

Validation of Reference Genes for Studying Different Abiotic Stresses by RT-qPCR in Oat (*Avena Sativa* L.)

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Abstract

Background: Oat (*Avena sativa* L.) is a widely cultivated cereal with high nutritional value growing mainly in temperate regions. The number of studies dealing with gene expression changes in oat increases and to obtain reliable RT-qPCR results it is essential to use reference genes that are least influenced by experimental conditions. However, no detailed study was conducted on reference genes in different tissues of oat under diverse abiotic stress conditions.

Results: In our work 9 candidate reference genes (ACT, TUB, CYP, GAPD, UBC, EF1, TBP, ADPR, PGD) were chosen and analysed by 4 statistical methods (GeNorm, Normfinder, BestKeeper, RefFinder). Samples were taken from two tissues (leaves and roots) of 13-day-old oat plants exposed to 5 abiotic stresses (drought, salt, heavy metal, low and high temperatures). ADPR was the top-rated reference gene for all samples, while different genes proved to be the most stable depending on tissue type and treatment combinations. TUB and EF1 were most affected by the treatments in general. Validation of reference genes was carried out by PAL expression analysis which further confirmed their reliability.

Conclusions: Our main goal was to identify reference genes with stable expression in oat under different abiotic stress conditions in different tissues. These results can contribute to reliable gene expression studies for future researches in cultivated oat.

Background

Oat (*Avena sativa*) is cultivated throughout the world as a unique cereal which serves as an excellent livestock due to its high protein and essential mineral level [1]. It is also a good source of dietary fiber, especially β -glucan with the potential to improve human health in many ways [2]. However, this crop is less profitable than maize, soybean or wheat crops, so it is often cultivated on such areas, which have a number of disadvantages, like drought or high salinity [3]. Furthermore, this species is sensitive to changes in light and temperature as well [4]. The current understanding of the stress-adaptive mechanisms in oat on a molecular level is still limited, and the main reason behind is probably, that the hexaploid oat genome sequencing program has only recently been completed (in 2020). Therefore, the discovery of genes playing role in abiotic stress response of oat is ahead of us.

Quantitative real-time PCR (RT-qPCR) is a powerful and sensitive method which is widely used for gene expression studies [5,6]. Although microarrays and RNAseq represent a preferred choice when it comes to the investigation of gene function on a wider scale [7], RT-qPCR is still of great importance when the methods presented above need further validation or if only a small number of genes has to be analysed, because of its cost-effectiveness and relative simplicity. However, it is only the case, when the experimental settings are properly executed and appropriate normalisation methods are used [8]. In addition to the sample integrity and the specificity of the reaction, the stability of the applied reference gene decisively determinates the reliability of the measurements [6,9]. Therefore, it is crucial to identify the most stable reference genes for gene expression studies, whose expressions are not influenced by the experimental conditions.

In the last years a number of studies were conducted to investigate the reference gene stability in oat under various circumstance. Jarošová and Kundu (2010) examined the stability of different reference gene candidates in virus-infected wheat, barley and oat. Furthermore, stable reference genes under herbicide stress were identified in wild relatives of cultivated oat [10,11] while the effects of different tissues and developmental stages were also examined [11,12]. Reference gene stability under salt stress was investigated by [13]. However, no detailed study was prepared how the stability of possible reference gene candidates is affected by various abiotic stresses. In our study the expression stabilities of 9 generally used candidate reference genes, namely ACT (Actin), TUB (α -Tubulin), CYP (Cyclophylin), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), UBC (Ubiquitin conjugating enzyme), EF1 (Elongation factor 1- α), TBP (TATA-binding protein II subunit), ADPR (ADP-ribosylation factor) and PGD (Phosphogluconate dehydrogenase) were tested in the leaves and roots of two oat cultivars (winter and spring varieties) under drought, salt, heavy metal, cold and heat treatments. 4 different statistical methods (GeNorm, Normfinder, BestKeeper, RefFinder) were used to identify the most stable reference genes. For final validation of candidate reference genes PAL (Phenylalanine ammonia lyase) was chosen as target gene, which encodes a highly stress responsive enzyme playing key role in the biosynthesis of a major stress hormone, salicylic acid (SA). Our results provide a basis for the normalization of gene expression in oat.

Results

Verification of amplification products, primer specificity and amplification specificity

Amplicon sizes of 9 reference genes (ACT, TUB, CYP, GAPDH, UBC, EF1, TBP, ADPR, PGD, PAL) and target gene (PAL) were checked by running 5-5 μ l of PCR products on 1.5% agarose gel. All PCR products were clearly amplified with the expected amplicon sizes, with no impurities or primer dimers (**Fig. 1**).

Melt curve analysis of the 9 reference genes revealed that in every case a single peak was observable under different abiotic stresses in both oat genotypes and the amplification curves showed a good repeatability (**Fig. 2**), which means that the primers amplified a single PCR product, therefore they are appropriate for detailed RT-qPCR studies.

C_q (quantification cycle) values of 9 reference genes in all oat samples are shown in the boxplot (**Fig. 3**). C_q value range in the investigated cultivars was very similar when observing a certain reference gene. The range of C_q values in all samples varied from 14.83 to 26.54, which shows that the mRNA transcript levels of the reference genes are high enough for gene expression analysis (<35). However, C_q values were influenced by treatments, tissue type and in some cases by genotype so it was necessary to analyse them under different circumstances.

Evaluation of stability ranking of candidate reference genes

GeNorm analysis

The M values were calculated by GeNorm to determine the average expression stability of the 9 reference genes in Mv Pehely and Mv Hópehely (**Table 1**). A reference gene with an M value under the threshold 1.5 can be considered as stable. In both genotypes ADPR had relatively high stability in most treatments and tissue types, while EF1 and TUB usually located in the end of the ranking order. For drought stress, ACT and ADPR were the most stable in leaves, while in roots ADPR and GAPDH ranked the highest in terms of stability. Interestingly, UBC had the lowest M values in Mv Hópehely, but it was less stable in Mv Pehely. PGD and CYP were among the most stable genes in salt stressed leaves in both genotypes, while in roots UBC and ADPR had the lowest M values. Under Cd stress ACT and ADPR had the highest stability values in leaves, while GAPDH was the most stable in roots. For cold stress, ADPR was in the beginning of ranking order in both cultivars in all tissue types. ACT was the most stable in the leaves under cold in Mv Pehely; however, it had lower stability in Mv Hópehely. UBC was among the first three most stable genes in the roots of both genotypes under cold stress. In the leaves of heat stressed samples, ACT and ADPR had the lowest M values in hydroponic cultures, but in pot experiment CYP proved to be the second most stable in both cultivars. In the roots ADPR and EF1 had the highest stabilities in both genotypes under heat stress.

GeNorm can be used to determine the optimal number of reference genes needed for normalisation by pairwise variation measurement (V_n/V_{n+1}). A V_n/V_{n+1} with 0.15 cutoff value indicates that the addition of an extra reference gene is not necessary. In our study, the V_2/V_3 values in both genotypes were lower than 0.15 in both tissue types under different abiotic stresses (**Fig. 4**) with the exception of all treatment/tissue combination, which means that 2 genes are enough for normalisation when dealing with a certain abiotic stress. If all the samples need to be analysed together, 5 reference genes are required for optimal normalisation for Mv Pehely, because only the V_5/V_6 value was lower than 0.15. However, for Mv Hópehely the V_8/V_9 value was still higher than 0.15, indicating that all the 9 reference genes could be necessary for normalisation under various stress conditions in this genotype. In conclusion, it may be worth to choose the most appropriate reference genes according to the applied experimental conditions.

NormFinder analysis

Normfinder evaluates the stability value by determining inter- and intragroup variations and the lower stability value indicates a more stable reference gene. When drought stress was applied, both the genotype and tissue type had an effect on the expression stability. In drought-stressed leaves of Mv Pehely PGD was the most stable, while it was ranked only as fourth in the leaves of Mv Hópehely. GAPDH was less stable in the leaves of both investigated cultivars, while in roots it was on the second place in Mv Pehely, while on the first place in Mv Hópehely in terms of ranking order. However, under drought stress all the stability values were very low, which indicates that the treatment only slightly influenced the stability in general. Under salt stress GAPDH was the most stable in leaves, while PGD and ADPR had the highest stability in roots. In Cd-stressed leaves GAPDH and ADPR had the top ranking, while in the roots the genotype influenced the stability of the investigated genes, in Mv Pehely EF1 was the most stable, while in Mv Hópehely GAPDH had the lowest stability values. For temperature stresses, ADPR was the most stable reference gene candidate in both cultivars (**Table 2**).

