scientific reports

Check for updates

OPEN

Identification of reference genes for gene expression assessment in *Avena sativa* under biotic stress triggered by *Blumeria graminis*

Sylwia Sowa✉, Joanna Toporowska & Edyta Paczos-Grzęda✉

A repeatable and reliable reverse transcription quantitative PCR (RT-qPCR) experiment depends upon proper reference genes (RGs) selection. This study aims to examine the expression stability of nine candidate RGs for the *Avena sativa* – *Blumeria graminis* experimental setup. *B. graminis* causes powdery mildew - the most devastating and economically important fungal disease of crops worldwide. RGs were evaluated in *Pm3* and *Pm4* oat differential lines and the susceptible cultivar Fuchs during compatible and incompatible interactions with different pathotypes of *Blumeria graminis* f. sp. *avenae* in six-time points post inoculation. The identification of genes exhibiting high expression stability was done by four algorithms (geNorm, NormFinder, BestKeeper and deltaCt). The results indicated that regardless of the analysed group, two most stable RGs are required for data normalization. The most sufficient RGs combination was *HNR* (heterogeneous nuclear ribonucleoprotein 27 C) + *EIF4A* (eukaryotic initiation factor 4 A-3). *ARF* (ADP-ribosylation factor) could also be pondered as demonstrating high expression stability. These genes can be considered universal candidates for RT-qPCR normalization to study interaction with *B. graminis* as well as *Puccinia coronata* and *Puccinia graminis*, as confirmed by our previous research. The worst candidate for data standardisation was TUA (α- tubulin). To our best knowledge, this is the first report regarding RGs' selection in this pathosystem. Identified RGs are proper normalisation candidates for gene expression studies in the *A. sativa* infected by *B. graminis* as well as other related pathogens.

Keywords RT-qPCR, Reference genes, *Blumeria graminis*, *Avena sativa*

Abbreviations

RG	Reference gene
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
ARF	ADP-Ribosylation factor
CYP	Cyclophilin
EF1A	Elongation factor 1-alpha
EIF4A	Eukaryotic initiation factor 4 A-3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HNR	Heterogeneous nuclear ribonucleoprotein 27 C
HSP70	Heat shock protein
TUA	Alpha tubulin
UBC	Ubiquitin conjugating enzyme (E2)
PAL	Phenylalanine ammonia lyase

Powdery mildew caused by the foliar pathogen, *Blumeria graminis* f. sp. *avenae* is among the most devastating, economically important fungal diseases of oat (*Avena sativa* L.). Developing resistance to powdery mildew is a crucial component of oat breeding and research. To date, 13 *Pm* resistance genes have been catalogued and genetically mapped in oat[1–5]. Based on *B. graminis* virulence analyses, attempts are being made to identify, introduce and pyramidize effective powdery mildew resistance genes (*Pm*)[6–8]. However, resistance conditioned by major genes may be short-lived, gradually overcome by new pathogen races therefore, the aim is to obtain durable resistance, independent of environmental factors[9]. Successful introduction of effective resistance

Institute of Plant Genetics, Breeding and Biotechnology, University of Life Sciences in Lublin, Akademicka 13, Lublin 20-950, Poland. ✉email: sylwia.sowa@up.lublin.pl; edyta.paczos@up.lublin.pl

strategies into oat breeding depends on understanding the host-pathogen interaction processes. The *Pm* genes have not yet been cloned, and the molecular mechanisms underlying the *A. sativa – B. graminis* interaction remain poorly understood. Lack of knowledge in this area constitutes the main issue facing modern phytopathology and resistance breeding.

RNA sequencing (RNAseq) and transcriptome analysis using high-throughput next-generation sequencing approaches (NGS) are nowadays the most frequently used tools for studying complex plant resistance mechanisms. Accurate validation and quantification of gene expression derived from in silico NGS data is performed by RT-qPCR. This study attempts to establish the most stable RGs for the *A. sativa – B. graminis* experimental setup to ensure obtaining repeatable and reliable RT-qPCR data. Thus far, research involving a selection of reference genes has focused on *Avena fatua* L.[10,11] and *Avena ludoviciana* L.[12] in response to herbicide stress. Additionally, RGs were selected for molecular investigation of *A. fatua* caryopses dormancy[13] and in response to *Trichoderma polysporum* infection[14]. The only study of reference genes' stability on *A. sativa* was performed by Yang et al.[15] on different types of oat tissues at various growth stages, Duan et al.[16] under salt stress and Tajti[17] under five abiotic stresses. Our research team was the first to investigate the effect of fungal infection (*Puccinia coronate* f. sp. *avenae and Puccinia graminis* f. sp. *avenae*) on candidate RGs expression in *A. sativa*[18,19]. In this study, the RGs selected based on literature and used in the previous analysis were also validated as an internal control for studying *A. sativa – B. graminis* interaction. The results allowed to verify the universality of the selected genes and enabled to improve the RTq-PCR protocol for *A. sativa – B. graminis* experimental setup.

Methods

Plant material and pathogen inoculation

In the study, we used *Pm3* carrier - *A. sativa* cultivar Mostyn with resistance originally derived from *A. sterilis* L. var. *ludoviciana* Cc 4346[20] and *Pm4* carrier - Cc6490 line – drived from the cross of cultivar Manod with *A. sativa* × *A. barbata* derivative possessing irradiation-induced translocation from highly resistant *A. barbata* genotype collected in Algeria[21]. Both *Pm3* and *Pm4* are major genes ensuring resistance to *B. graminis* f. sp. *avenae*. The common oat cultivar Fuchs without documented resistance to powdery mildew infection was a susceptible control. Oat seeds were kindly provided by Dr Matthias Herrmann from Julius Kuehn Institute, Federal Research Centre for Cultivated Plants, Institute for Breeding Research on Agricultural Crops, Sanitz, Germany. Oat seeds were grown in plug trays filled with a universal substrate containing peat for 10 days in a phytotron at 18 °C for a 16-h photoperiod.

For pathogen inoculation two *B. graminis* f. sp. *avenae* race isolates (Pol_18, Kar_21, Table 1) were chosen with a virulence profile defined based on a set of nine differential oat lines carrying a single powdery mildew resistance genes (*Pm1*, *Pm3*, *Pm4*, *Pm5*, *Pm6*, *Pm7*, *Pm3*&*8*, *Pm9*, *Pm10*). To generate compatible interaction (infection and disease development), 'Fuchs' was treated with *B. graminis* race Pol_18 and *Pm3* was treated with Kar_21. Incompatible interaction (a host-type resistance response) was obtained for *Pm3* inoculated with Pol_18 and *Pm4* inoculated with Kar_21 (Fig. 1).

