Transcriptome-based identification and validation of optimal reference genes for quantitative real-time PCR normalization in Psathyrostachys huashanica

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Research

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Transcriptome-based identification and validation of optimal reference genes for quantitative real-time PCR normalization in *Psathyrostachys huashanica*

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Abstract

Background:

P. *huashanica* (*Psathyrostachys huashanica*), known as an important resistance resource reservoir, is a rare and endangered plant growing suitably in Huashan mount region and would be urgently exploited in wheat genetic improvements sooner. During the utilization process, different IRGs (internal reference genes) need to be appropriately selected as standards based on biotic and abiotic stress conditions. It is crucial that Real-time RT-qPCR with combination of bioinformatics were adopted to explore the reliable IRGs from transcriptome of P. *huashanica.*

Results:

The present work reported new 3 species of IRGs, *UBC2*, *UBC17,* 18S *rRNA*, which were screened from transcriptome of P. *huashanica* under biotic and abiotic stress conditions, using RT-qPCR and four algorithms, including geNorm, NormFinder, BestKeeper, and RefFinder, to analyse expression of sixteen candidate reference genes. These genes appear as following *18S rRNA* (18S ribosomal RNA), *EF1-α* (eukaryotic elongation factor 1 alpha), *UBC2* (ubiquitin-conjugating enzyme E2-2), *UBC17* (ubiquitin-conjugating enzyme E2-17), *α-TUB2A*

(alpha tubulin-2A), *β-TUB3* (beta tubulin 3), *ADF4* (Actin-depolymerising factor 4), *ACTIN* (actin), *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase), *60SARP* (60S acidic ribosomal protein), *UBQ* (polyubiquitin), *SamDC* (S-Adenosylmethionine decarboxylase), *EIF4A* (eukaryotic initiation factor 4A), *ARF* (ADP-ribosylation factor), *HIS1* (histone H1), and *HIS2B* (histone H2B). Analysis of gene expression demonstrated that the expression of *UBC2* gene was most stable under ABA hormone stress, low temperature stress and high temperature stress, similarly, *UBC17* gene under IAA hormone stress, salinity stress and drought stress, both *UBC17* genes and 18S *rRNA* genes under abiotic and biotic stress, respectively. The most stable gene was *UBC2* gene in the root, *UBC17* gene in stem and leaf. In this study, *α-TUB2A*, *UBC* and *ACTIN* genes were verified as the suitable reference genes across all tested samples. To further validate the suitability of the selected reference genes, we evaluated the relative expression of *PsaCPK3* (Calcium-dependent protein kinase) and *PsaHSP70-1* (heat shock protein 70-1), which are stress-related genes that may be involved in response to adversity.

Conclusions:

This study has identified a set of the most stable IRGs suiting for RT-qPCR detection of a few target gene expressions from P. *huashanica* in different experimental conditions. In addition, this study should provide the accuracy information for gene expression analysis in P. *huashanica*.

Keywords: Internal reference genes, *Psathyrostachys huashanica*, Gene expression, RT-qPCR, Normalization

Background

P. *huashanica* (2n=2x=14,NsNs) is a new species of genus *Psathyrostachys* plants, and that is a rare and endangered plant growing suitably in Huashan mount region in China, known as an important resource reservoir of resistance to Barley yellow dwarf virus, wheat stripe rust wheat take-all, wheat fussarium head blight and drought. P. *huashanica* belongs to the genus *Psathyrostachys*, *Triticeae* of *Poaceae*, a wildly perennial relative of common wheat, and possesses numerous potentially valuable agronomic traits, such as tolerance to drought and salinity, resistance to stripe rust, wheat yellow dwarf virus and wheat take-all fungus [1-5]. Thus, in recent years, an increasing number of researchers have been studying these potentially valuable characteristics, which could be incorporated into common wheat [6]. Recently, a series of *Triticum*

aestivum-P. huashanica Keng monosomic and disomic addition lines were created, and some stress response factors were identified at the molecular level to enhance disease resistance [7-8]. Understanding the expression patterns of stress response genes is the key to elucidate the mechanisms involved in various stresses of P. *huashanica*. Although some studies have been carried out on identification of gene function of P. *huashanica*, there have been no studies conducted for selecting suitable reference genes.