Bestkeeper analysis

Bestkeeper analyses the expression of the candidate reference genes by the calculation of the standard deviation (SD) and the coefficient of variance (CV) using the untransformed Cq values. The reference gene with the lowest $CV \pm SD$ value can be considered the most stable. Bestkeeper ranked in most cases PGD as the most stable reference gene, while TUB was mostly in the end of the ranking order. In drought stressed samples PGD was ranked in the first 3 places in leaves and in roots as well. For salt stress, TBPII could be considered as a stable reference gene. Under Cd stress PGD was in the first three places of the ranking order in both genotypes and organs. However, the stability of UBC highly depended on the tissue type, it was very stable in leaves while unstable in roots. TBPII could be considered as a stable gene in Cd-stressed leaves while CYP in roots in both genotypes. During temperature stresses PGD and TBPII were amongst the top rated genes (**Table 3**).

RefFinder analysis

RefFinder is a user-friendly online tool, which combines the so far presented statistical methods (geNorm, Normfinder, BestKeeper and the dCt method) in order to calculate a final comprehensive ranking. ADPR proved to be the most stable and TUB the most unstable in most treatments in both tissue types in general, while the stability of the other reference genes was influenced to a greater extent by the applied treatment, tissue and genotype combinations. In drought stressed leaves of Mv Pehely PGD was the most stable, while in Hópehely ACT was the top ranked, in roots GAPDH and ADPR were the most stable genes. When applying salt stress, in leaves GAPDH and CYP were the most stable, while in roots ADPR and PGD were in the beginning of the ranking order. For heavy metal stress, GAPDH and ADPR were the most stable genes in leaves, but PGD and CYP in roots. Under cold and heat stresses ADPR was almost always the top ranked one in both genotypes and tissue types (**Table 4**). Furthermore, ADPR proved to be stable under different growing conditions as well, when the plants were exposed to temperature stresses.

Validation of the reference gene candidates

The relative expression level of PAL (phenylalanine ammonia lyase) was used to validate the candidate reference genes in our study. PAL is one of the key enzymes during the synthesis of the well-known stress hormone, SA [14]. Elevated PAL activity or mRNA level was found in different plant species after the exposure of various abiotic stresses [15–17]. The relative expression level of PAL was normalized with the two most stable reference genes and the least stable reference gene in oat according to the applied stress factor. Reference genes were carefully chosen after comparing the results of different evaluation programs while taking into account the effects of genotype and tissue type as well. As shown in **Fig. 5A**, in response to heat in the leaves of Mv Hópehely growing in soil, the relative expression of PAL increased significantly, when using the most stable reference genes (ADPR+UBC), while the relative transcript level did not reach a 2-fold increase when using the least stable reference gene, TUB. When applying salt stress in the leaves of Mv Pehely hydroponically (**Fig. 5B**), a 5-fold increase was measured when applying the most stable reference genes alone or in combination (GAPDH+CYP), but the relative expression level was overestimated, when UBC was used as reference gene. In conclusion, if the applied reference gene has a very high stability, one gene is sufficient for

normalisation. (The relative expression values of PAL in all tested tissue and genotype under different abiotic stresses are available as supplementary material, in **Additional file 1**).

Discussion

RT-qPCR is a widely used method for gene expression analysis because of its relative simplicity and high sensitivity. However, the reliability of the results is greatly determined by reference gene selection used for normalization [5,8]. Ideally, the stability of the reference gene should not be influenced by the experimental conditions. Nevertheless, several studies point out, that the mRNA transcript levels could be affected by tissue type [11,12], developmental stage [18] treatment type [19,20] and genotype [18] as well. Furthermore, the expression stability of the same reference gene may also depend on the investigated species [21]. Therefore, it is always necessary to analyse and validate the potential reference genes prior to their applications.

In our study, 9 candidate reference genes (ACT, TUB, CYP, GAPD, UBC, EF1, TBP, ADPR, PGD) were screened from the leaves and roots of two oat genotypes under different abiotic stresses (drought, salt, heavy metal, cold and heat), and their expression stabilities were analysed by 4 different statistical programs (GeNorm, NormFinder, BestKeeper, RefFinder). The general rating by the different programs had a substantial agreement, which were the least stable genes for each treatment/tissue/genotype combinations so they could be easily excluded. However, the most stable gene determined by the applied programs were not always the same due to their different calculations. It was especially remarkable when using BestKeeper, which gave a higher ranking to a certain gene compared to the other programs in some cases. That was especially true for TBPII in drought stressed roots of Mv Hópehely, and for PGD in cold stressed leaves of both cultivars, respectively. The difference between the ranking order of BestKeeper and the rest of the softwares is also mentioned by other studies [22–24]. The stability of the reference genes were influenced by all the experimental conditions, such as tissue type, genotype and treatment type, but the applied stress treatments had the most pronounced effect on stability in general.

For drought stress, all the stability values were very low, independently from the applied program which indicates that gene expression stabilities of the reference genes was not particularly affected by this treatment in general. The suitability of ADPR under drought stress was confirmed by our experiments and it was also mentioned earlier in barley (*Hordeum vulgare*, L.) [25]. Furthermore, CYP was found to be stable in barley when exposed to drought, while in our experiment it was one of the least stable genes. Interestingly, a study in durum wheat (*Triticum durum*, L.) identified GAPDH as a stable reference gene under drought [26], although, under our experimental conditions it only showed high stability in roots, while it was unstable in the leaves of the investigated oat cultivars. In addition to our study, the applicability of ACT as reference gene in drought stressed leaves was also suggested in barley [25]. Interestingly, the stability values of UBC were genotype and tissue type dependent. Namely, it was the least stable gene in the leaves of Mv Pehely, while it was one of most stable ones in the leaves of Mv Hópehely. Furthermore, it had moderate stability in the roots of Mv Pehely while it was stably expressed in the roots of Mv Hópehely.

When applying salt stress, tissue type greatly affected the expression stability. GAPDH, CYP and PGD had high stability in leaves, while in roots UBC and ADPR were the most stably expressed genes in both cultivars. GAPDH was a proposed reference gene in *Triticum durum* [26] under salt stress as well. However, Duan et al. (2020) also investigated the effects of salt stress in oat and they found the expression of GAPDH unstable. In contrast, the same study identified EF1 and TBP as the best combination for normalization, but in our experimental setup both genes had relatively low stabilities. ACT exhibited low expression stability in oat both in our study and in the work of Duan et al. Furthermore, α -TUB was also in the end of ranking order in our analysis and in the above mentioned study as well. The expression stability of UBC showed genotype and tissue type dependence under salt stress similarly to drought stress, which is also an osmotic stress.

Under Cd stress, GAPDH was found to be stably expressed according to the different evaluating softwares in our study. However, in giant reed (*Arundo donax* L.), which also belongs to the *Poaceae* family, GAPDH was amongst the least stable genes when the plants were treated with Cd [27]. ACT1 showed high stability in leaves of both investigated oat cultivars. In agreement with this, another members of the Actin gene family showed high expression stabilities under Cd exposure, ACT12 in switchgrass (*Panicum virgatum* L.) [24] and ACT3 in soybean (*Glycine max* L.). PGD was a stable reference gene in the roots of both oat genotypes, however, in soybean its expression was greatly influenced by Cd stress [28]. TUB exhibited low expression stability in our oat samples and in Cd-treated soybean plants [28] as well.

When looking for stably expressed genes under temperature stresses, two oat homologs of wheat ADPR (Ta2291) and PGD (Ta30797) were also tested, since they were suggested as suitable reference genes candidates by Paolacci et al. (2008). The overall applicability of ADPR for cold and heat stresses in different genotypes, tissue types and growing media was confirmed since it was in the first three places of the ranking order calculated by every software with the exception of BestKeeper. The high expression stability of ADPR under cold and heat stress was also indicated in *Hordeum brevisubulatum* [29] and it was the best reference gene in cold stressed barley [25]. However, PGD only exhibited high expression stability in the roots of Mv Pehely under cold stress, while it was less stable in leaves and under heat stress. The stability of ACT was influenced by genotype, tissue type and growing medium, namely, it showed the high stability in the leaves, when cold stress was applied in Mv Pehely, and under heat stress, but only when the plants were grown hydroponically. ACT is a commonly used reference gene in different plant species [22] but these differences suggest a caution approach when choosing this gene as reference gene. EF1 was usually amongst the least stable genes under temperature stresses with the exception of the heat stressed roots of Mv Pehely where it was the most stable gene. EF1 was also ranked the highest in terms of stability in *Hordeum brevisubulatum* under heat stress [29].