Before inoculation, spores of isolates were multiplied on leaf fragments of 'Fuchs'[22] using the host-pathogen method of Hsam et al.[23]. Inoculation and incubation were performed on the first leaves of 10-day-old seedlings according to the methodology described by Sowa et al.[18,19].

Plant experiments were performed in accordance with relevant guidelines and regulations.

RNA extraction

Total RNA extraction from plant material was performed after 0 (uninoculated 'Fuchs, *Pm3* and *Pm4*), 6, 12, 24, 48, 72 and 96 hours post-inoculation (hpi). Three biological replicates, each comprised of five leaves pooled from different seedlings from the analysed group were sampled and grind with a sterile mortar and pestle in liquid nitrogen. The RNA isolation was performed with TRIzol reagent (Invitrogen) following the manufacturer's protocol. RNA quality and quantity were assessed with NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific Inc., USA) and electrophoretically on 1.5% agarose gel (Table S1, Fig. S1).

Two-step RT-qPCR

The cDNA synthesis and DNase treatment were performed with Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase (Thermo Scientific™) according to the manufacturer's instructions. The reverse transcription was conducted in 20 µl reactions containing 1 µg of RNA. Obtained cDNA diluted before use to 30 ng/µl served as a template in the qPCR reactions.

In our preliminary research regarding RGs validation for studying *A. sativa* interaction with *Puccinia* pathogens[18,19], a wider set of candidate genes was tested. Only nine RGs (*ARF* - ADP-ribosylation factor, *CYP* - cyclophilin, *EF1A* - elongation factor 1-alpha, *EIF4A* - eukaryotic initiation factor 4 A-3, *GAPDH* - glyceraldehyde-3-phosphate dehydrogenase, *HNR* - heterogeneous nuclear ribonucleoprotein 27 C, *HSP70* - heat shock protein, *TUA* - alpha tubulin, *UBC* - ubiquitin conjugating enzyme (E2)) met all amplification criteria

Race no.	Virulence to Pm differentials	Avirulence to Pm differentials
Pol_18	*Pm1*, *Pm6*, *Pm9*, *Pm10*	*Pm3*, *Pm3*&8, *Pm4*, *Pm5*, *Pm7*
Kar_21	*Pm1*, *Pm3*, *Pm3*&*8*, *Pm6*, *Pm9*, *Pm10*	*Pm4*, *Pm5*, *Pm7*

Table 1. Virulence profile of *B. graminis* f. sp. *avenae* pathotypes used for inoculation.

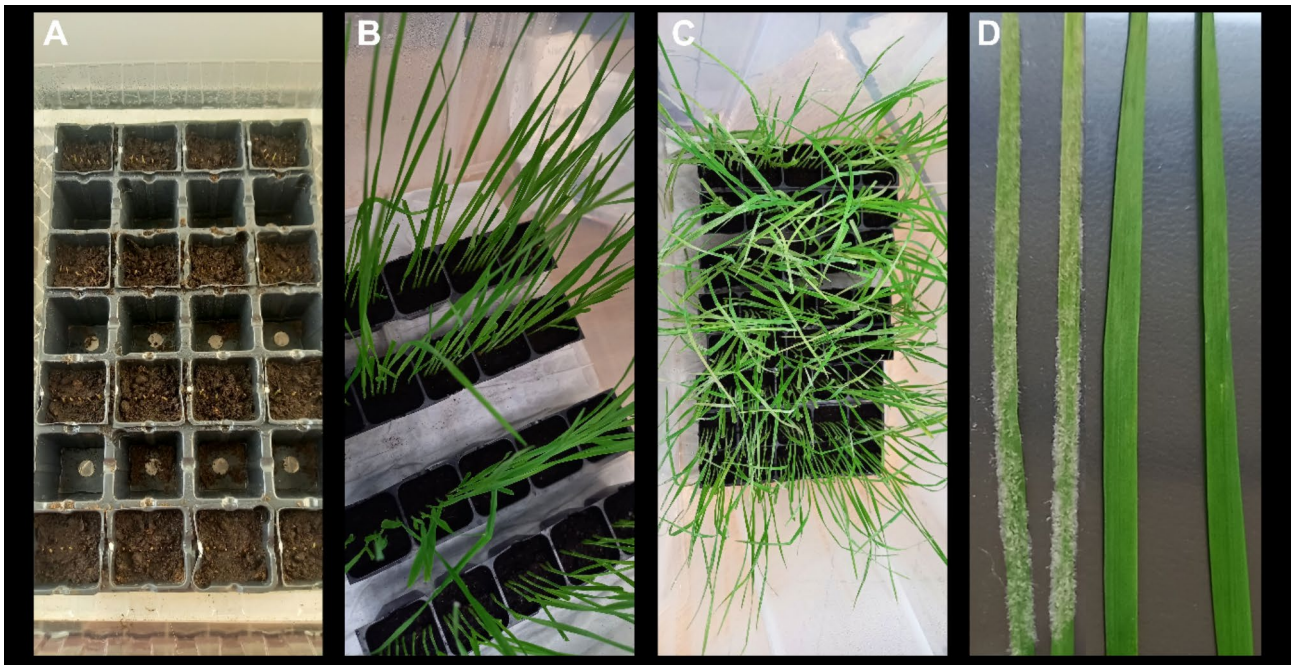

Fig. 1. *Pm3* and *Pm4* oat differential lines and cultivar Fuchs during compatible and incompatible interactions with different *Blumeria graminis* f. sp. *avenae* races.(**A**) Oat seeds grown in plug trays. (**B**) Plant material collection at different time intervals after *B. graminis* inoculation (**C**) Powdery mildew symptoms in susceptible oat genotype 'Fuchs' treated with *B. graminis* race Pol_18 twelve days after inoculation. (**D**) Comparison of powdery mildew symptoms on leaves of *Pm3* treated with Kar_21 (susceptible reaction) and Pol_18 (resistant reaction) *B. graminis* races.

(amplification efficiency between 90 and 110%, regression coefficients ≥ 0.990, presence of a single amplicon on the dissociation curve) and were used for gene expression analyses in the present study (Table S2).

All qPCR reactions were performed on QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, USA) with Power Track SYBR Green Master Mix (Thermo Fisher Scientific Inc., USA). The 20 µL reaction mixture consisted of 30 ng of cDNA, 1 × qPCR Mix and 400 nM of each primer. The cycling program was as follows: 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To confirm the specificity of PCR products, melting curve analysis was performed after each run with continuous data collection from 60 to 95 °C. The reactions were performed in three biological replicates along with no template control (NTC) and no-reverse transcription control (NRT). To confirm amplification efficiencies for each primer pair, standard curves generated from serial dilution of pooled cDNA were used.