Real-time quantitative polymerase chain reaction (RT-qPCR) has become one of the most commonly used techniques to analyse mainly gene expression because of its high sensitivity, accuracy, convenience, reproducibility and specificity [9-11]. However, there are many variable factors could easily affected the accuracy of experimental data obtained by qRT-PCR, including differences in RNA sample quality, extraction method, reverse transcription efficiency and genomic DNA contamination [12-14]. Using suitable IRGs could help eliminate the variability introduced by different tissue samples and various experimental conditions, thus ensuring the accuracy and reliability of RT-qPCR results.

In previous studies, traditional housekeeping genes, such as GAPDH, 18S rRNA, ACT, EF1-α，β-TUB, and UBQ, are often used as IRGs without evaluating the expression stability because of their indispensable role in maintaining plant growth and development in the face of different conditions [15-18]. They were assumed to be stably expressed in various tissues at different developmental stages and under a wide range of experimental conditions [19]. However, these traditional housekeeping genes have proved not to be always stably expressed in all tissues, development stages or experimental treatments [20-21]. The unstable expression of reference genes may affect final experimental results, thus leading to the deviation of target gene expression. Therefore, there is urgent need to select suitable reference genes that have stable expression levels under a variety of experimental conditions.

At present, several studies have been performed on the evaluation and validation of reference genes under biotic and abiotic stresses in a wide variety of plant species, such as Arabidopsis [22], rice [23], wheat [24], barley [25], Panax [26], soybean [27], melon [28], kiwifruit [29], pear [30], tomato [31] and cassava [32]. However, systematic evaluation of the selection of suitable reference genes for RT-qPCR data normalisation in P. *huashanica* under various experiments has not been reported.

RNA-seq has become a powerful high-throughput tool for quantitative gene expression levels and exploring gene transcriptional regulation in different organisms under diverse conditions and treatments [33-34]. In previous studies, RNA-seq datasets have also been used to identify transcriptional changes under various abiotic and biotic stresses, and hence a series of stable expression reference genes were identified and validated by analysing the large data sets [35-37].

In this study, 16 reference genes (18S rRNA, EF1-α, UBC2, UBC17, α-TUB2A, β-TUB3, ADF4, ACTIN, GAPDH, 60SARP, UBQ, SamDC, EIF4A, ARF, HIS1, and HIS2B), were selected based on the RNA-seq datasets of P. *huashanica*. The expression stability of these selected reference genes was evaluated by RT-qPCR to identify potential reference genes suitable for target gene normalisation in experiments under abiotic conditions, including drought, salinity, cold and heat stress, hormonal stimuli (IAA and ABA) and biotic conditions, including fungal and viral inoculation in different plant tissues. Three software products (NormFinder, geNorm and BestKeeper) were used to systematically evaluate the qRT-PCR data, and the results were delivered to RefFinder for further analysis. Furthermore, the expression levels of two stress-related genes, *PsaHSP70-1* and *PsaCPK3*, were conducted to validate the suitability of the selected reference genes. This work provides a series of suitable IRGs for future gene expression studies under different experimental conditions in P. *huashanica*.

Results

Verification of the amplification efficiency and specificities of the primers for RT-qPCR

We selected 16 protein-coding genes in *P. huashanica* as candidate reference genes for quantitative detection of mRNAs in leaf and stem tissue and 14 protein-coding genes in roots due to the low expression of *GAPDH* and *SamDC* genes. These gene names, corresponding primer sequences and expected product sizes are presented in Table 1, and the amplification specificities of these primers were confirmed by the melting curves which were shown as a single peak and all primers were amplified with a single band after agarose gel electrophoresis (Additional file 1: Fig. S1 and Fig. 1). We made standard curves using cDNAs with 10-fold gradient dilutions to get the amplification efficiency and the regression coefficient for each pair of primers. The amplification efficiency ranged from 75.22% to 100.73%, and the regression coefficient ranged from 0.991 to 0.9994 (Table 1).

Expression level of the candidate reference genes in different treatments and different tissues

The expression level of each gene was detected in corresponding tissues (leaves、stems and roots) under different treatments (abiotic and biotic stress) by RT-qPCR technology. Lower CT values indicate high expression levels and good stability for the selected genes. Therefore, two candidate genes, *GAPDH* and *SAMDC*, were absent in the analysis of root tissues due to their high CT values. The average CT values of each gene ranged from 17 to 31 and all CT values were collected (Fig. 2 and Additional file 1: Table S1). The CT values indicated that transcription levels of reference genes are unstable under different experimental conditions.