In order to validate the stability of the investigated reference genes, PAL expression analysis was performed. Our study revealed, as long as a reference gene with very high stability is chosen (**Fig. 5**), one reference gene may be sufficient for accurate normalization. However, according to the GeNorm V/V value calculation the application of 2 reference genes are more suitable in leaves or in roots exposed to a certain abiotic stress. As it is shown in Additional file 2 on **Fig. S1-S4**, the expression of PAL was highly inducible by most of the treatments, especially in leaves. However, drought stress might be considered as a mild stress in this study, which is supported by the fact, that PAL expression was not induced by PEG treatment nor in leaves or roots of the two genotypes.

Correspondingly, the reference gene stability values calculated by the different software were very low in the case of all reference genes tested compared to the values under other abiotic stresses. In contrast, salt treatment, heavy metal exposure and low temperature caused a remarkable elevation of PAL transcript levels in both genotypes, which indicates increased stress effect for the plants. Accordingly, the stability of the reference gene candidates changed to a higher extent depending on the individual genes. Interestingly, heat stress only induced PAL expression in the leaves of Mv Hópehely when grown in soil but not in hydroponic culture; however, this treatment greatly affected reference gene stability in general. In agreement with this observation, the growing media influenced the stability of certain reference genes as well. While ADPR kept its high stability under both growing conditions as indicated by the different software, the ranking order of the rest of the reference gene candidates changed according to the growing medium. For example, ACT showed high stability in the leaves of heat stressed Mv Pehely in hydroponic culture, however, in soil it was less stable. Furthermore, when investigating the stability in the cold stressed leaves of Mv Hópehely, CYP was only stable in soil but its expression was influenced by cold temperature under hydroponic growing conditions.

Conclusions

In conclusion, 9 candidate reference genes were chosen in two oat genotypes. Our results indicated that the stability of certain reference genes could be greatly influenced by the different experimental conditions. Furthermore, the possible effect of growing media under temperature stresses on reference gene stability was also revealed. For all samples, ADPR was the top-ranked gene in both cultivars. For individual stress treatments the most stable reference genes varied according to the tissue type and genotype. However, efforts were made to propose the most appropriate reference gene candidates according to the treatment type. Under drought stress, ADPR was a suitable reference gene in leaves, while GAPDH in roots. For salt stress, GAPDH and PGD were appropriate reference genes in leaves, while ADPR in roots. When exposed the plants to heavy metal stress, GAPDH was stably expressed in leaves and PDG had high stability in roots. ADPR performed the best in cold and heat stressed samples. Nevertheless, α -TUB and EF1- α were influenced greatly by the experimental conditions and they were rarely suitable for normalization. For the first time, stably expressed reference genes for different tissues were identified in a spring and winter oat genotype under five different abiotic stresses. Our study provides a suitable reference for selecting stable internal reference gene candidates to investigate gene expression under various abiotic stresses in oat. These results can contribute to better understand the molecular mechanisms playing role in abiotic stress response of this species.

Methods

Plant materials, growth conditions, and stress treatments

Two oat (*Avena sativa* L.) cvs. 'Mv Pehely (spring type) and 'Mv Hópehely' (winter type) were selected for the present experiments. Seeds were germinated for 3 days on wet filter under dark conditions at 25°C then they were grown either hydroponically using modified Hoagland solution [30] or in plastic pots filled with filled with a 3:1 (v:v) mixture of loamy soil and sand at 22°C/20°C with 16-h/8-h light/dark photoperiod, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity and 75% relative humidity in a Conviron PGV-36 phytochamber (Controlled Environments, Winnipeg, Canada). The above mentioned parameters are referred later as control conditions. For hydroponic growing glass beakers were used with 10 plants per beaker, for pot experiment also 10 plants were sown in one pot. Nutrient solution for hydroponic culture was changed in every second day, while for the plants growing in pot regular water supply was provided. 13-day-old plants were exposed to different abiotic stresses for 24 hours. Stress conditions were chosen based on earlier findings, where stress responses were clearly observable, but they were not very serious causing the death of the plants. For drought stress plants were treated with 15% PEG-6000 hydroponically [31] while salinity stress was induced by adding 250 mM/L NaCl [32] to the hydroponic medium. For heavy metal treatment plants were treated with modified Hoagland solution containing 250 $\mu\text{M/L}$ $\text{Cd}(\text{NO}_3)_2$ [33]. In order to determinate if different growing conditions can cause any difference in the stability of reference genes when applying temperature stresses, hydroponically grown and soil-grown plants were also tested. Cold stress was imposed at 4°C while heat stress was applied at 35 °C in growing chambers where all the settings was the same like the control conditions mentioned above but the temperature. Meanwhile, one part of the hydroponic cultures were treated with Hoagland solution (hydroponic control) and were kept together with untreated plants of the soil experiment (soil control) under control conditions for 24 hours. For leaf samples the second, fully developed leaves were collected while root tissues were sampled only from hydroponically grown plants after washing the roots with distilled water. Thereafter all samples were frozen immediately in liquid nitrogen and stored at -80°C until further analyses.

Isolation of RNA and cDNA synthesis

Total RNA was extracted from leaf and root samples with TRI Reagent. Samples were further cleaned with Direct-Zol RNA MiniPrep Kit (Zymo Research, USA) including on-column Dnase I treatment. RNA quantification was carried out with a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., USA) while its integrity and the lack of gDNA contamination was checked on 1.5% agarose gel. 1000 ng of total RNA was reversely transcribed into cDNA with M-MLV Reverse Transcriptase (Promega Corporation) and oligo(dT)₁₈ (Thermo Fisher Scientific) according to manufacturer's instructions. 4-fold dilution products were stored at -20 °C until RT-qPCR studies.

Reference gene selection and PCR primer design

The majority of candidate reference genes were chosen based on their description being stable internal control genes for qRT-PCR analysis in different plant species ([10,13,19,34]. The applicability of ADPR (Unigene cluster: Ta30797) and PGD (Unigene cluster: Ta2291) (candidate reference genes proposed by [20]) were confirmed previously by our research group in wheat (*Triticum aestivum* L.) under different stress conditions [33,35–37]. For primer design, known wheat sequences were chosen either from NCBI Gene Bank or in the case of ADPR and PGD the corresponding sequences were obtained from NCBI-Unigene database. Homologous oat sequences were retrieved from the *Avena sativa* v1.0 genome database (<https://avenagenome.org>, BioProject 541449) via BLASTn search using highly conserved regions (identification of conserved domains was performed via NCBI Conserved Domain Finder [38] of the corresponding

Triticum aestivum cDNA sequence with the following criteria: aligned sequence size being >300 bp in length, an E-value < $1e^{-5}$ and minimum 87% sequence homology to the query sequence (detailed informations about sequence alignments, E-values, percentage of identity are available in **Additional file 2**).

For primer design Primer3 software [39] was used and primers were also checked with Oligoanalyzer [40] to avoid primer dimerization. The primer design conditions were as follows: Tm, 59–62 °C; amplicon size, 90–200 bp; primer length, 20–24 bp; GC content, 45–60%. PCR products were also run on 1.5% agarose gel in order to confirm the presence of a single amplicon with the expected amplicon size. Primer sequences are available in **Table 5**.

Real time quantitative PCR and amplification efficiency determination

Measurements were performed on a Biorad CFX96 Touch Real-Time Detection System in 96-well microtiter plates. Mastermix was prepared using a final volume of 5 μ l, which consisted of 1 μ l 4-fold diluted cDNA, 0.1–0.1 μ l forward and reverse primers (from 10 μ M stock solution), 2.5 μ l PCR BIO SyGreen Mix (PCR Biosystems) and 2.5 μ l molecular grade water. PCR cycling consisted of three steps as follows: 3 min initial pre-incubation at 95°C, followed by 39 cycles of 5 s at 95°C for denaturation, and 30 s at 60°C for annealing and extension. Melting curve analysis was also performed to verify PCR specificity by constant increase in temperature from 65°C to 95°C, at increment of 0.5°C. 5-step dilution series of cDNA pool (including cDNA samples from different genotypes and tissue types exposed to 5 abiotic stresses) was used for standard curve preparation. PCR efficiency and correlation coefficient (R^2) were determined for each gene by CFX Maestro program. All reactions were run using three biological and three technical replicates. No template controls were also included to check the absence of primer dimers and random contaminations.