Gene expression stability analysis

Raw data was processed using a dedicated module from Thermo Fisher Cloud (Thermo Fisher Scientific). The expression stability of candidate RGs was assessed by geNorm[24], NormFinder[25], BestKeeper[26] and delta Ct method[27]. The Cq values included reaction efficiency corrections. The analysis was conducted according to the following datasets – separately for *A. sativa* – *B. graminis* compatible and incompatible interactions, independently for Fuchs cultivar, *Pm3* and *Pm4* oat line and additionally as full dataset consisting of all experimental samples[28]. Overall final, comprehensive ranking was generated according to the method proposed by Velada et al.[29] based on the geometric mean of the ranks.

To verify the reliability of the selected RGs, the relative expression analysis of *PAL* gene (phenylalanine ammonia lyase) was measured and standardized using the most stable and least stable RGs with the $2^{-\Delta\Delta Ct}$ method.

Results

Primers' characteristics

Primers' efficiency and specificity were retested on a pool of all cDNA of the examined oat genotypes. All primer pairs of nine RGs identified in our preliminary research (*ARF*, *CYP*, *EF1A*, *EIF4A*, *GAPDH*, *HNR*, *HSP70*, *TUA* and *UBC*) generated single amplicons confirmed by melting curves analysis (Fig. S2) and no peak was detected in the NRT nor NTC samples. Primer's efficiency varied from 100.76% (*EIF4A*) to 112.71% (*GAPDH*) with the regression coefficients above 0.99. The primer pairs melting temperature (Tm) was between 83.51 °C (*EF1A*) and 91.9 °C (*CYP*), and the amplicon sizes varied from 88 bp (*EIF4A*) and 158 bp (*EF1A*) (Table 2). To determine the gene expression levels, raw quantification cycle (Cq) values were estimated. The mean Cq ranged between 18.1 (*EF1A*) and 22.5 (*HNR*) (Fig. S3).

Gene	Slope	R^2	Reaction efficiency (%)	Tm (°C)
ARF	−3.1367	0.9966	108.36	85.39
CYP	−3.2261	0.9967	104.16	91.90
EF1	−3.2532	0.9994	102.95	83.51
EIF4A	−3.3039	0.9994	100.76	86.24
GAPDH	−3.0508	0.9973	112.71	85.82
HNR	−3.2321	0.995	103.89	83.72
HSP70	−3.1204	0.9982	109.16	86.39
TUA	−3.0647	0.9975	111.98	83.71
UBC	−3.0678	0.9979	111.82	84.15
PAL	−3.1167	0.9965	109.34	86.45

Table 2. Primer parameters derived from RT-qPCR analysis. R^2— regression coefficient, Tm - melting temperature of the amplicon.

All primer pairs were used for expression stability analysis using four algorithms, BestKeeper, ΔCt, geNorm and NormFinder. For each algorithm, RGs were ranked from the most to least stable.

Gene expression stability analysis

BestKeeper

BestKeeper uses untransformed Cq values and determines the most stable genes based on the highest correlation coefficients (r) and the lowest standard deviations (SD)[26]. The highest coefficient of correlation in all experimental sets, regardless of the sample subgroups being analysed separately or together, was estimated for *GAPDH* and *HNR*. The lowest r was recorded for *UBC*. All tested genes displayed SD below 1. The lowest mean SD was observed within *EIF4A* and *HNR* results. The highest mean SD was within *TUA*, *CYP* and *GAPDH* results (Fig. S3; Fig. 2A, B).

ΔCt

The ΔCt method compares the relative expression of gene pairs within each sample to determine which reference gene is the most stable. A low average SD (mean SD) indicates a low level of variability. According to the ΔCt, HNR and EIF4A were the most stable candidate RGs in the overall dataset and other analysed subgroups. Regardless of the dataset, TUA had the largest mean SD recorded (Fig. 2C).

geNorm

The stability measure (M value) of all tested RGs, was calculated by the GeNorm algorithm based on relative Cq values. A reference gene is considered stable if its M value is less than the cutoff of 1.5[24]. In the evaluated datasets, every candidate gene showed expression stability lower than 0.95. The best-performing RG in the full dataset and all analysed subgroups was *HNR*. When individual subgroups were analysed, the second most stable gene was *EIF4A*, however, in the case of the full dataset, slightly lowest M value was shown by *ARF*. The worst performing RG in almost each analysed sample set was *TUA*. In the case of *A. sativa – B. graminis* incompatible interaction, the highest M value was recorded for *CYP* (Fig. 2D).

NormFinder

In NormFinder based on relative Cq quantities, intra- and intergroup variation within each analysed dataset was calculated and a stability value (SV) for each gene was estimated[25]. Genes with low SV associated with low expression variation are considered best candidates for RT-qPCR data normalization. In this experiment, SV of all tested candidate RGs was below 0.4. The lowest SV was presented by *HNR*, with the exception of *Pm4* oat line dataset, where a slightly lower value was recorded for *GAPDH* and *HNR* was the second best-performing RG. In the remining analysed subgroups *EIF4A* was evaluated as the second most stable gene, however, when the full dataset was considered, *ARF* performed better. The highest SVs representing high variation in expression was reported for *TUA* and *CYP* (Fig. 2E).

Reference genes selection

The number of RGs ideal for correct normalization of qPCR data was determined by applying the geNorm algorithm to calculate pairwise variation (Vn/Vn + 1). Vn/Vn + 1 < 0.15 suggests that n genes are adequate to produce trustworthy results and adding $n+1$ more genes would not enhance the data analysis. In this study, $V_{2/3}$ values obtained within all analysed datasets were below 0.10 (Fig. 3) indicating that, regardless of the analysed group, only two most stable RGs are required for effective data normalization.

Due to the differences between SD and r results generated by BestKeeper, a comprehensive ranking was performed by separating these results as well as completely excluding the BestKeeper results (Table S3). Overall, the most stable expression regardless of the sample subgroups being analysed separately or together, was recorded for *HNR*. The most appropriate combination of RGs for obtaining reliable qPCR results in a tested plant-pathogen system was *HNR* + *EIF4A*. High level of expression stability was also recorded for *ARF*. The worst gene candidate for normalization showing the highest expression variation was *TUA*.