Expression stability of the candidate reference genes

Different candidate genes have different expression levels in different plant tissues and under different stresses. To evaluate the expression stability of the candidate reference gens accurately, four programs (geNorm、NormFinder、Bestkeeper and refFinder) and Delta CT method were applied to the analysis, which suggest a stability ranking from the most expression-stable gene to the least stable gene (Additional file 2: Table S2).

Bestkeeper analysis. The rankings of the expression stabilities of the selected genes were variable in different tissues and treatments. Bestkeeper recommended reference genes expressing stably by SD and CV values. In abiotic stress treatments, the top five stable candidate genes in all tested samples under abiotic stress evaluated by Bestkeeper were *18S, ELF4A, βTUB, UBC2* and *UBC17*, and *18S* was the most stable gene (Table 2), whereas the two least stable genes were *αTUB* and *EF1α*. In stem tissue, *18S, ELF4A, UBC2* and *UBC17* were ranked as the top four most stable genes in different treatments. Bestkeeper suggested that *UBC17, UBC2, UBI* and *ADF4* expressions were relatively stable in leave tissue, whereas the *GAPDH* and *60SRP* were evaluated as two least stable genes. Bestkeeper recommended a stability ranking of *18S> ARF> UBC2> βTUB* in root tissue. All the tested samples were classified into six abiotic treatments and one biotic treatments. In the IAA hormone-stressed treatment, *18S, ELF4A, HIS1* and *ARF* were selected by Bestkeeper as the most stable genes, and *EF1α* was the least stable gene. In the ABA hormone-stressed group, the stable gene ranking was *18S> HIS1> UBC17> ELF4A> UBC2*, while *EF1α* and *αTUB* were two least stable genes. For the heat-stressed treatment, Bestkeeper suggested that *18S, ELF4A, βTUB* and HIS1 were the four most stable genes, with *αTUB* and *HIS4* ranked as the least stable genes. Expression stability ranking of candidate reference genes

under cold, salinity stress and drought-stressed treatments are shown in Table 3. The *18S* and *UBC17* genes were evaluated as the most stable ones in root, stem and leaf tissues, respectively, under biotic stress (Table 3). Additionally, we recommend the stability ranking under abiotic stress in different tissues, which is shown in Table 3.

GeNorm analysis. The geNorm program offered the optimal number of reference genes by calculating the pairwise variations Vn/Vn+1 to normalise the RT-qPCR data. The V2/3 value and V3/4 value (Fig. 3) in stem tissues under abiotic and biotic stress, respectively, was below the cut-off value of 0.15, which means two of the most stable genes were suitable for normalising the qRT-PCR data under abiotic stress and three were suitable in the biotic stress treatment. The stability ranking calculated by the geNorm program is shown in Table 2, Table 3 and Additional file 2: Table S2.

Normfinder analysis. Results from the Normfinder program showed that the *UBC17* and *18S* genes were the most stable genes under abiotic and biotic stress treatment, respectively. The expression stability rankings under different treatments in different tissues are shown in Additional file 2: Table S2.

RefFinder analysis. The RefFinder program is an online tool for normalising the RT-qPCR data, and it combines the results calculated by three other programs and finally presents the comprehensive expressional stability ranking for all selected genes. RefFinder recommended the gene expressional stability rankings of *UBC2>βTUB>HIS4>ELF4A* and *UBC2>ACTIN>UBI>UBC17* in roots under abiotic and biotic stress, respectively. In stem tissue, the rankings were *UBC17>UBC2>EF1α>ELF4A* and *18S>ADF4>ELF4A>ARF* under abiotic and biotic stress, respectively. In leaf tissue, the expressional stability rankings under abiotic and biotic stress were *UBC17>ELF4A>UBC2>βTUB* and *UBC17>ELF4A>EF1α>18S,* respectively. The Ct values from all tested samples under abiotic stress were calculated by RefFinder, and the stability ranking was *UBC17>UBC2>βTUB>ELF4A*. In addition, in biotic-stressed treatment, data from stem and leave tissue was analysed, and *18S, ELF4A, UBC17* and *UBC2* were ranked as the top four stable genes. The ranking in different tissues under different treatments is shown in Additional file 2: Table S2.