Stability ranking of reference genes

To evaluate the relative expression stabilities of candidate reference genes under different abiotic stresses, 4 different statistical softwares, GeNorm [5], Normfinder [41], BestKeeper [6] and an online data analysis tool, RefFinder [42] were used. Cq values obtained from CFX Maestro program were exported into Microsoft Excel 2016 and used as input for further analyses. The GeNorm algorithm was applied as a built-in module of qBasePLUS software to evaluate the stability of reference genes based on stability value (M). Genes with an M value below the threshold of 1.5 are considered as stably expressed. The software can also determine the optimal number of reference genes for target gene expression normalisation with pairwise variation calculation (V_n/V_{n+1}). A V_n/V_{n+1} cutoff value of 0.15 or lower means that the addition of a further reference gene is not necessary. NormFinder was used as an Excel-based algorithm to identify stable reference genes based on intra- and inter-group variations amongst the tested genes and the lowest stability value indicates the highest stability. The program needs the raw Ct values to be transformed using the formula $2^{-\Delta Cq}$. Bestkeeper is also an Excel-based tool which uses raw Cq values as input. It can rank the stability values by calculating the coefficient of variation (CV) and standard deviation (CV \pm SD). Reference genes with the lowest CV \pm SD values can be considered as most stable. A comprehensive ranking of the above mentioned evaluating programs was prepared with RefFinder which assigns an appropriate weight to an individual gene and calculates the geometric mean of their weights for the overall final ranking.

Validation of reference genes by expression analysis of PAL under different abiotic stresses

In order to validate the reliability and the stability of the candidate reference genes determined by the applied softwares, PAL was selected as target gene to analyse the gene expression pattern using the two most stable reference genes and the least stable reference gene. Relative transcript level was calculated with $2^{-\Delta\Delta Cq}$ method [43]. The expression level of PAL was determined with forward primer 5'-GCAACTTCCAGGGCACCC-3' and reverse primer 5'-CTCCGAGAACTGAGCGAACAT-3' (reference sequence MT150275.1, amplicon size 95 bp, amplification efficiency 93%).

Declarations

Authors' Contributions

Conceptualization, Methodology, Validation, Writing-original draft, J.T.; Supervision, Writing-review & editing T.J., M.P. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

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

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Tables

Table 1. Average expression stability values (M) of 9 reference gene candidates calculated by GeNorm. The two types of growing media are indicated with h (hydroponic) and s (soil), respectively.

GeNorm analysis												
Ranking order			1	2	3	4	5	6	7	8	9	
Drought stress	Mv Pehely	Leaves (h)	ADPR	ACT	TBP11	CYP	PGD	GAPDH	EF1	TUB	UBC	
			0.101	0.102	0.106	0.150	0.177	0.212	0.228	0.236	0.254	
		Roots (h)	GAPDH	ADPR	ACT	UBC	PGD	TBP11	CYP	TUB	EF1	
			0.079	0.081	0.092	0.134	0.156	0.188	0.216	0.242	0.271	
	Mv Hópehely	Leaves (h)	UBC	ACT	ADPR	PGD	TBP11	TUB	GAPDH	EF1	CYP	
			0.105	0.113	0.127	0.149	0.168	0.191	0.212	0.227	0.241	
		Roots (h)	UBC	ADPR	GAPDH	EF1	PGD	ACT	CYP	TUB	TBP11	
			0.078	0.079	0.090	0.131	0.151	0.175	0.218	0.267	0.314	
Salt stress	Mv Pehely	Leaves (h)	GAPDH	CYP	PGD	ACT	ADPR	EF1	TUB	TBP11	UBC	
			0.106	0.110	0.117	0.219	0.253	0.325	0.370	0.452	0.507	
		Roots (h)	UBC	ADPR	CYP	TBP11	EF1	PGD	GAPDH	ACT	TUB	
			0.193	0.195	0.203	0.215	0.244	0.290	0.396	0.449	0.565	
	Mv Hópehely	Leaves (h)	PGD	UBC	CYP	GAPDH	ADPR	ACT	TUB	TBP11	EF1	
			0.116	0.130	0.134	0.339	0.465	0.531	0.569	0.615	0.706	
		Roots (h)	UBC	ADPR	PGD	EF1	GAPDH	ACT	TUB	TBP11	CYP	
			0.134	0.135	0.167	0.232	0.394	0.453	0.511	0.572	0.628	
Heavy Metal Stress	Mv Pehely	Leaves (h)	ACT	CYP	ADPR	GAPDH	PGD	EF1	TBP11	UBC	TUB	
			0.126	0.133	0.145	0.154	0.192	0.241	0.311	0.360	0.413	
		Roots (h)	GAPDH	ACT	CYP	PGD	EF1	ADPR	TBP11	UBC	TUB	
			0.182	0.199	0.239	0.269	0.298	0.373	0.450	0.497	0.583	
	Mv Hópehely	Leaves (h)	ADPR	ACT	EF1	TUB	GAPDH	CYP	PGD	UBC	TBP11	
			0.146	0.150	0.177	0.217	0.272	0.430	0.489	0.531	0.571	
		Roots (h)	GAPDH	PGD	ADPR	CYP	EF1	ACT	UBC	TBP11	TUB	
			0.177	0.208	0.232	0.262	0.294	0.323	0.395	0.449	0.525	
Cold stress	Mv Pehely	Leaves (h)	ACT	ADPR	UBC	CYP	TUB	GAPDH	EF1	PGD	TBP11	
			0.175	0.181	0.196	0.230	0.355	0.415	0.476	0.565	0.632	
		Leaves (s)	ACT	ADPR	CYP	TBP11	TUB	PGD	UBC	GAPDH	EF1	
			0.151	0.159	0.166	0.309	0.352	0.371	0.410	0.517	0.591	
		Roots (h)	GAPDH	ADPR	UBC	PGD	TBP11	ACT	EF1	TUB	CYP	
			0.145	0.150	0.163	0.175	0.206	0.264	0.294	0.322	0.378	
	Mv Hópehely	Leaves (h)	ADPR	UBC	ACT	CYP	EF1	TUB	PGD	TBP11	GAPDH	
			0.111	0.125	0.140	0.170	0.217	0.265	0.384	0.466	0.580	
		Leaves (s)	ADPR	CYP	PGD	TBP11	TUB	ACT	UBC	GAPDH	EF1	
			0.218	0.229	0.271	0.304	0.314	0.369	0.396	0.495	0.579	
		Roots (h)	ADPR	PGD	UBC	GAPDH	CYP	EF1	ACT	TUB	TBP11	
			0.121	0.123	0.124	0.190	0.250	0.312	0.348	0.413	0.491	
Heat stress	Mv Pehely	Leaves (h)	ACT	ADPR	GAPDH	UBC	CYP	PGD	TBP11	EF1	TUB	
			0.188	0.189	0.192	0.209	0.236	0.300	0.340	0.460	0.528	
		Leaves (s)	TUB	CYP	ACT	EF1	ADPR	UBC	TBP11	GAPDH	PGD	
			0.145	0.156	0.159	0.204	0.319	0.373	0.448	0.481	0.544	
		Roots (h)	ADPR	EF1	PGD	ACT	TUB	CYP	TBP11	UBC	GAPDH	
			0.286	0.298	0.328	0.391	0.428	0.524	0.56	0.664	0.795	

	Mv Hópehely	Leaves (h)	UBC	ACT	ADPR	GAPDH	CYP	TUB	TBP11	PGD	EF1
			0.129	0.140	0.146	0.181	0.220	0.246	0.352	0.408	0.502
		Leaves (s)	UBC/ADPR	CYP	ACT	GAPDH	TBP11	PGD	EF1	TUB	
			0.197	0.206	0.227	0.276	0.388	0.481	0.647	0.772	
		Roots (h)	EF1	ADPR	PGD	TUB	ACT	CYP	UBC	TBP11	GAPDH
			0.183	0.191	0.21	0.339	0.431	0.521	0.666	0.733	0.806
All samples	Mv Pehely		TBP11	UBC	ADPR	ACT	EF1	GAPDH	TUB	CYP	PGD
			0.503	0.533	0.552	0.706	0.785	0.861	0.934	1.048	1.143
	Mv Hópehely		ACT	ADPR	EF1	GAPDH	TBP11	UBC	CYP	PGD	TUB
			0.517	0.572	0.601	0.723	0.949	1.027	1.106	1.197	1.299

Table 2. Expression stability values of 9 oat candidate reference genes calculated by NormFinder. The two types of growing media are indicated with h (hydroponic) and s (soil).