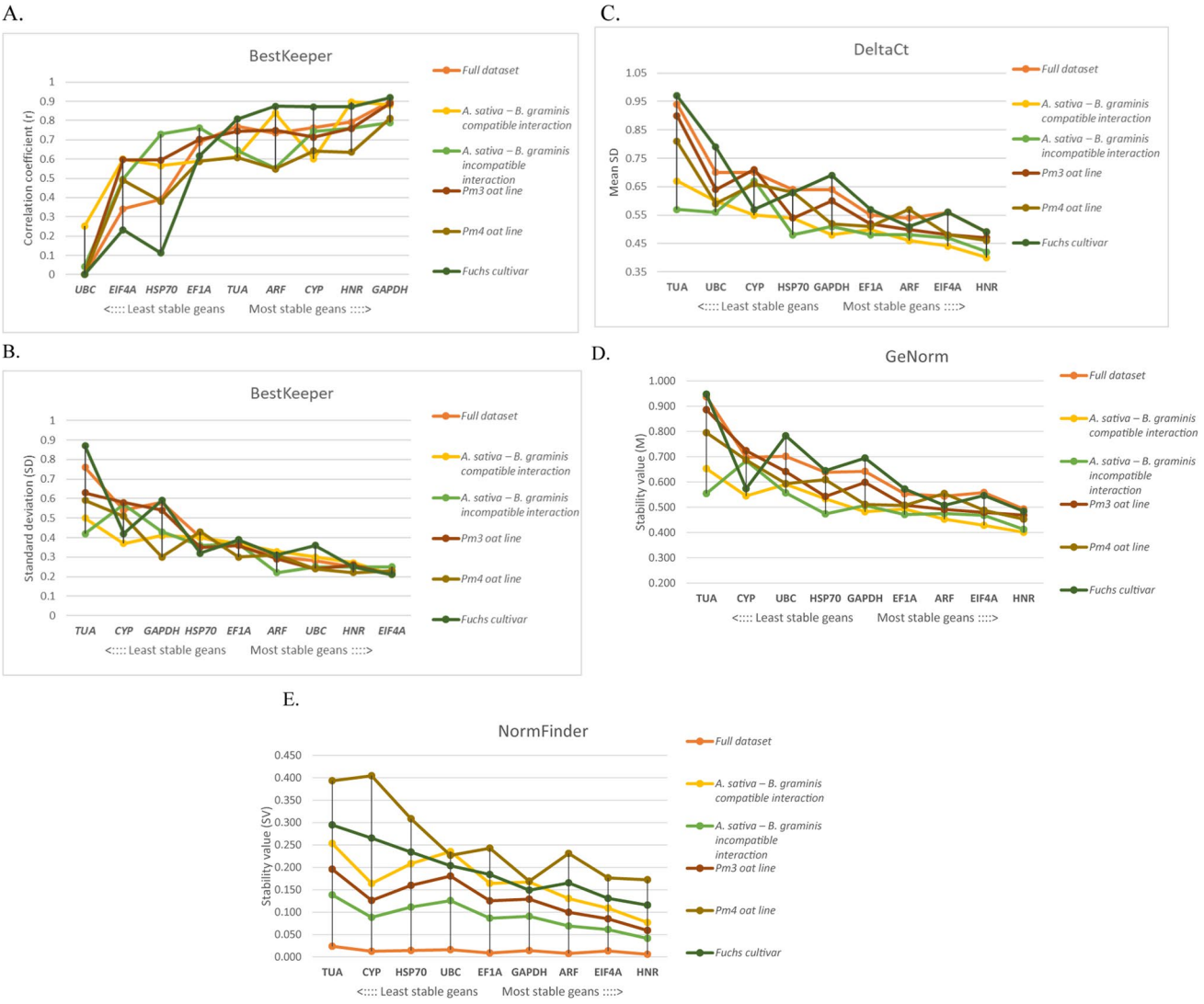

Fig. 2. Expression stability of candidate reference genes determined by BestKeeper (**A**, **B**), ΔCt (**C**), geNorm (**D**) and NormFinder (**E**) algorithms for full dataset, compatible and incompatible interaction dataset, Pm3 and Pm4 oat line as well as Fuchs cultivar dataset.

Reference genes validation

The relative gene expression profile scan of *PAL* was carried out to demonstrate how inaccurate data normalization may affect the results and to verify the reliability of the chosen RGs. The examination was conducted within four datasets: Fuchs cultivar – *B. graminis* compatible interaction (Fig. 4), *Pm4* – *B. graminis* incompatible interaction as well as *Pm3* – *B. graminis* compatible and incompatible interaction (Fig. S4) against the lowest-performing RG (*TUA*) as well as two sets of the highest-performing RGs (*HNR* + *ARF* and *HNR* + *EIF4A*), either independently or together. With best-performing RGs used either singly or in pairs, consistent expression patterns were seen at each time point across all examined groups. *TUA* normalized data showed a significant overestimation of *PAL* transcript level reaching up to seven times in samples of *Pm3* – *B. graminis* incompatible interaction harvested 72 and 96 hpi, indicating inaccurate transcription upregulation (Fig. S4).

Discussion

Crown rust (caused by *P. coronata)*, stem rust (*P. graminis*) and powdery mildew (*B. graminis*) are the most widespread and damaging oat diseases, however, the molecular basis of oat resistance to these pathogens remains poorly understood. Phytopathological studies often rely on examining gene expression patterns using molecular biology techniques, with RT-qPCR emerging as a favoured method due to its sensitivity and specificity. To ensure the reproducibility of results, the MIQE guideline recommends the use of reference genes (RGs)[30], however the RG's expression stability may be strictly dependent on the type of the performed experiment[31–35]. Our previous studies provided the first data regarding RGs' selection for *A. sativa* under *P. coronata* and *P. graminis* infection[18,19]. Both studies examined the same set of nine genes selected based on literature and enabled the choice of a universal qPCR normalization set suitable to use in both compatible and incompatible interactions.

