gene sequences are available in public databases. Currently, there are limited molecular resources publicly available for forage and turf grass analysis.

In this study we amplified and sequenced regions of nine housekeeping genes from the model grass species *L. temulentum*, designed primers for quantitative RT-PCR and evaluated these genes for normalizing quantitative RT-PCR assays in plants subjected to seven different forms of abiotic stress. The utility of these housekeeping genes as internal control genes for quantitative RT-PCR is presented.

2. Materials and methods

2.1. Plant materials

L. temulentum L. (Darnel ryegrass) cv. Ceres seeds were planted in 4 in.^{2} pots (volume approximately 750 mL) in SB40 Sunshine Growing Mix (Sun Gro Horticulture, Canada). Plants were fertilized weekly using Technigro 20-18-20 all-purpose fertilizer (Sun Gro Horticulture, Canada). Plants were grown in a Conviron E15 (Conviron, Winnipeg, Canada) growth chamber under 8 h photoperiod at 21 °C day and 18 °C night.

2.2. Stress treatments

Plants to be treated with different abiotic stresses were grown for 6 weeks as described above. Pots containing 9–10 plants/pot were then used for various stress treatments described below. The aerial portions of the plants were collected at the described time (below), immediately frozen in liquid nitrogen and stored at −80 °C.

2.2.1. Drought

To induce drought stress, watering was stopped and pots were allowed to dry out overnight. After 24 h some very mild wilting was observed. After 36 h plants displayed signs of wilting and were collected.

2.2.2. UV stress

Plants were laid on their side and irradiated for 5 min. Two hand held 254 nm UV shortwave devices (Model UVG-11, Ultra-violet Products Inc, USA) were held 5–6 in. above the stems and leaves. Plants displayed signs of flaccidity after irradiation and at time of tissue collection. Samples from 6 to 8 plants were collected 1 and 8 h post-treatment.

2.2.3. Heat stress

Plants were subjected to 40 °C in a Conviron E15 Growth Chamber to simulate heat stress. The plants were well watered and placed in a shallow pan of water to maintain adequate hydration during heat stress treatments. Tissue from 6 to 8 plants was collected after 2 and 8 h of heat stress.

2.2.4. Wounding

Plants were mechanically wounded by closing a hemostat perpendicularly across the leaves and stems 3–5 times. Tissue was collected 12 h and 24 h after wounding.

2.2.5. Salt stress

Plants were subjected to salt stress by treating the soil with 500 mL of 500 mM NaCl. Plants showed signs of mild wilting in the leaf blades after 1 h and throughout the duration of the experiment. The aerial portions of 6–7 plants were collected 12 and 24 h after salt treatment.

2.2.6. Osmotic stress

Plants were subjected to osmotic stress by treating the soil with 500 mL of 12.87% polyethylene glycol 6000 solution (PEG). Plants showed mild wilting of leaf blades after 1 h of treatment and more severe wilting as time progressed. The aerial portions of 6–7 plants were collected 12 and 24 h after stress treatment.

2.2.7. Cold stress

Plants were subjected to 4 °C for 24 h. Tissue was collected from plants after 24 h in the cold.

Control tissue was collected from plants that were untreated and watered normally. All tissues were collected, immediately frozen in liquid nitrogen and stored at −80 °C.

2.3. RNA gel blot analysis of genes

Genes coding for delta 1-pyrroline-5-carboxylate synthetase (Accession # EB709850) and *COR413* (Accession # 891238) were subjected to further analysis by RNA blot analysis. Harvested tissue was ground to a powder in liquid nitrogen using a precooled mortar and pestle. Total RNA extractions from these ground tissues were performed using TRIzol reagent (Life Technologies, Gaithersburg, MD) following manufacturer's instructions. Ten micrograms of total RNA isolated from selected/treated *L. temulentum* plant tissues was electrophoretically separated on 1.2% denaturing formaldehyde agarose gels and blotted onto a Hybond N nylon membrane [27]. Inserts of selected cDNAs were amplified using T7 and SP6 primers and a standard three-step PCR protocol with an annealing temperature of 55 °C. The amplified fragments were ethanol precipitated and rehydrated at a concentration of 25 ng/μL. Amplified fragments were used to generate ^{32}P-labeled DNA probes using the Ambion DECAprime II DNA labeling kit (Ambion, USA) for RNA blot analysis. The membranes were hybridized overnight with a ^{32}P-labeled probe in a solution containing 50% formamide, 5× Denhart's solution, 0.1% (w/v) SDS, 6× SSPE, and 100 μg/mL denatured salmon-sperm DNA at 42 °C. Filters were initially washed in 2× SSC and 0.1% SDS at 42–50 °C for 30 min, then subjected to a wash starting at 50 °C and allowing to cool to RT with shaking, and briefly in 0.2× SSC and 0.1% SDS at RT before autoradiography.