NormFinder analysis													
Ranking order				1	2	3	4	5	6	7	8	9	
Drought stress	Mv Pehely	Leaves (h)	PGD	TBP11	CYP	ADPR	ACT	EF1	GAPDH	TUB	UBC		
			0.096	0.112	0.119	0.124	0.127	0.137	0.139	0.151	0.173		
		Roots (h)	ADPR	GAPDH	PGD	UBC	ACT	TBP11	CYP	TUB	EF1		
			0.032	0.053	0.067	0.074	0.102	0.120	0.138	0.153	0.157		
	Mv Hópehely	Leaves (h)	ACT	UBC	ADPR	PGD	GAPDH	TUB	TBP11	EF1	CYP		
			0.034	0.036	0.082	0.094	0.100	0.113	0.120	0.124	0.132		
		Roots (h)	GAPDH	UBC	ADPR	EF1	PGD	ACT	CYP	TUB/TBP11			
			0.008	0.014	0.021	0.025	0.041	0.072	0.117	0.175			
Salt stress	Mv Pehely	Leaves (h)	GAPDH	CYP	PGD	ADPR	ACT	EF1	TUB	TBP11	UBC		
			0.049	0.196	0.218	0.251	0.276	0.330	0.369	0.382	0.408		
		Roots (h)	PGD	ADPR	TBP11	GAPDH	ACT	UBC	CYP	EF1	TUB		
			0.090	0.216	0.257	0.268	0.307	0.308	0.308	0.349	0.587		
	Mv Hópehely	Leaves (h)	GAPDH	ADPR	ACT	PGD	CYP	UBC	TUB	TBP11	EF1		
			0.137	0.243	0.317	0.342	0.344	0.354	0.385	0.570	0.615		
		Roots (h)	PGD	EF1	ADPR	UBC	GAPDH	ACT	TBP11	CYP	TUB		
			0.081	0.091	0.183	0.253	0.337	0.441	0.518	0.551	0.638		
Heavy Metal Stress	Mv Pehely	Leaves (h)	CYP	GAPDH	ADPR	ACT	EF1	PGD	TBP11	UBC	TUB		
			0.120	0.126	0.145	0.171	0.188	0.213	0.270	0.302	0.354		
		Roots (h)	EF1	PGD	CYP	GAPDH	ADPR	ACT	TBP11	UBC	TUB		
			0.103	0.108	0.191	0.243	0.247	0.284	0.417	0.444	0.584		
	Mv Hópehely	Leaves (h)	GAPDH	ACT	ADPR	PGD	CYP	UBC	EF1	TUB	TBP11		
			0.184	0.219	0.253	0.278	0.304	0.336	0.367	0.431	0.452		
		Roots (h)	GAPDH	ADPR	PGD	EF1	CYP	ACT	UBC	TBP11	TUB		
			0.055	0.110	0.132	0.161	0.223	0.270	0.349	0.431	0.493		
Cold stress	Mv Pehely	Leaves (h)	ADPR	UBC	CYP	ACT	TUB	GAPDH	EF1	PGD	TBP11		
			0.121	0.148	0.152	0.216	0.338	0.357	0.463	0.478	0.493		
		Leaves (s)	ACT	CYP	ADPR	UBC	TBP11	TUB	PGD	GAPDH	EF1		
			0.070	0.089	0.127	0.166	0.348	0.392	0.408	0.450	0.492		
	Mv Hópehely	Roots (h)	ADPR	EF1	PGD	GAPDH	ACT	UBC	TUB	TBP11	CYP		
			0.117	0.142	0.146	0.173	0.178	0.219	0.232	0.241	0.349		
		Leaves (h)	ADPR	UBC	ACT	CYP	TUB	EF1	PGD	TBP11	GAPDH		
			0.057	0.071	0.134	0.184	0.192	0.283	0.410	0.538	0.617		
Mv Hópehely	Leaves (s)	ADPR	UBC	CYP	ACT	PGD	TUB	TBP11	GAPDH	EF1			
		0.102	0.149	0.163	0.174	0.339	0.370	0.385	0.395	0.561			
	Roots (h)	ADPR	PGD	CYP	GAPDH	UBC	EF1	ACT	TUB	TBP11			
		0.090	0.125	0.149	0.171	0.188	0.248	0.282	0.418	0.457			
Heat stress	Mv Pehely	Leaves (h)	ADPR	CYP	GAPDH	UBC	ACT	TBP11	PGD	EF1	TUB		
			0.091	0.133	0.155	0.173	0.218	0.383	0.409	0.437	0.461		
		Leaves (s)	ADPR	UBC	ACT	TBP11	TUB	GAPDH	CYP	EF1	PGD		
			0.079	0.113	0.211	0.279	0.280	0.293	0.332	0.380	0.507		
		Roots (h)	ADPR	EF1	PGD	ACT	CYP	TBP11	TUB	UBC	GAPDH		
			0.091	0.169	0.215	0.337	0.406	0.413	0.427	0.694	0.769		

	Mv Hópehely	Leaves (h)	ADPR	GAPDH	UBC	ACT	CYP	TUB	PGD	TBP11	EF1
			0.034	0.107	0.146	0.148	0.159	0.224	0.399	0.413	0.528
		Leaves (s)	CYP	ADPR	UBC	ACT	GAPDH	TBP11	PGD	EF1	TUB
			0.087	0.095	0.137	0.208	0.237	0.512	0.575	0.609	0.795
		Roots (h)	ADPR	EF1	PGD	TUB	CYP	ACT	UBC	GAPDH	TBP11
			0.041	0.052	0.146	0.394	0.401	0.479	0.583	0.619	0.662
All samples	Mv Pehely		ADPR	TBP11	UBC	ACT	EF1	GAPDH	CYP	TUB	PGD
			0.178	0.357	0.374	0.400	0.496	0.588	0.691	0.743	0.884
	Mv Hópehely		ADPR	ACT	TBP11	UBC	EF1	GAPDH	CYP	PGD	TUB
			0.207	0.393	0.467	0.527	0.581	0.662	0.714	0.890	0.945

Table 3. Expression stability values of 9 oat candidate reference genes calculated by Bestkeeper. The two types of growing media are indicated with h (hydroponic) and s (soil).