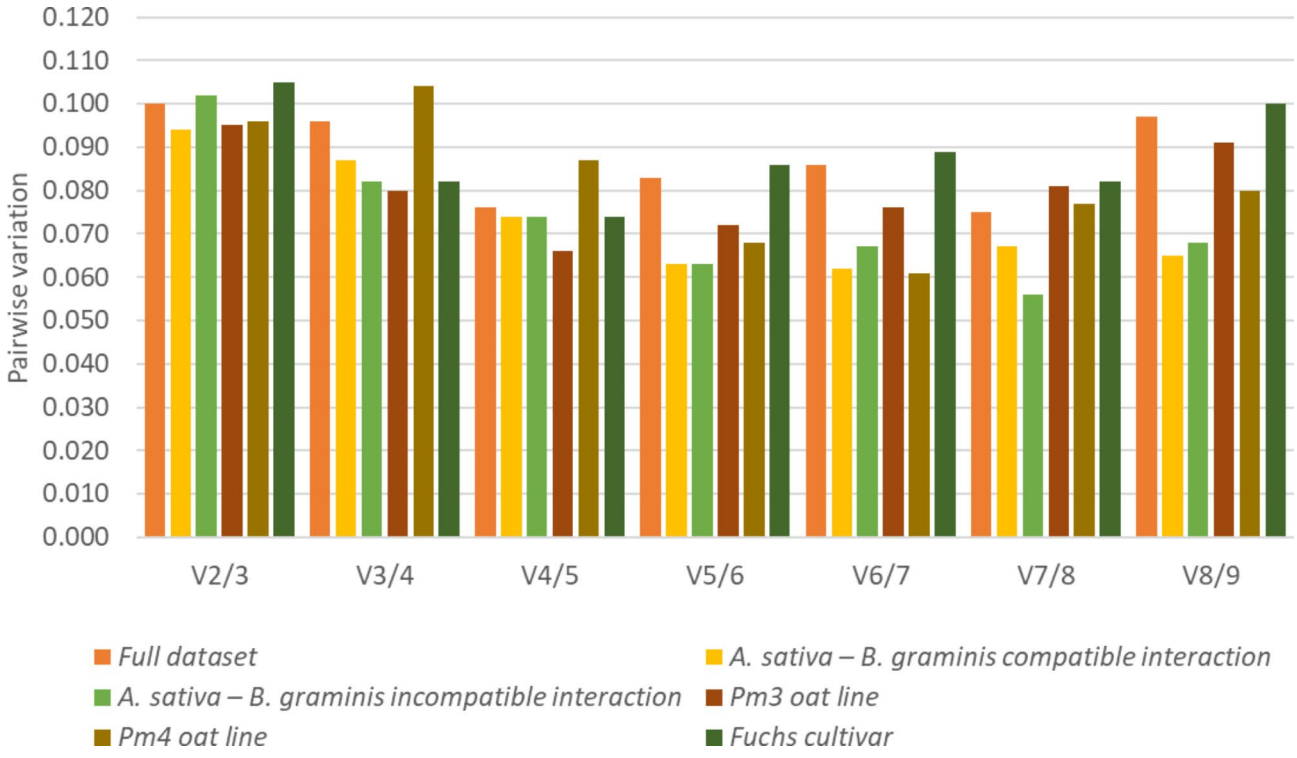

Fig. 3. The optimal number of RGs for accurate RT-qPCR data normalization calculated by the geNorm algorithm. Pairwise variation (V_n/V_{n+1}) value < 0.15 indicates no need for an additional reference gene.

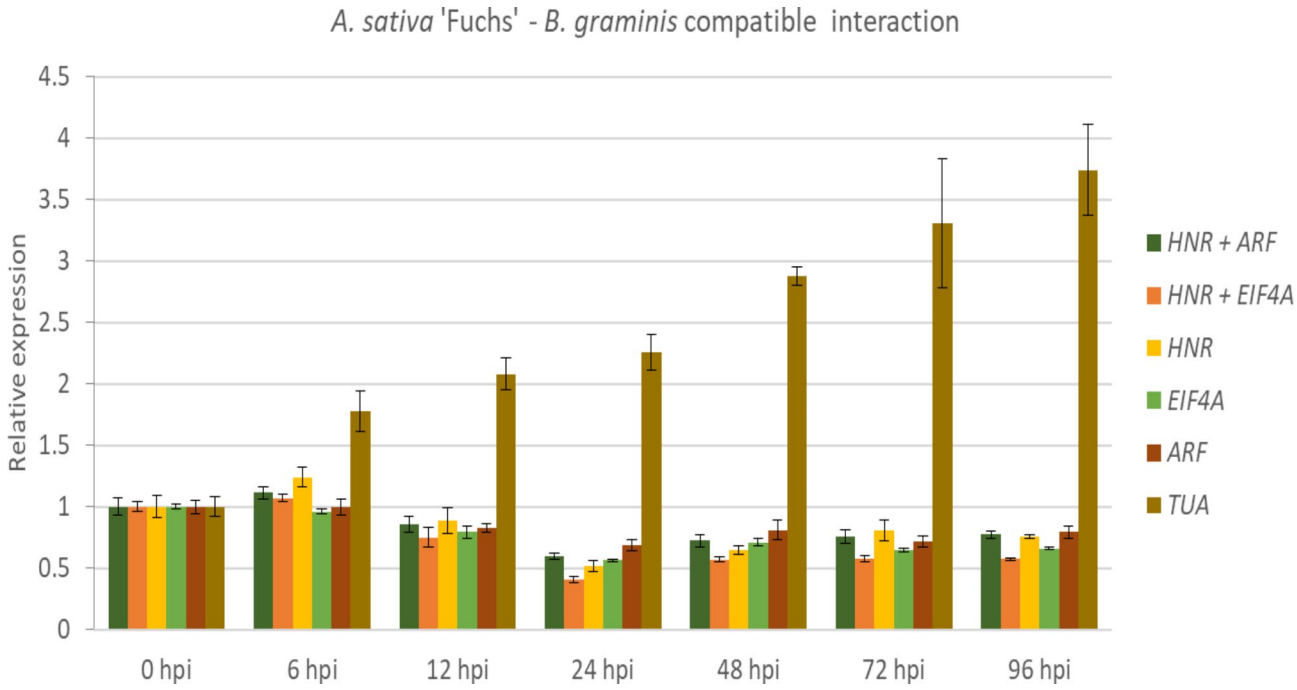

Fig. 4. Gene expression profile of the *PAL* (phenylalanine ammonia lyase) in *A. sativa* Fuchs cultivar – *B. graminis* compatible interaction. The evaluation was done against the lowest-performing reference gene (*TUA*) as well as two sets of the highest-performing RGs (*HNR* + *ARF* and *HNR* + *EIF4A*), either independently or together. Data shown as mean ± SD.

In this research, the same nine genes' set was tested as an internal control for studying *A. sativa – B. graminis* interaction which allowed the verification of the selected genes' universality and the selection of genes most suitable for this pathosystem.