Validation of reference genes

CPK genes encoding calcium-dependent protein kinases and HSP70 genes encoding heat shock

protein 70 play an important role in response to environmental stress during plant growth [38-39]. To validate the effectiveness of candidate reference genes, the relative expression levels of *PsaHSP70-1 and PsaCPK3* were calculated using selected reference genes under salinity stress and BYDV-GAV infection, and the corresponding transcriptome data is shown in Fig 4. The selected genes were as follows: *18S, elf4A* and *18S+UBC17* under BYDV-GAV infection in stem and leaf tissue; *UBC17, UBC2* and *βTUB+UBC2* for salinity stress in stem and leaf tissue; and we also selected *αTUB* as the least stable gene to evaluate the relative expression levels of *HSP70* and *CPK-3*. Under BYDV-GAV infection, the expression levels of *PsaHSP70-1 and PsaCPK3* were higher, and reached their peak after 11 days of virus infection when normalised by *18S, elf4A* and *18S+UBC17*, while the expression of the two genes was much lower when normalised by *αTUB* (Fig. 4). In the salinity stress group, the expression of *PsaHSP70-1 and PsaCPK3* both increased under salinity stress in leaf tissue and the expression peak appeared at 36 h after the stress. In contrast, the expression level decreased when using *αTUB* as a reference gene. The expression level of *PsaHSP70-1* in stem tissue under salinity stress is also shown in Fig 4, and the transcriptome data was not acquired. According to the normalisation results above, significant differences were observed when using the most stable candidate genes and least stable genes to normalise the *PsaHSP70-1 and PsaCPK3*, which validated the effectiveness of the most stable reference genes.

Discussion

P. *huashanica*, as one of the rare wheat-relative species, contains abundant resistance resources [40]. Along with a growing body of research conducted in P. *huashanica*, gene expression regulation studies are becoming more important, and quantitative gene expression measurement requires appropriate reference genes. RT-qPCR has become indispensable for the detection and quantification of gene expression because of its numerous advantages and great flexibility [41]. Moreover, validating appropriate reference genes is crucial precondition for quantifying gene expression using RT-qPCR, and hence, the stable reference genes of P. *huashanica* urgently needed to be validated. Besides, using RT-qPCR technique to investigate the gene expression profiles in P. *huashanica* not only allows the better understanding of the underlying molecular mechanism in regulation of expression of resistance genes, but it also provides insights into the

complex regulatory networks involved under different stresses. To obtain reliable and accurate quantification results in RT-qPCR analysis, it is fundamental to select and validate of appropriate reference genes for normalising RT-qPCR data.

RNA-seq has become a powerful tool for high throughput analysis the change of gene transcriptional level under different experimental conditions [42]. This technique has already been widely used in the plant research field for the selection of stably expressed reference genes for RT-qPCR in tomato, *Nicotiana benthamiana,* grape, soybean, potato and *Lycoris aurea* [35, 37, 43-46]. In our study, we took advantage of the RNA-seq dataset generated from BYDV-GAV infected P. *huashanica*, including inoculated and mock-treated samples. From the dataset, 16 candidate reference genes with low coefficients of variation were selected for further evaluation. This strategy led to the identification of a set of stably expressed genes in P. *huashanica.*

Four different statistical algorithms (geNorm, NormFinder, Bestkeeper and DeltaCt) and a web-based comprehensive software tool RefFinder were used to evaluate the expression stability of 16 candidate reference genes under various abiotic and biotic stress conditions in P. *huashanica*. Obvious differences were observed in gene stability rankings generated by these algorithms and tools. For example, under abiotic stress, 18S was ranked first by Bestkeeper, whereas it was ranked seventh by RefFinder. Of course, there was also the ranking that could be observed in *UBC17* generated by NormFinder, Bestkeeper and DeltaCt at the same time. Differences could also be found under biotic stress; 18S was ranked at the top by geNorm, NormFinder, RefFinder and DeltaCt, but it was ranked in the middle position by Bestkeeper. The deviation illustrated above is probably due to the statistical algorithms exploited to calculate stability. The geNorm algorithm procedure is similar to Normfinder software, which uses minimum Ct values to calculate the ΔCt values and the stability of each candidate reference gene in the total experimental samples [47-48].

Despite these differences, according to the five algorithms, *18S* and *UBC17* were considered the two most stable reference genes in all the sample sets. Furthermore, our research results are consistent with previous studies, which showed that 18S rRNA was identified as the most stable reference gene in plants under biotic and abiotic stresses, for example, *P. graminis* f. sp. *tritici*-infected wheat [49], *Vigna mungo* under drought treatments [50] and *Hibiscus cannabinus* under excess salinity and drought [51]. *UBC* is a kind of ubiquitin-conjugating enzyme that targets

protein for degradation via the proteasome, and is an indispensable component in the life of plants [52]. In addition, UBC was the most stable reference gene identified in *Lycoris aurea* under NaCl and cold stresses [46], *Pennisetum glaucum* under drought, salt, heat, cold and ABA stresses [53] and *Corchorus capsularis L.* under PEG stress [54]. Moreover, our study results found that *βTUB* and *ELF4A* performed stable expression in all experimental sets.