Table 1
Reference genes selected for study.

Gene description	Accession numbers of genes used for degenerate primers (primer position based on underlined sequence)	Primer sequences	Amplicon length (bp)	Accession no.
ACT11, Actin 11	AY107106 (*Z. mays*), AY742219 (*S. officinarum*), AY145451 (*H. vulgare*), and AK100267 (*O. sativa*)	^{13}5′-GAGGAYATCCAGCCYCTYGTCTG-3′ ^{1114}5′-CAYTTCCTGTGVACRATNGMBGG-3′	1102	EU328529
CAP, cap binding protein CPB20	PC0131956 (*Z. mays*), XM 466689 Locus OJ1004_A05.24 (*O. sativa*)	^{1835}5′-CAATGGCGTCCCTYTTCA-3′ ^{8875}5′-CSTCTTCTTCATCAGARTCACC-3′	705	EU328530
eEF-1α, eukaryotic elongation factor 1-α	M90077 (*T. aestivum*), D63583 (*O. sativa*), AF136824 (*Z. mays*), Z50789 (*H. vulgare*)	^{5305}5′-GACTCTCGCAAGTCGAC-3′ ^{1671}5′-GGCTTGGTGGGAATCATC-3′	1142	EU328532
eIF-4a eukaryotic initiation factor 4a	AY104966 (*Z. mays*), DQ013263 (*P. glaucum*) AK073620 (*O. sativa*), BT013166 (*L. esculentum*) and X79141 (*N. tabacum*)	^{14}5′-CACCVGARGGYTCYCAGTTYGATGC-3′ ^{1076}5′-GTKGCSAGRTCRTAGTTGATGAC-3′	1063	EU328531
GAPDH, glyceraldehyde-3-phosphate dehydrogenase	M36650 (*H. vulgare*), X60343 (*H. vulgare*), U45856 (*Z. mays*), AK064960 (*O. sativa*), AY857765 (*T. monococcum*), AF251217 (*T. aestivum*)	^{1875}5′-ACCCSTTCATCASCACCG-3′ ^{9745}5′-RAAGATGCTDGACCTGYTGTC-3′	788	EU328533
β-TUB, beta-tubulin	AY742902 (*L. perenne*)	^{15}5′-ACGTCTACTACAACGAGGCC-3′ ^{1759}5′-GCCAATGAAGGTTGATGCC-3′	965	EU328534
UBC, ubiquitin-conjugating enzyme E2	AK059694 (*O. sativa*), BT016339 (*Z. mays*)	^{1465}5′-GCATCTAGRGCWAGGCTYTTYAAG-3′ ^{6095}5′-CCTTTCTTTGGCATGGCAGC-3′	464	EU328535
UBQ5, ubiquitin 5	M60175 (*H. vulgare*), AK061988 (*O. sativa*), BT017442 (*Z. mays*), AY290730 (*T. aestivum*)	^{5565}5′-AAGATGCAGATCTTCGTG-3′ ^{1025}5′-TAMGCCTKCTGRTTGTAGA-3′	470	EU328536
25S, 25S ribosomal RNA	AF479136.1 (*D. zibethinus 26S rRNA*), AF479135.1 (*O. pyramidale 26S rRNA*), AF479199.1 (*V. officinalis 26S rRNA*), AY095471.1 (*B. succulenta 26S rRNA*), and AK119809.1 (*O. sativa 25S rRNA*)	^{1071}5′-ATAACCGCATCAGGTCTCCA-3′ ^{1933}5′-ACCCTTYTGTTCCACACGAG-3′	862	EU328537

Accession numbers of closely related genes from other monocot species which were aligned to design degenerate primers shown in column 3. Primer sequence numbers are based on gene sequence of underlined homolog in column 2. Expected amplicon length and GenBank accession numbers assigned to partial clones obtained from *L. temulentum* utilizing these primers.