In *A. sativa* studies, there are many obstacles related to the development of a universal research methodology resulting from the complexity of the oat genome[36]. Although the amount of *Avena* sequencing data is continually growing[37–39], Yang et al.[15] drew attention to the difficulty in designing PCR primers optimal for gene expression analysis. Oat polyploid genome mainly contains duplicated genes and each gene copy may not be uniformly expressed in different samples[40,41]. Although "single-copy" genes should ideally be used as a reference, the study of Yang et al.[15] shows that using duplicated RG's in polyploid oat is also reasonable and valid. The authors evaluated the expression of eleven genes in *A. sativa* developing seeds and corresponding endosperm, as well as shoots and roots of seedlings including four duplicated genes. The best-performing potential RG set in all investigated samples and developing endosperms has been determined to be *EIF4A + HNR*. The pairing of *EP + EF1A* was the most effective RGs' set for developing seeds. The four-copy duplicated RG, *UBC21* (Ubiquitin-Conjugating Enzyme 21) paired with *HNR* were identified as the most stable RGs set in shoots and roots of oat seedlings. Duan et al.[16] highlighted how particular sample subsets may affect the appropriate selection of RGs in *A. sativa*. According to their research, *EF1A* and *TBP* (TATA-binding protein) were the two RGs that performed the best in salt-stressed *A. sativa* samples. However, *TBP* exposed medium expression stability in the dataset composed of various tissues. *EF1A* and *PP2A* (serine/threonine protein phosphatase 2 A) were the top-ranked RGs selected in this subgroup. In another research involving RGs selection in *A. sativa* Tajti et al.[17] conducted expression assessments on the roots and leaves of oat seedlings exposed to different abiotic stressors. There was significant variation in the order of RGs in the comprehensive rankings produced for particular datasets. Depending on the experimental set, the same RG was ranked first or last (for example, *GAPDH* in salt-stressed leaves and cold-stressed leaves, respectively). However, the authors were able to identify *ARF* as the top-rated RG for all samples.

Apart from these studies, research involving the selection of RGs to analyse gene expression under different stress conditions has primarily focused on *Avena fatua* L. and *Avena ludoviciana* Dur. Wrzesińska et al.[10] assessed the expression of six genes in *A. fatua* biotypes resistant to various herbicides and identified *TBP* and *GAPDH* as the most stably expressed. Eight potential RGs of *A. fatua* were evaluated by Liu et al.[11]. The authors selected *GAPDH* and *EF1A* as the best RGs to investigate *A. fatua* gene expression profiles of various tissues, developmental stages, and herbicide treatments. Similar investigations were conducted on *A. ludoviciana* herbicide-treated stem and leaf tissues[12]. Out of four genes, *TBP* was concluded to be the most stable reference for qPCR analysis. This gene along with *18 S* (18 S ribosomal RNA) and *UBC* were also found among the RGs with the greatest stability in the study of *A. fatua* in response to *Trichoderma polysporum* infection[14]. Moreover, in the case of molecular studies of *A. fatua* dormancy, *AfTBP2*, the homolog of *TBP*, along with two *UBC* homologs, *AfUBC1* and *AfUBC2*, as well as one homolog of *GAPDH*, *AfGAPDH1* appeared as more stably expressed than other tested RGs[13].

In this research, we assessed the expression stability of candidate RGs in *A. sativa – B. graminis* compatible and incompatible interactions over six-time points post-inoculation with four methods, ΔCt, geNorm, NormFinder and BestKeeper. The first three algorithms generated similar results with almost identical arrangements of the first three recommended genes. Differences appeared in the results generated by BestKeeper, as it is based on the highest r and the lowest SD[26]. Overall, the most stable gene expression, regardless of the sample subgroups being analysed individually or together, was recorded for *HNR*. The second RG of choice for the full dataset was *ARF*, however, when individual subgroups were analysed, the second most stable gene was *EIF4A*. These genes performed very well and can be considered universal candidates for RT-qPCR normalization to study interaction with *B. graminis* as well as *P. coronata* and *P. graminis*, as confirmed by our prior research[18,19]. The genes we selected appeared to also serve as proper RGs in previous studies of *A. sativa* as well as other species. In the abovementioned study of Yang et al.,[15] out of eleven putative RGs *EIF4A + HNR* exhibited maximum stability in *A. sativa* developing endosperms as well as all evaluated samples. Moreover in roots and leaves of *A. sativa* seedlings exposed to different abiotic stressors, Tajti et al. selected *ARF* as the most suitable for all samples[17]. Maksup et al.[42] examined the expression stability of seven candidate genes in three indica genotypes and one japonica genotype of *Oryza sativa* L., of which *HNR*, *EP* (expressed protein) and *TBC* (TBC1 domain family member 22[a]) served as the high-quality references. In the study of Jakobus et al.[43], six RGs were tested in wheat line during incompatible interaction with *Puccinia triticina*, *Puccinia striiformis* and *Puccinia graminis* f. sp. *tritici*. *ARF* along with *RLI* (RNase L inhibitor-like protein) expressed the best stability in wheat infected by *P. triticina*. Interestingly, researchers obtained diverse results dependent on the studied interaction. In our studies, we have managed to select genes suitable for RTqPCR normalization regardless of the analysed genotype or interaction. Computing pairwise variation ($V_{2/3}$) with the geNorm algorithm allowed to determine the number of RGs ideal for correct normalisation in *A. sativa – B. graminis* pathosystem. $V_{2/3}$ obtained within all analysed datasets was below 0.15 indicating that, regardless of the analysed group, only two most stable RGs are required for effective data normalization. Moreover, gene expression results obtained using *HNR*, *ARF* and *EIF4* for normalization independently, were comparable to those achieved by normalizing with two RGs. This could be a feasible alternative for extensive experimental studies or when facing budget limitations.

The expression profile scan of the *PAL* was performed to demonstrate how imprecise data normalisation can affect the results and to verify the reliability of the selected RGs. *PAL* encodes a highly stress-responsive enzyme that plays a key role in the biosynthesis of salicylic acid, a major stress hormone. With best-performing RGs, consistent expression patterns were seen at each time point across all examined groups. *TUA* normalized data revealed a considerable overestimation of *PAL* transcript level reaching up to seven times in samples of *Pm3 – B. graminis* incompatible interaction harvested 72 and 96 hpi, indicating substantial upregulation of transcription. This demonstrates, how inaccurate data normalisation may impact obtained results. *TUA* was the worst candidate for data standardisation not only in the present study but also in the *A. sativa – P. graminis*

pathosystem analysed in our previous research[19]. In the *A. sativa* – *P. coronata* interaction the worst RG was *CYP*[18] however, both these genes exhibited low expression stability in each of our studies. The housekeeping genes of basic cellular metabolism may show altered expression due to the plant reallocating resources towards defence mechanisms, making RGs less stable under these conditions. During biotic stress, plants often reinforce cell walls as a physical barrier, which requires the expression of genes involved in cell wall synthesis and modification. Reference genes related to structural components like *ACT* and *TUA* may show variability due to these changes, as the plant prioritizes resources toward cell wall defence over other cellular maintenance[44,45]. Cyclophilins are often chosen as reference genes for qPCR normalization[46] however, studies have indicated that they accumulate during fungal infection and play a significant role in signal transduction under stressful conditions. Changes in the expression of cyclophilins in response to fungal attack have been recorded e.g. in cultivated grapevine (*Vitis vinifera* L.)[47,48], potato (*Solanum tuberosusm* L.)[49], pepper (*Capsicum annuum* L.)[50] and Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*)[51]. This confirms the pointlessness of choosing *CYP* as the reference gene to study plant-pathogen interaction. Furthermore, both *UBC* and *HSP70*, which in our studies had unstable expression levels, also seem to be poor choices for this type of analysis. *UBC* expression may vary due to stress-induced changes to accommodate increased pathogenesis-related protein production. Due to localized defence responses in infected or nearby tissues, plants may increase protein degradation and turnover mechanisms impacting ubiquitination or the proteasome pathway[52]. Although *HSP70* is often considered as a candidate RG[46] it has also been proven that this gene may be involved in plant-biotic stress interaction by e.g. stabilizing key proteins, assisting in defence signalling and regulating cell death[53,54].