In this study, 16 candidate reference genes were selected from large-scale transcriptome data (unpublished) and previous studies serving as the source of the reference gene selection for evaluation of expression stability. The expression levels and stabilities of 16 candidate reference genes were analysed in various tissues submitted to different stress treatments as well as at different development periods in P. *huashanica*. To the best of our knowledge, this is the first report on the identification and validation of suitable reference genes for normalising RT-qPCR analysis in P. *huashanica* under various experimental conditions.

Conclusions

In this study, with three computer algorithms, geNorm, BestKeeper and NormFinder, and a comprehensive ranking tool RefFinder, we identified a series of stably expressed RG sets (including 18S+UBC17 under biotic stress and βTUB+UBC2 under abiotic stress) from 16 candidate reference genes from different tissue samples and different conditions at different time points in P. *huashanica*. A further expression analysis of *PsaCPK3* and *PsaHSP70-3* in leaves and stems subjected to both abiotic and biotic stress confirmed the suitability of the novel reference gene set. The results provide valuable information for more precise RT-qPCR data normalisation and further gene expression studies in P. *huashanica*.

Materials and methods

Plant Materials and Stress Treatments

The P. *huashanica* plants used in this study were grown in a greenhouse. For drought and salinity treatments, the plants were supplemented with Hoagland solution containing 15% PEG_{6000} and 200mM NaCl, respectively, for 0, 12, 36 and 72 h. For cold and hot treatments, the plants were exposed to low and high temperature in an incubator at 4°C and 40°C, respectively, for 0, 6, 12 and 24 h. For hormone treatments, plants were sprayed with 200 mM ABA and IAA solutions for

0, 12, 36 and 72 h. For virus treatments, leaves were inoculated with viruliferous aphids carrying BYDV-GAV, control plants (mock-infected) were inoculated with non-viruliferous aphids, and samples were collected at 0, 3, 7 and 14 d, consisting of five aphids per leaf [55]. For fungal treatments, leaves inoculated with Pst virulent isolate CYR32 [56] were collected at 0, 3, 7, and 14 d. All treatments were performed with three biological replications, immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction.

Total RNA Isolation and cDNA Synthesis

Total RNA was isolated with a Biospin Plant Total RNA Extract Kit, which purifies all sizes of total RNA, and genomic DNA was removed by DNase I (RNase free) (bioer, Hangzhou, China) according to the manufacturer's instructions with some modifications. The integrity of total RNA was assessed by 1.5% agarose-gel electrophoresis, and the quantity and quality of RNA was further measured by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). Only RNA samples with an OD260/280 value between 1.8 and 2.2 were used for cDNA synthesis. A total of 1.0 μg RNA was performed into cDNA synthesis in a 20-μl reaction mixture using a PrimeScript™ RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. The cDNA was diluted 4-fold with nuclease-free water to 100 μl for RT-qPCR.

Reference Gene Selection and Primer Design

Taking advantage of newly generated RNA-seq data that P. *huashanica* leaves challenged with BYDV-GAV, we chose those with high expression (mostly, RPKM > 10) and ranked them based on their coefficient of variation (CV) (Additional file 2: Table S2). Finally, 16 genes were selected as the candidate reference genes. The corresponding reference genes coding sequences were extracted based on the transcriptome sequencing database built by our lab (unpublished). The primers were designed for RT-qPCR using the Primer 3 Plus program (www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) according to the following parameters: the amplicon product size was set as 80–150 bp, primer length was 18–24 bp, melting temperature (Tm) was 55–62°C and GC content was 40–70%. Then, the amplification specificity of primers was confirmed by RT-PCR with a 1.2% agarose-gel electrophoresis.

Real-Time Quantitative PCR Analysis

RT-qPCR analysis was performed using UltraSYBR Mixture (cwbiotech, Jiangsu, China), and the reactions were conducted in a final volume of 25 μl containing 12.5 μl of 2x UltraSYBR Mixture,

0.2 uM of each primer, 1.0 µl of diluted cDNA and nuclease-free water. A Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA) was used to perform RT-qPCR reaction with the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and a melting-curve program was generated by the instrument. Each real-time PCR reaction was performed with three technical and biological replicates.