2.4. Isolation of candidate housekeeping genes from L. temulentum

Nine candidate housekeeping genes were selected for analysis in this study (see Table 1). All of the genes have known nucleotide sequences in various plant species including two *Lolium* species, but not in *L. temulentum*. Sequences from closely related species were analyzed and used to design degenerate primers (Table 1). Homologous genes from *L. temulentum* were amplified by polymerase chain reaction (PCR) using leaf cDNA as a template. The sequences of the amplified products were subjected to BLAST searches for identity verification.

2.5. RNA isolation and cDNA synthesis

Two samples (biological replicates) representative of each stress treatment were extracted from pooled leaf tissue of several plants. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to determine RNA concentration and quality. The quality of RNA was also assessed by agarose gel electrophoresis. Samples were treated with DNase using the Turbo DNA-free kit (Ambion, Austin, TX) as directed in the manufacturer's protocol. RNA was quantified again after the DNase treatment using the NanoDrop ND-1000 spectrophotometer.

DNase-treated total RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. To produce cDNA for *25S rRNA* expression analysis, 0.5 μg RNA from each sample was reverse transcribed with random primers provided in the kit in a total volume of 20 μL. For expression analysis of the remaining housekeeping genes, 0.9 μg RNA from each sample was reverse transcribed using the kit's oligo-dT primer in a 20 μL reaction.

2.6. Primer design and real-time PCR analysis

Based on the DNA sequences of the PCR products we cloned, real-time PCR primers were designed using Primer3 software [28]. The primer sequences are shown in Table 2. Each PCR reaction contained 10 μL 2× iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 600 nM of each primer and 1 μL diluted cDNA (corresponding to 1 ng of RNA for 25S, and 10 ng of RNA for all other genes) in a total volume of 20 μL. For each primer pair, no-template controls were also run. The real-time PCR reactions were performed using the Bio-Rad iQ5 Real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Primer annealing temperatures were optimized using gradient PCR with annealing temperatures at 55, 57 and 59 °C (5, 3 and 1 °C below primer melting temperature) within the cycling parameters listed below. We chose the annealing temperature with the highest amplification and best product specificity, as determined by melt curve analysis, to use in subsequent reactions. Amplification efficiency for each set of primers was tested prior to the expression studies. For each primer pair, amplification efficiency was calculated as $E = -1 + 10^{(-1/\text{slope})}$ [29], where the slope is derived from a dilution series standard curve of aerial tissue samples of the control treatment. All amplification efficiencies were between 95 and 101% (Table 2). The expression stability reactions were performed under the following conditions: 3 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 55–59 °C (optimal temperature for each primer pair; see Table 2) in a 96-well reaction plate. Dissociation curve analysis was performed for each sample [30] to ensure the specificity of PCR products. One sample from each plate was also analyzed by agarose gel electrophoresis to verify that the product was a single band of the correct size. Two biological replicates for

Table 2
Primers designed for real-time polymerase chain reaction, expected amplicon size and primer pair efficiencies.