This study confirms our previous findings, that *HNR* and *EIF4A* may be sufficient, universal RGs with a stable level of expression not only in *P. coronata* and *P. graminis*[18,19], but also in *B.* graminis compatible and incompatible interaction with *A. sativa*. All those pathogens have obligate biotrophic lifestyles depending entirely on live plant cells for nutrition and survival[55]. Many plant-biotroph interactions follow the gene-for-gene model, where specific pathogen effector genes (*Avr* genes) and corresponding plant resistance (*R*) genes interact[55]. In our studies, along with genotypes without documented resistance to pathogen infection, oat lines with major gene resistance were evaluated. Perhaps identified RGs are proper normalization candidates for gene expression studies in the *A. sativa* infected not only by *B. graminis*, but also by other obligate biotrophs against which resistance relies mostly on the classical gene-for-gene model. However, it cannot be ruled out that the results can be extended to interactions involving other types of immunity. The obtained results will significantly simplify the development of RT-qPCR research methodology and facilitate a comprehensive analysis of gene expression in oat.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Received: 12 August 2024; Accepted: 26 November 2024
Published online: 03 December 2024

References

1. Admassu-Yimer, B., Esvelt Klos, K., Griffiths, I., Cowan, A. & Howarth, C. Mapping of crown rust (Puccinia coronata f. sp. avenae) resistance gene Pc54 and a novel quantitative trait locus effective against powdery mildew (Blumeria graminis f. sp. avenae) in the oat (Avena sativa) line Pc54. *Phytopathology*. https://doi.org/10.1094/phyto-10-21-0445-r (2022).
2. Herrmann, M. H. & Mohler, V. Locating two novel genes for resistance to powdery mildew from Avena byzantina in the oat genome. *Plant. Breed.* **137**, 832–838 (2018).
3. Hsam, S. L. K., Mohler, V. & Zeller, F. J. The genetics of resistance to powdery mildew in cultivated oats (Avena sativa L.): Current status of major genes. *J. Appl. Genet.* **55**, 155–162 (2014).
4. Ociepa, T. et al. Molecular identification and chromosomal localization of new powdery mildew resistance gene Pm11 in oat. *Theor. Appl. Genet.* **133**, 179–185 (2020).
5. Ociepa, T. & Okoń, S. Chromosomal location of Pm12 — a novel powdery mildew resistance gene from avena sterilis. *Genes (Basel)* **13**, 1–10 (2022).
6. Okoń, S. M. & Ociepa, T. Virulence structure of Blumeria Graminis f. sp. avenae populations in Poland across 2014–2015. *Eur. J. Plant. Pathol.* **149**, 711–718 (2017).
7. Cieplak, M., Terlecka, K., Ociepa, T., Zimowska, B. & Okoń, S. Virulence structure of Blumeria Graminis f. sp. avenae populations in Poland across 2014–2015. *Plant. Pathol. J.* **37**, 115–123 (2021).
8. Cieplak, M., Nucia, A., Ociepa, T. & Okoń, S. Virulence structure and genetic diversity of blumeria graminis f. sp. avenae from different regions of Europe. Plants 11, (2022).
9. Cabral, A. L. et al. Oat fungal diseases and the application of molecular marker technology for their control. in Future challenges in crop protection against fungal pathogens (eds Goyal, A. & Manoharachary, C.) 343–358 (Springer Science + Business Media, New York, https://doi.org/10.1007/978-1-4939-1188-2. (2014).
10. Wrzesińska, B., Kierzek, R. & Obrępalska-Stęplowska, A. Evaluation of six commonly used reference genes for gene expression studies in herbicide-resistant Avena fatua biotypes. *Weed Res.* **56**, 284–292 (2016).
11. Liu, J. et al. Selection and evaluation of potential reference genes for gene expression analysis in Avena fatua. *Plant. Prot. Sci.* **55**, 61–71 (2019).
12. Akbarabadi, A., Ismaili, A., Kahrizi, D. & Firouzabadi, F. N. Validation of expression stability of reference genes in response to herbicide stress in wild oat (Avena ludoviciana). *Cell. Mol. Biol.* **64**, 113–118 (2018).
13. Ruduś, I. & Kępczyński, J. Reference gene selection for molecular studies of dormancy in wild oat (Avena fatua L.) caryopses by RT-qPCR method. *PLoS One*. **13**, e0192343 (2018).
14. Zhu, H., Ma, Y. & Guo, Q. Expression stability of internal reference gene in response to Trichoderma polysporum infection in Avena fatua L. *Curr. Genet.* https://doi.org/10.1007/s00294-021-01200-4 (2021).
15. Yang, Z., Wang, K., Aziz, U., Zhao, C. & Zhang, M. Evaluation of duplicated reference genes for quantitative real-time PCR analysis in genome unknown hexaploid oat (Avena sativa L). *Plant. Methods*. **16**, 1–14 (2020).
16. Duan, Z. L., Han, W. H., ., Yan, L. & Wu, B. Reference gene selections for real time quantitative PCR analysis of gene expression in different oat tissues and under salt stress. *Biol. Plant.* **64**, 838–844 (2020).