BestKeeper analysis											
Ranking order			1	2	3	4	5	6	7	8	9
Drought stress	Mv Pehely	Leaves (h)	PGD	TUB	CYP	EF1	TBP11	GAPDH	ADPR	ACT	UBC
			0.36±0.08	0.52±0.11	0.64±0.13	0.64±0.14	0.67±0.17	0.80±0.16	1.01±0.22	1.05±0.23	1.14±0.26
		Roots (h)	TBP11	PGD	UBC	ADPR	CYP	GAPDH	ACT	TUB	EF1
		0.23±0.06	0.43±0.09	0.76±0.16	1.03±0.19	1.32±0.25	1.35±0.22	1.37±0.24	1.71±0.28	2.04±0.37	
	Mv Hópehely	Leaves (h)	ADPR	TBP11	PGD	UBC	TUB	ACT	EF1	GAPDH	CYP
			0.50±0.11	0.51±0.13	0.67±0.14	0.72±0.16	0.97±0.21	0.98±0.22	1.05±0.25	1.31±0.28	1.71±0.34
Roots (h)		PGD	CYP	EF1	UBC	GAPDH	ADPR	TBP11	ACT	TUB	
	1.21±0.26	1.45±0.28	1.46±0.27	1.51±0.33	1.57±0.25	1.80±0.34	1.94±0.48	2.34±0.41	2.85±0.45		
Salt stress	Mv Pehely	Leaves (h)	CYP	TBP11	PGD	UBC	GAPDH	ACT	ADPR	EF1	TUB
			0.58±0.11	0.72±0.18	0.79±0.17	1.15±0.26	1.17±0.24	2.72±0.57	2.77±0.58	3.25±0.71	3.57±0.75
		Roots (h)	CYP	UBC	TBP11	EF1	ADPR	PGD	ACT	GAPDH	TUB
		0.99±0.19	1.00±0.22	1.10±0.27	1.23±0.23	1.57±0.30	2.20±0.48	4.51±0.83	4.68±0.80	7.20±1.24	
	Mv Hópehely	Leaves (h)	TBP11	PGD	UBC	CYP	GAPDH	ADPR	ACT	TUB	EF1
			0.67±0.17	0.81±0.17	0.83±0.19	1.04±0.20	3.01±0.62	4.00±0.85	4.69±0.98	5.07±1.05	6.38±1.41
Roots (h)		TBP11	UBC	PGD	ADPR	CYP	EF1	GAPDH	ACT	TUB	
	1.04±0.25	1.06±0.23	1.18±0.25	1.28±0.24	1.51±0.28	2.21±0.41	4.64±0.78	4.72±0.84	6.10±1.00		
Heavy Metal Stress	Mv Pehely	Leaves (h)	UBC	TBP11	PGD	CYP	ACT	EF1	ADPR	GAPDH	TUB
			0.60±0.13	0.78±0.19	1.09±0.23	1.67±0.32	2.33±0.49	2.40±0.53	2.55±0.53	2.74±0.56	3.76±0.79
		Roots (h)	PGD	CYP	EF1	ADPR	TBP11	UBC	GAPDH	ACT	TUB
		0.66±0.14	0.98±0.19	1.15±0.21	1.40±0.26	1.69±0.40	2.36±0.50	2.43±0.41	2.49±0.44	4.46±0.75	
	Mv Hópehely	Leaves (h)	TBP11	UBC	PGD	CYP	GAPDH	ACT	ADPR	EF1	TUB
			0.62±0.16	0.99±0.22	1.08±0.23	1.20±0.23	3.53±0.73	3.99±0.84	4.30±0.91	4.66±1.04	5.24±1.09
Roots (h)		CYP	PGD	ADPR	GAPDH	TBP11	EF1	UBC	ACT	TUB	
	0.78±0.15	1.11±0.24	1.36±0.25	1.40±0.22	1.82±0.44	1.89±0.34	1.91±0.40	1.97±0.34	3.59±0.57		
Cold stress	Mv Pehely	Leaves (h)	PGD	TBP11	UBC	ACT	ADPR	CYP	TUB	GAPDH	EF1
			0.80±0.17	1.06±0.27	1.35±0.30	1.57±0.33	1.80±0.38	1.90±0.37	3.36±0.71	4.31±0.86	4.47±0.96
		Leaves (s)	TUB	TBP11	PGD	ADPR	ACT	CYP	UBC	EF1	GAPDH
		0.40±0.08	0.47±0.12	0.69±0.15	1.25±0.26	1.66±0.35	1.97±0.38	2.56±0.57	4.77±1.04	4.86±0.93	
	Roots (h)	PGD	TBP11	UBC	EF1	ADPR	GAPDH	CYP	ACT	TUB	
		0.67±0.14	0.94±0.23	1.14±0.24	1.49±0.28	1.52±0.29	1.83±0.30	1.85±0.36	2.15±0.38	2.37±0.38	
	Mv Hópehely	Leaves (h)	PGD	TBP11	TUB	UBC	ADPR	ACT	CYP	EF1	GAPDH
			1.09±0.23	1.10±0.28	1.32±0.28	1.99±0.44	2.25±0.48	2.82±0.60	2.94±0.57	3.02±0.69	6.06±1.22
		Leaves (s)	TBP11	PGD	TUB	ADPR	CYP	ACT	UBC	GAPDH	EF1
	0.30±0.08	0.35±0.07	0.97±0.21	1.64±0.35	1.83±0.36	2.66±0.57	2.90±0.63	5.11±1.00	5.14±1.15		
Roots (h)	PGD	UBC	ADPR	CYP	TBP11	GAPDH	EF1	ACT	TUB		
	0.63±0.13	1.01±0.22	1.07±0.20	1.19±0.23	1.40±0.34	1.73±0.28	2.17±0.40	2.58±0.45	3.97±0.64		
Heat stress	Mv Pehely	Leaves (h)	PGD	TBP11	UBC	ADPR	GAPDH	CYP	ACT	TUB	EF1
			0.49±0.11	0.75±0.19	1.40±0.31	1.95±0.41	2.00±0.41	2.63±0.51	2.65±0.56	4.33±0.91	4.36±0.94
		Leaves (s)	TBP11	GAPDH	UBC	PGD	ADPR	ACT	TUB	EF1	CYP
	0.22±0.06	0.46±0.09	1.02±0.23	1.13±0.24	1.40±0.29	2.93±0.62	3.13±0.64	4.06±0.90	4.10±0.78		
Roots (h)	ADPR	PGD	EF1	TBP11	CYP	ACT	TUB	UBC	GAPDH		
	1.16±0.22	1.23±0.27	1.49±0.27	1.71±0.41	2.11±0.40	2.78±0.50	3.11±0.51	3.98±0.82	6.04±1.05		

	Mv Hópehely	Leaves (h)	TBPII	PGD	CYP	ADPR	GAPDH	UBC	TUB	ACT	EF1
			0.70±0.18	0.97±0.21	1.32±0.26	1.61±0.35	1.76±0.37	1.94±0.43	2.31±0.49	2.34±0.50	4.45±1.00
		Leaves (s)	TBPII	PGD	GAPDH	UBC	ADPR	CYP	ACT	EF1	TUB
			0.45±0.11	0.64±0.14	1.87±0.38	2.32±0.51	2.93±0.62	3.55±0.68	4.01±0.84	6.5±1.44	8.19±1.65
		Roots (h)	EF1	ADPR	CYP	PGD	TBPII	UBC	TUB	ACT	GAPDH
			0.90±0.16	0.93±0.17	1.11±0.21	1.46±0.32	2.58±0.62	3.21±0.66	3.32±0.53	4.16±0.74	5.77±0.98
All samples	Mv Pehely		PGD	CYP	TBPII	UBC	ADPR	ACT	EF1	GAPDH	TUB
			1.49±0.32	2.44±0.47	2.78±0.69	3.12±0.68	4.75±0.95	6.91±1.36	7.32±1.48	7.88±1.48	9.62±1.84
	Mv Hópehely		PGD	CYP	TBPII	UBC	ADPR	ACT	EF1	GAPDH	TUB
			1.45±0.31	2.31±0.44	2.76±0.69	2.78±0.60	5.92±1.19	7.29±1.44	8.20±1.69	8.95±1.69	11.43±2.1

Table 4. Comprehensive ranking of 9 oat candidate reference genes calculated by RefFinder

RefFinder analysis												
Ranking order			1	2	3	4	5	6	7	8	9	
Drought	Stress	Mv Pehely	Leaves (h)	PGD	TBPII	EF1	TUB	CYP	ADPR	GAPDH	ACT	UBC
			Roots (h)	ADPR	GAPDH	PGD	UBC	TBPII	ACT	CYP	TUB	EF1
	Mv Hópehely	Leaves (h)	ACT	UBC	ADPR	PGD	TBPII	TUB	GAPDH	EF1	CYP	
		Roots (h)	GAPDH	UBC	ADPR	EF1	PGD	CYP	ACT	TUB	TBPII	
Salt Stress	Mv Pehely	Leaves (h)	GAPDH	CYP	PGD	ADPR	ACT	EF1	TBPII	TUB	UBC	
		Roots (h)	ADPR	PGD	CYP	UBC	TBPII	EF1	GAPDH	ACT	TUB	
	Mv Hópehely	Leaves (h)	GAPDH	CYP	PGD	ADPR	UBC	ACT	TBPII	TUB	EF1	
		Roots (h)	UBC	PGD	ADPR	EF1	TBPII	GAPDH	CYP	ACT	TUB	
Heavy Metal Stress	Mv Pehely	Leaves (h)	GAPDH	ADPR	CYP	ACT	PGD	UBC	TBPII	EF1	TUB	
		Roots (h)	PGD	CYP	EF1	GAPDH	ACT	ADPR	TBPII	UBC	TUB	
	Mv Hópehely	Leaves (h)	ACT	GAPDH	ADPR	PGD	UBC	CYP	TBPII	EF1	TUB	
		Roots (h)	GAPDH	PGD	CYP	ADPR	EF1	ACT	UBC	TBPII	TUB	
Cold	Stress	Mv Pehely	Leaves (h)	ADPR	UBC	ACT	CYP	PGD	TUB	TBPII	GAPDH	EF1
			Leaves (s)	ADPR	ACT	CYP	TUB	TBPII	UBC	PGD	GAPDH	EF1
			Roots (h)	ADPR	PGD	GAPDH	UBC	EF1	TBPII	ACT	TUB	CYP
	Mv Hópehely	Leaves (h)	ADPR	UBC	ACT	TUB	PGD	CYP	EF1	TBPII	GAPDH	
		Leaves (s)	ADPR	CYP	PGD	TBPII	UBC	TUB	ACT	GAPDH	EF1	
		Roots (h)	ADPR	PGD	UBC	GAPDH	CYP	EF1	ACT	TBPII	TUB	
Heat	Stress	Mv Pehely	Leaves (h)	ADPR	UBC	GAPDH	CYP	PGD	TBPII	ACT	TUB	EF1
			Leaves (s)	UBC	TBPII	ADPR	GAPDH	ACT	TUB	CYP	PGD	EF1
			Roots (h)	ADPR	EF1	PGD	ACT	CYP	TUB	TBPII	UBC	GAPDH
	Mv Hópehely	Leaves (h)	ADPR	UBC	GAPDH	ACT	CYP	TBPII	PGD	TUB	EF1	
		Leaves (s)	UBC	ADPR	CYP	TBPII	GAPDH	ACT	PGD	EF1	TUB	
		Roots (h)	ADPR	EF1	PGD	CYP	TUB	ACT	UBC	TBPII	GAPDH	
All samples	Mv Pehely		ADPR	TBPII	UBC	ACT	PGD	CYP	EF1	GAPDH	TUB	
	Mv Hópehely		ADPR	ACT	TBPII	UBC	PGD	CYP	EF1	GAPDH	TUB	