Gene	Primer sequence designed for quantitative RT-PCR (5′–3′)	Amplicon length (bp)	Optimal annealing temperature	Efficiency
ACT11	^{774}CCTTTTCCAGCCATCTTTCA ^{873}GAGGTCCTTCCTGATGTCCA	100	57	101%
CAP	^{251}CTCCAGGGAAGATGCTGAAG ^{345}CTTGAAAGCCCCAATCAAAA	95	57	97%
eEF-1α	^{573}CCTTGCTTGAGGCTCTTGAC ^{675}GTTCCAATGCCACCAATCTT	103	57	95%
eIF-4a	^{570}TGCTTTCACGTGGTTTCAAG ^{664}AGGAGGCATGGTAGCAGAGA	95	55	99%
GAPDH	^{682}CTGCGATCAAGGAGGAGTCT ^{776}CCTGTTGTCACCCTGGAAGT	95	57	97%
β-TUB	^{452}GTGCATGGTTCTTGACAACG ^{554}GGTGGCAGAGATGAGATGGT	103	57	95%
UBC	^{318}CAGAGCCATTATTGCGTTGA ^{424}CCATGGACTGGTAGCCTCTG	107	57	95%
UBQ5	^{190}AAGGAGTCAACCCTCCACCT ^{298}TCACCTTCTTGTGCTTGTGC	109	59	97%
25S	^{391}CCCAGTGCTCTGAATGTCAA ^{489}ATGACGAGGCATTTGGCTAC	99	59	96%

each sample were used for real-time PCR analysis and three technical replicates were analyzed for each biological replicate.

2.7. Data analysis

We used iQ5 Optical System Software V. 2.0 (Bio-Rad Laboratories, Hercules, CA) to collect the fluorescence data. The cycle threshold, C_T (the cycle at which the fluorescent signal is significantly different from background), was determined for each reaction. Average C_T and standard deviation of C_T for each sample were estimated based on pooled biological replicates. Outliers were detected using Grubbs' test [31] and were removed from the average C_T calculation. Any replicate showing non-specific products in the dissociation curve analysis were also removed. At least two of the three technical replicates and five of the total replicates (from two biological samples) were included in the average C_T calculations. geNorm v3.4 software [21] was used to analyze gene expression stability. Raw expression values were calculated in Microsoft Excel using the average C_T values and primer efficiencies according to Vandesompele et al. [21].

3. Results and discussion

In order to determine if the plants/tissues to be examined by qPCR were responding to the various stress treatments, two previously characterized stress-related genes [18] were analyzed by Northern blot analysis. The cold responsive *COR413* gene encodes for a membrane protein that may act as a putative G-protein-coupled receptor and is believed to be involved in either stress signaling or membrane stability during cold stress [32,33]. The delta 1-pyrroline-5-carboxylate synthetase gene (*P5CS*) encodes a key enzyme in proline biosynthesis [34]. Proline is an important osmoprotectant produced in plants in response to water related stresses [35–37].

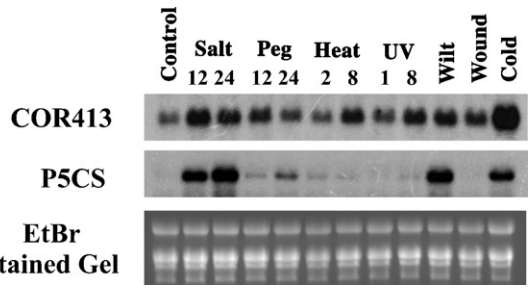

Fig. 1. RNA blot analysis of *P5CS* and *COR413* gene expression in response to different abiotic stresses. Total RNA was extracted from tissue collected from the aerial portions of plants that had been subjected to different stresses. Control represents plants that were untreated and watered normally. Numbers above the lanes represent the number of hours of exposure to the stress (unless stated otherwise). Salt: 500 mM NaCl; PEG: 12.87% PEG 6000; heat: 40 °C; UV: time after 5 min exposure to shortwave ultraviolet light; wilt: no watering for 36 h; wound: 12 h after mechanically wounded 3–5 times perpendicularly across the leaves; cold: 4 °C for 24 h. Each lane was loaded with 10 μg total RNA and run on an agarose gel. Equal loading of RNA per lane was confirmed by visualization with ethidium bromide of the gel before blotting and the nylon membrane after blotting. The RNA blots were hybridized with ^{32}P-labeled DNA probes for *P5CS* and *COR413* genes.