17. Tajti, J., Pál, M. & Janda, T. Validation of reference genes for studying different abiotic stresses in oat (Avena sativa L.) by RT-qPCR. *Plants* **10**, 1–19 (2021).
18. Sowa, S., Sozoniuk, M., Toporowska, J., Kowalczyk, & K. Paczos-Grzęda, E. Validation of reference genes as an internal control for studying Avena sativa–Puccinia coronata interaction by RT–qPCR. *Sci. Rep.* **12**, 1460 (2022).
19. Sowa, S., Sozoniuk, M., Toporowska, J., Kowalczyk, K. & Paczos-Grzęda, E. Reference genes expression stability in Avena sativa L. during compatible and incompatible interactions with Puccinia graminis. *Sci. Rep.* **12**, 18369 (2022).
20. Hayes, J. D. & Jones, I. T. Variation in the pathogenicity of Erysiphe Graminis D.C. f. sp. avenae, and its relation to the development of mildew-resistant oat cultivars. *Euphytica* **15**, 80–86 (1966).
21. Aung, T. & Thomas, H. Ransfer of mildew resistance from the wild oat Avena barbata into the cultivated oat. *Nature* **260**, 603–604 (1976).
22. Sowa, S. & Paczos-Grzęda, E. A study of crown rust resistance in historical and modern oat cultivars representing 120 years of Polish oat breeding. *Euphytica* **216**, 1–10 (2020).
23. Hsam, S. L. K. et al. Genetic studies of powdery mildew resistance in common oat (Avena sativa L.) I. cultivars and breeding lines grown in Western Europe and North America. *Euphytica* **96**, 421–427 (1997).
24. Vandesompele, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, 1–12 (2002).
25. Andersen, C. L., Jensen, J. L. & Ørntoft, T. F. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245–5250 (2004).
26. Pfaffl, M. W., Tichopad, A., Prgomet, C. & Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol. Lett. 266 26, 509–515 (2004). (2004).
27. Silver, N., Best, S., Jiang, J. & Thein, S. L. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol. Biol.* **7**, 1–9 (2006).
28. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* **25**, 402–408 (2001).
29. Velada, I., Ragonezi, C., Arnholdt-Schmitt, B. & Cardoso, H. Reference genes selection and normalization of oxidative stress responsive genes upon different temperature stress conditions in Hypericum perforatum L. *PLoS One*. **9**, e115206 (2014).
30. Bustin, S. A. et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611–622 (2009).
31. Thellin, O. et al. Housekeeping genes as internal standards: Use and limits. *J. Biotechnol.* **75**, 291–295 (1999).
32. Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* **3**, 1101–1108 (2008).
33. Gutierrez, L. et al. The lack of a systematic validation of reference genes: A serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant. Biotechnol. J.* **6**, 609–618 (2008).
34. Guenin, S. et al. Normalization of qRT-PCR data: The necessity of adopting a systematic, experimental conditions-specific, validation of references. *J. Exp. Bot.* **60**, 487–493 (2009).
35. Kozera, B. & Rapacz, M. Reference genes in real-time PCR. *J. Appl. Genet.* **54**, 391–406 (2013).
36. Bekele, W. A., Wight, C. P., Chao, S., Howarth, C. J. & Tinker, N. A. Haplotype based genotyping-by-sequencing in oat genome research. *Plant. Biotechnol. J.* **16**, 1452–1463 (2018).
37. Avena sativa – OT3098 v2. PepsiCo. https://wheat.pw.usda.gov/jb?data=/ggds/oat-ot3098v2-pepsico
38. Peng, Y. et al. Reference genome assemblies reveal the origin and evolution of allohexaploid oat. *Nat. Genet.* **54**, 1248–1258 (2022).
39. Kamal, N. et al. The mosaic oat genome gives insights into a uniquely healthy cereal crop. *Nature* **606**, 113–119 (2022).
40. Chaffin, A. S. et al. A consensus map in cultivated hexaploid oat reveals conserved grass synteny with substantial subgenome rearrangement. *Plant. Genome*. **9**, 1–21 (2016).
41. Zeng, L., Deng, R., Guo, Z., Yang, S. & Deng, X. Genome-wide identification and characterization of glyceraldehyde-3-phosphate dehydrogenase genes family in wheat (Triticum aestivum). *BMC Genom.* **17**, 1–10 (2016).
42. Maksup, S., Supaibulwatana, K. & Selvaraj, G. High-quality reference genes for quantifying the transcriptional responses of Oryza sativa L. (ssp. indica and japonica) to abiotic stress conditions. *Chin. Sci. Bull.* **58**, 1919–1930 (2013).
43. Scholtz, J. J. & Visser, B. Reference gene selection for qPCR gene expression analysis of rust-infected wheat. *Physiol. Mol. Plant. Pathol.* **81**, 22–25 (2013).
44. Hardham, A. R. Microtubules and biotic interactions. *Plant. J.* **75**, 278–289 (2013).
45. Livanos, P., Galatis, B. & Apostolakos, P. The interplay between ROS and tubulin cytoskeleton in plants. *Plant. Signal. Behav.* **9**, (2014).
46. Janská, A. et al. The choice of reference gene set for assessing gene expression in barley (Hordeum vulgare L.) under low temperature and drought stress. *Mol. Genet. Genomics*. **288**, 639–649 (2013).
47. Figueiredo, A. et al. Transcriptional and metabolic profiling of grape (Vitis vinifera L.) leaves unravel possible innate resistance against pathogenic fungi. *J. Exp. Bot.* **59**, 3371–3381 (2008).
48. Monteiro, F., Sebastiana, M., Pais, M. S. & Figueiredo, A. Reference gene selection and validation for the early responses to downy mildew infection in susceptible and resistant Vitis vinifera cultivars. *PLoS One* **8**, (2013).
49. Godoy, A. V., Lazzaro, A. S. & Casalongué, C. A. San Segundo, B. expression of a Solanum tuberosum cyclophilin gene is regulated by fungal infection and abiotic stress conditions. *Plant. Sci.* **152**, 123–134 (2000).
50. Kong, H. Y., Lee, S. C. & Hwang, B. K. Expression of pepper cyclophilin gene is differentially regulated during the pathogen infection and abiotic stress conditions. *Physiol. Mol. Plant. Pathol.* **59**, 189–199 (2001).
51. Lee, J. R. et al. Molecular and functional characterization of a cyclophilin with antifungal activity from Chinese cabbage. *Biochem. Biophys. Res. Commun.* **353**, 672–678 (2007).
52. Gao, W. et al. Genome–wide identification and expression analysis of the UBC gene family in wheat (Triticum aestivum L). *BMC Plant. Biol.* **24**, 1–17 (2024).
53. Berka, M., Kopecká, R., Berková, V., Brzobohatý, B. & Černý, M. Regulation of heat shock proteins 70 and their role in plant immunity. *J. Exp. Bot.* https://doi.org/10.1093/jxb/erab549 (2022).
54. Zhou, Z. et al. Comprehensive genomic analysis and expression Profile of Hsp70 Gene Family related to Abiotic and biotic stress in Cucumber. *Horticulturae* **9**, 1–13 (2023).
55. Lorrain, C., dos Gonçalves, K. C., Germain, H., Hecker, A. & Duplessis, S. Advances in understanding obligate biotrophy in rust fungi. *New. Phytol.* **222**, 1190–1206 (2019).

Author contributions

SS Conceptualization, methodology, data curation, resource managing, formal analysis, original draft writing, review and editing. JT Experimentation. EPG Resources, supervision, review and editing. All authors read and approved the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-81348-4.

Correspondence and requests for materials should be addressed to S.S. or E.P.-G.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.