Table 5. Primer sequences of 9 candidate reference genes used in RT-qPCR analysis

Gene Abb.	Gene Name	Primer sequence and Reverse	Forward	Amplicon Length (bp)	Efficiency	R ²	Accession No.
ACT	Actin1	CGAGCGGGAAATTGTAAGGG CGATCATGGATGGCTGGAAG		191	92%	0.994	MF405765.1
TUB	α -Tubulin	AGGTCTTCTCCCGCATCG CCTCCTCCATGCCCTCAC		90	98%	0.995	U76558.1
CYP	Cyclophilin	AGTCCATCTACGGCGAGAAGT GGGACGGTGCAGATGAAGAA		120	93%	0.998	EU035525.1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	GTTTGGCATCGTTGAGGGTT TGCTGCTGGGAATGATGTTG		131	93%	0.998	KR029492.1
UBC	Ubiquitin conjugating enzyme	CAAGCTGACCCTGCAATTCA GGGCTCCACTGGTTCTGTA		135	91%	0.992	M62720.1
EF1	Elongation factor 1- α	AAGGAGGCAGCCAACCTTCA AGCTCAGCAAACCTTGACAGC		122	96%	0.999	M90077.2
TBP1I	TATA-binding protein II subunit	GATGAGGCAGCCGAAGATTG TCCAAAGTCAACCATCATTGCT		156	91%	0.993	L07604.1
ADPR	ADP-ribosylation factor	CTCATGGTTGGTCTCGATGC ACATCCCAAACAGTGAAGCT		143	94%	0.998	Ta2291 (Unigene cluster)
PGD	Phosphogluconate dehydrogenase	GCAAAGATGAAACTGGTGGTCA CAACCCACTTTTGTCCGCC		90	93%	0.995	Ta30797 (Unigene cluster)

Figures

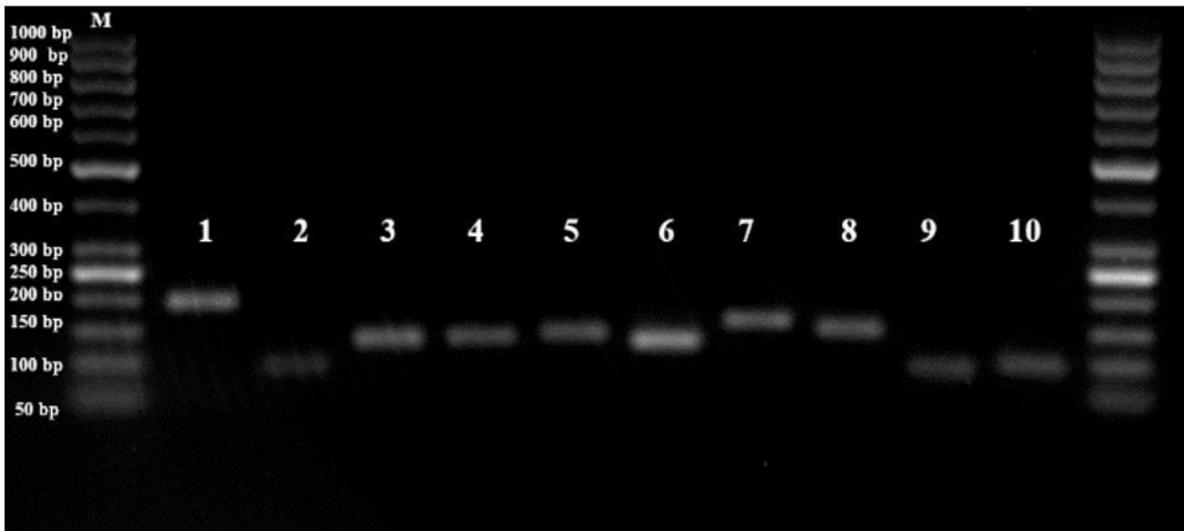


Figure 1
 PCR products of reference genes (1-9) and gene of interest (10). M: 250 bp DNA Ladder, 1: ACT, 2: TUB, 3: CYP, 4: GAPDH, 5: UBC, 6: EF1, 7: TBP1I, 8: ADPR, 9: PGD, 10: PAL.