We examined the expression patterns of *P5CS* and *COR413* genes in response to various abiotic stress conditions. Both genes were activated by salt, drought and cold as shown in Fig. 1. Not surprisingly, the cold responsive gene *COR413* showed its highest level of induction in response to the cold treatment. Osmotic stress (PEG treatment) resulted in the activation of the *COR413* gene in a pattern similar to that observed with salt stress, high levels of expression after 12 h of treatment that decreased after 24 h, whereas the *P5CS* gene displayed a very low level of induction after 24 h. It should be noted that symptoms of osmotic stress, such as loss in leaf stature and the initiation of leaf curling in the foliage of tillers, were clearly visible in treated plants 12 h after the PEG treatment. The *COR413* gene was induced by heat stress, UV light and mechanical wounding, while the *P5CS* gene showed no induction in response to these stress treatments. The differences in expression patterns displayed by the *COR413* and *P5CS* genes demonstrated that the plants responded to the various stress treatments and validated the plant tissues that were used for the qPCR analysis.

We utilized homology-based PCR cloning and sequencing of nine genes from *L. temulentum* that have been used as reference genes for quantitative RT-PCR analysis in other species (Table 1). Internal primers for quantitative RT-PCR were developed and tested for specificity and efficiency using SYBR-green-based quantitative RT-PCR on the Bio-Rad iQ5 Real-time PCR detection system. All primers were gene-specific and efficiencies ranged from 95 to 101% (Table 2). Efficiencies were used in calculations for subsequent experimental data. Specificity of real-time PCR products was verified by representative dissociation curves and agarose gel electrophoresis as shown in Fig. 2. All dissociation curves for the gene products displayed similar single peak patterns. To determine the optimal reference gene(s) for stress-related gene expression studies, we measured the expression levels of nine housekeeping genes in 12 different stress samples, representing 7

Table 3
Stress conditions used for gene expression analysis.

Sample	Description
1	Control: 6-week-old plant
2	Cold, 4 °C for 24 h
3	Heat, 40 °C for 2 h
4	Heat, 40 °C for 8 h
5	PEG, 12 h
6	PEG, 24 h
7	Salt, 12 h
8	Salt, 24 h
9	UV, 1 h
10	UV, 8 h
11	Wound, 12 h
12	Wound, 24 h
13	Wilt, 12 h