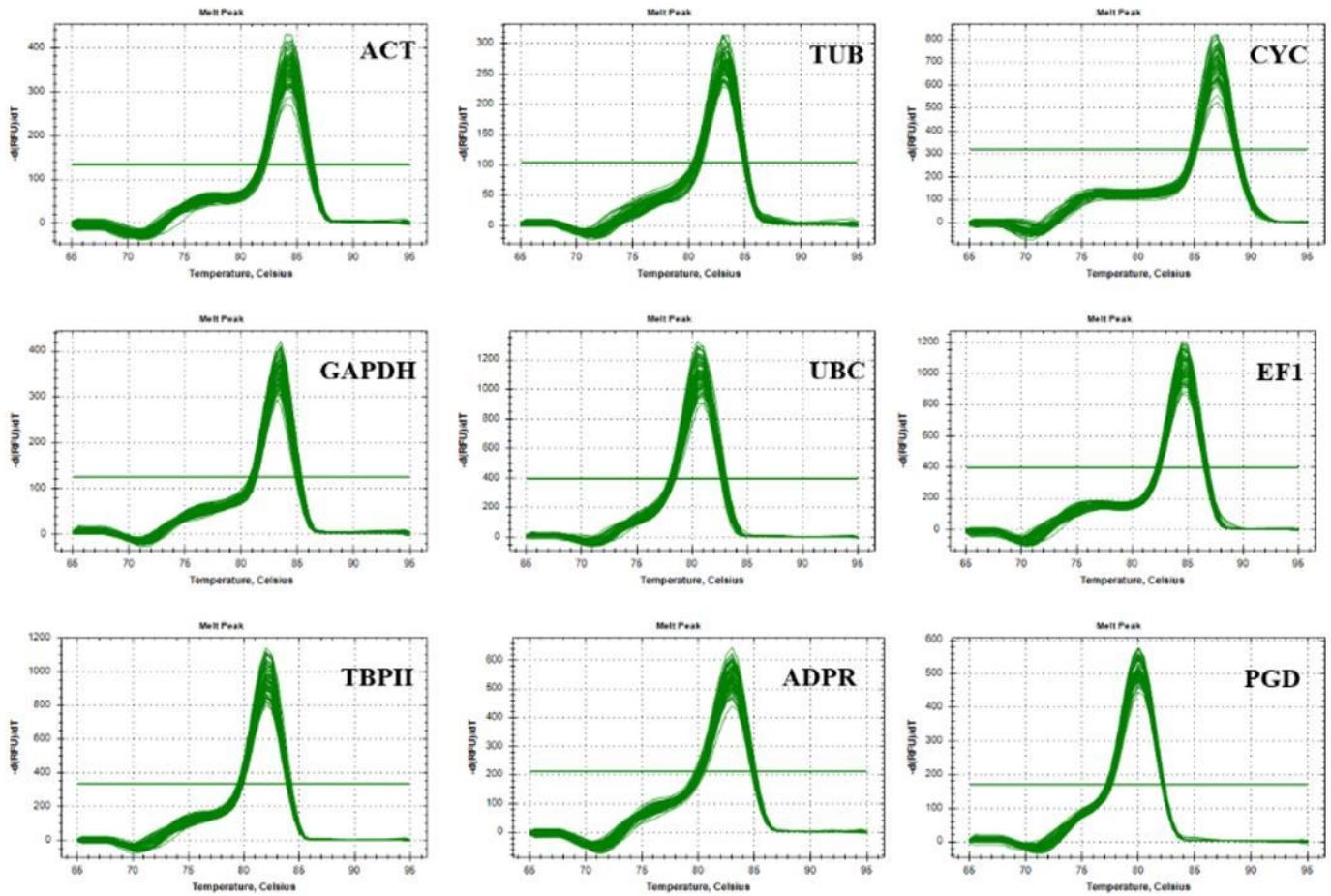


Figure 2

Melt curves of 9 oat reference genes candidates

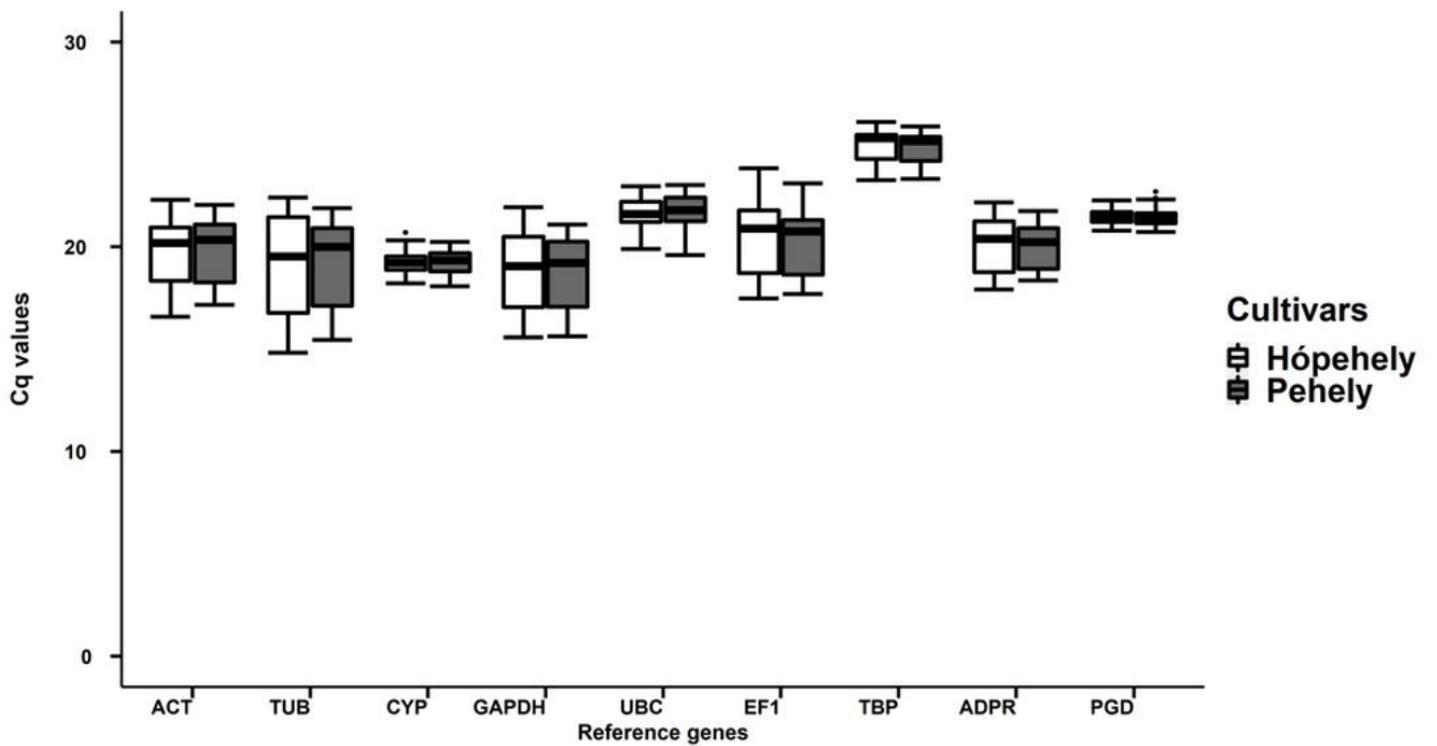


Figure 3

qRT-PCR Cq values for 9 oat candidate reference genes in leaf and root samples under different abiotic stresses in cultivars 'Mv Pehely' and 'Mv Hópehely'

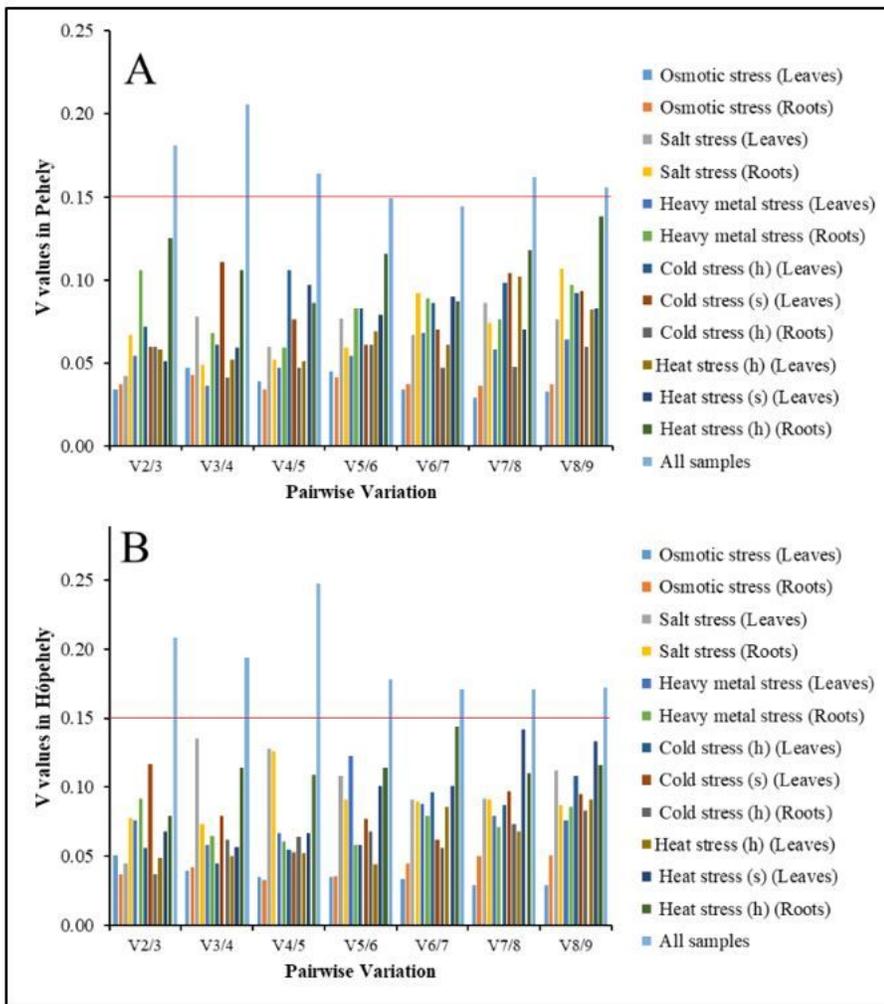


Figure 4
Pairwise variation measure of candidate reference genes in Mv Pehely (A) and Mv Hópehely (B) genotypes. The two types of growing media are indicated with h (hydroponic) and s (soil).

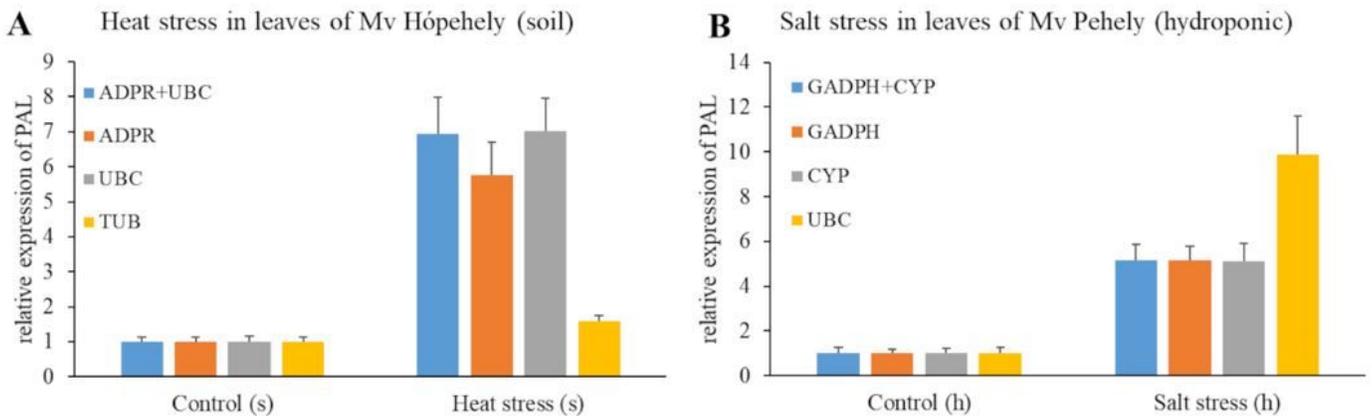


Figure 5

Relative expression levels of the target gene. A) Relative expression level of phenylalanine ammonia lyase (PAL) under heat stress in leaves of Mv Hópehely in pot experiment using the two most stable (ADPR and UBC) and the least stable (TUB) genes. B) Relative expression level of PAL under salt stress in leaves of Mv Pehely in hydroponic culture using the two most stable (GAPDH+CYP) and the least stable (UBC) genes.

Supplementary Files

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- [Additionalfile1.pdf](#)
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