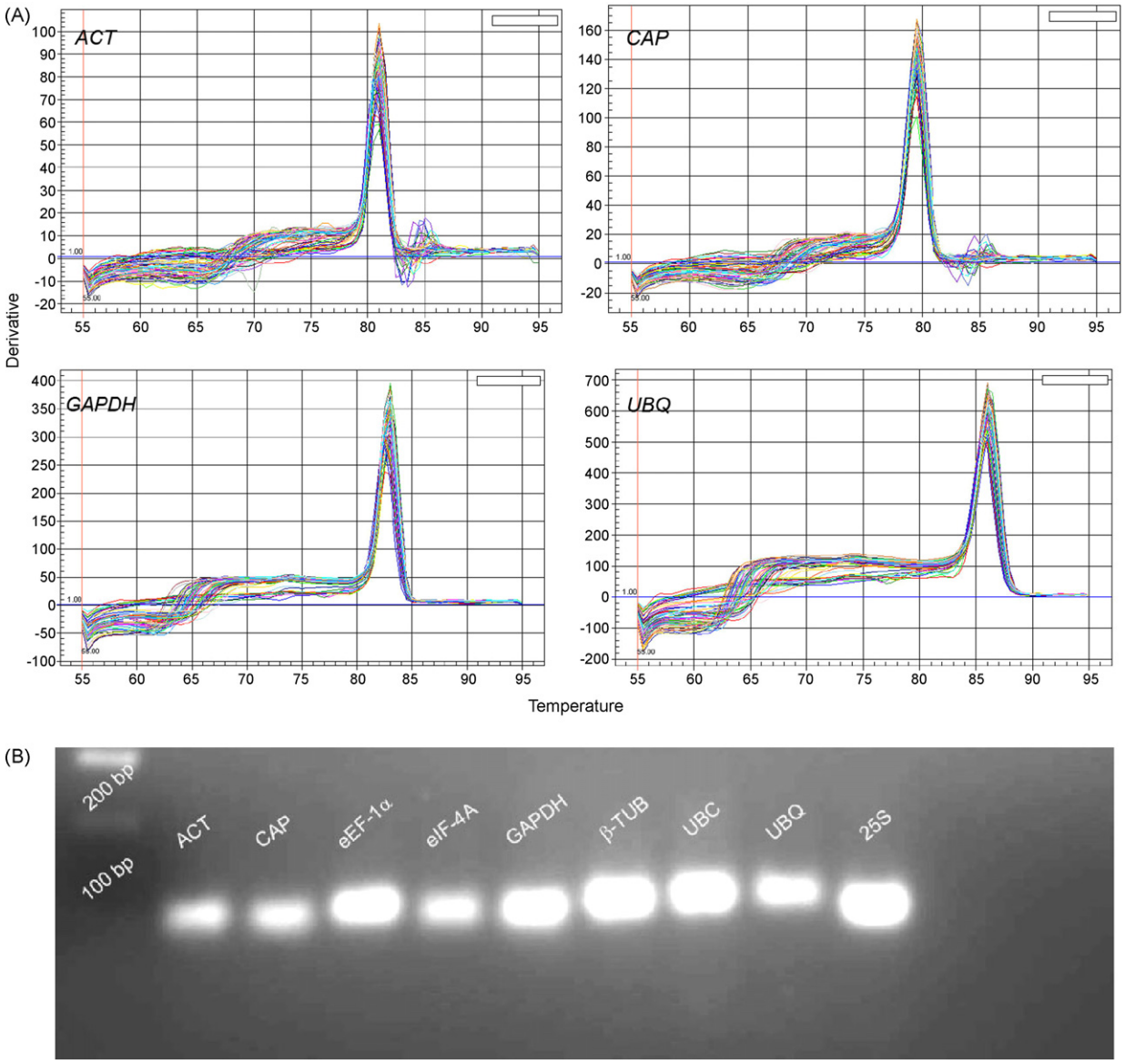

Fig. 2. Verification of specific products from real-time RT-PCR reactions. (A) Representative dissociation curve analysis for *ACT*, *CAP*, *UBQ* and *GAPDH* showing a single peak. All genes tested displayed single peaks. (B) 1.5% agarose gel image showing specific PCR products of expected size for each housekeeping gene tested.

different forms of abiotic stress (Table 3). Average C_T values for all transcripts except *25S rRNA* ranged from 17.6 to 24.6 (Table 4). The average C_T value for *25S rRNA* was 8.6. Therefore, abundance of *25S rRNA* was several orders of magnitude greater than that of other genes, especially if one considers that the amount of cDNA used in the real-time PCR reaction was only 1/10th of that used for the other genes. Of the remaining transcripts, *UBC* was the least abundant and *eEF-1α* was the most abundant.

The geNorm v3.4 software was used to calculate gene expression stability (*M*) for each internal control gene tested. For each gene, the program determines the pairwise variation with all other housekeeping genes as the standard deviation of the logarithmically transformed expression ratios. *M* is the average pairwise variation of a particular gene with all other control genes in a set. A lower *M*-value denotes a more stable gene expression. The program then performs stepwise exclusion of the gene with the highest *M*-value (lowest stability) until the two most stable genes are left [21]. For our data set, *eEF-1α* and *UBQ5* had the lowest *M*-values indicating that they were the most stably expressed of the internal control genes tested (Fig. 3). The *M*-value for *ACT* was the highest indicating that it was the least stably expressed of the genes tested in our samples (Fig. 3).

It is often desirable or even necessary to use more than a single internal control gene for normalization in expression studies. Therefore, an additional function of geNorm is to calculate a normalization factor (NF*n*, *n* = the number of genes included) for the most stable control genes, then for other genes by stepwise inclusion of the next most stable gene. Afterwards, pairwise variations ($Vn/n+1$) between two subsequent normalization factors are calculated, indicating the effect of including one additional gene for normalization. If *V* is large, then inclusion of the subsequent gene for normalization has a significant effect. The recommended cut-off value of 0.15 for *V* to be significant was used in our study [21]. When analyzing housekeeping genes for our samples, the inclusion of the two most stably expressed housekeeping genes as internal control genes is optimal (Fig. 4). Inclusion of additional housekeeping genes for internal control genes beyond the two most stably expressed genes would not have a significant effect on the results.

Table 4
Average C_T values of different housekeeping genes from different stress conditions.

Sample	*ACT*	*CAP*	*EEF1α*	*EIF4a*	*GAPDH*	*β-TUB*	*UBC*	*UBQ5*	*25S*
1	21.770	23.230	18.440	21.090	19.550	21.230	25.330	21.970	8.800
2	21.800	22.580	18.040	20.240	19.000	20.890	24.940	20.920	8.330
3	21.960	21.740	17.570	19.840	18.530	20.700	24.890	21.280	8.610
4	22.180	21.660	17.520	20.660	18.810	20.600	25.140	20.920	9.180
5	21.370	21.340	17.610	20.350	19.930	21.000	24.530	21.120	8.790
6	20.860	21.750	17.590	20.090	18.790	20.690	24.240	21.330	8.920
7	23.050	21.830	18.150	19.870	19.600	21.970	24.540	21.690	9.150
8	22.620	22.230	17.760	18.910	19.270	22.110	24.550	21.450	8.730
9	21.050	21.470	17.040	19.930	18.490	19.960	24.460	20.530	8.650
10	21.830	21.280	16.980	19.850	18.840	20.750	24.580	20.760	8.260
11	21.850	21.640	16.910	19.960	19.570	21.190	24.360	20.830	7.960
12	22.080	22.430	17.580	20.050	19.390	20.880	24.590	21.120	8.320
13	23.550	21.590	17.960	20.110	20.280	21.630	24.090	21.810	8.500
Average C_T	21.998	21.905	17.627	20.073	19.235	21.046	24.634	21.210	8.631
S.D.	0.746	0.561	0.459	0.501	0.547	0.588	0.352	0.432	0.355

Stress conditions used for gene expression analysis: (1) control: 6-week-old plant; (2) cold, 4 °C for 24 h; (3) heat, 40 °C for 2 h; (4) heat, 40 °C for 8 h; (5) PEG, 12 h; (6) PEG, 24 h; (7) salt, 12 h; (8) salt, 24 h; (9) UV, 1 h; (10) UV, 8 h; (11) wound, 12 h; (12) wound, 24 h; (13) wilt, 12 h.

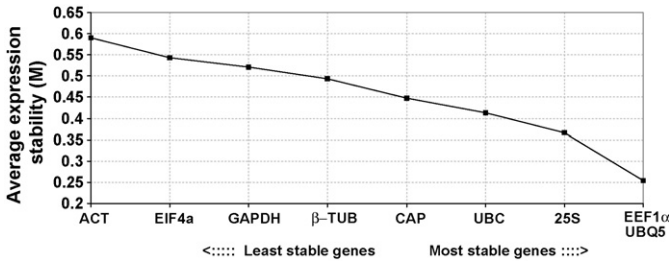

Fig. 3. Expression stability and housekeeping gene ranking based on geNORM calculations.

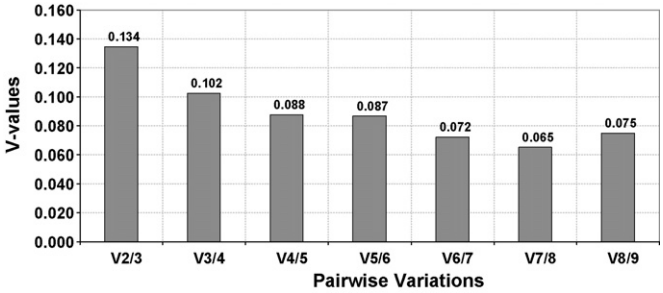

Fig. 4. Pairwise variation analysis to determine the optimal number of reference genes needed for accurate normalization, *V*-values less than 0.15 indicate no further genes are needed for calculation of a reliable normalization factor.

In conclusion, our study indicates that multiple internal control genes are necessary and that two housekeeping genes (*eEF-1α* and *UBQ5*) are sufficient for proper quantitative RT-PCR analysis of gene expression when analyzing the aerial tissues for different types of abiotic stress in *L. temulentum*.

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