Data Analysis

Expression levels of the 16 reference genes in all samples were determined by their cycle threshold values (Ct). The PCR amplification efficiency of each gene was evaluated using a standard curve generated by RT-qPCR using a fourfold dilution series of cDNA templates (1, 1/10, 1/100, 1/1000, 1/10000). The correlation coefficients (R^2) and slope values were obtained from standard curves. The PCR efficiency (E) was calculated using the slope value with the formula ($E = (10^{(-1/slope)}-1) X 100\%$) [57].

Five different statistical algorithms (geNorm [58], NormFinder [59], Bestkeeper [60], Delta CT method [61] and RefFinder [62]) were used to calculate the expression stability of candidate reference genes. Ct values were converted to relative quantities according to the formula ($2^{-\Delta Ct}$) for the geNorm and NormFinder analysis. RefFinder, a web-based tool, integrates the four computational programs (geNorm, NormFinder, BestKeeper, and the Delta CT method) to generate a comprehensive ranking of candidate reference genes according to the total experimental treatments.

Validation of Reference Genes

To validate the reliability of selected reference genes, the top two stable reference genes, an optimum reference gene combination and the most unstable reference gene were used to normalise the relative expression levels of *PsaHSP70-1* and *PsaCPK3* in biotic and abiotic experimental conditions. The relative expression levels at different time points were calculated using the $2^{-\Delta\Delta Ct}$ method [63]. The qPCR primer pairs designed by Primer 3 for *PsaHSP70-3* were 5'-AAGAAGGGCGGTGAGAAGAA-3' and 5'-CCCTCCTCAGCTTTCCAAGA-3', and for *PsaCPK3*, the primer pairs were 5'-GCGATGAGGCGACAATCAAA-3' and 5'-GTCACCTGTCTCAATGCGTC-3'.

Abbreviations

18S rRNA: 18S ribosomal RNA; *EF1-α*: eukaryotic elongation factor 1 alpha; *UBC2*: ubiquitin-conjugating enzyme E2-2; *UBC17*: ubiquitin-conjugating enzyme E2-17; *α-TUB2A:* alpha tubulin-2A; *β-TUB3:* beta tubulin 3; *ADF4*: Actin-depolymerising factor 4; *ACTIN*: actin; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *60SARP*: 60S acidic ribosomal protein; *UBQ*: polyubiquitin; *SamDC:* S-Adenosylmethionine decarboxylase; *EIF4A:* eukaryotic initiation factor 4A; *ARF*: ADP-ribosylation factor; *HIS1*: histone H1; *HIS2B:* histone H2B; RT-qPCR: Quantitative real-time polymerase chain reaction

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional fles.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CS, JL and YW designed the research. CS, JL, CW and XZ performed the experiments. CS, JL and YW contributed to data analysis and helped to draft the manuscript. All authors read and

approved the final manuscript.

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Not applicable

Additional files

Additional file 1: Fig. S1. Melt curve analysis of the selected 16 candidate reference genes.

Table S1. The CT values of the total 16 reference genes under different conditions.

Additional file 2: Table S2. Expression stability ranking under different tissues and different stresses.

Table S3. Selection of candidate reference genes from RNA-seq data based on RPKM expression and coefficient of variation (CV).

Table 1. The characteristics of candidate reference genes and corresponding primers

gene symbol	gene name	gene ID	Forward primer sequence(5'-3')	Reverse primer sequence(5'-3')	Size(bp)	E(%)	R^2
18S	18S rRNA	Isoform_40207	GTGAAGACGGTGAGATGTGC	TTCTGCCTCTTGTTCGACCT	115	95.71008	0.9987
EF1α	eukaryotic elongation factor 1 alpha	Isoform_18504	TTGAGATGCACCACGAGTCT	CAACAAACCCACGCTTGAGA	159	98.68284	0.9978
UBC2	ubiquitin-conjugating enzyme E2-2	Isoform_54942	TGCAAGGAAGAGGCTGATGA	TTGAACGTACCACCATCCCA	150	97.63233	0.9959
UBC17	ubiquitin-conjugating enzyme E2-17	Isoform_57085	TCTGCTTTCAATCTGCTCGC	TGTCGTCTCGTACTTGGACC	103	90.93045	0.9945
αTUB	alpha tubulin-2A	Isoform_12228	TTTCCTCCTATGCCCCAGTG	AGACAGCAGGCCATGTACTT	126	83.99646	0.9935
βTUB	beta tubulin 3	Isoform_10167	TGACATTCTCGGTGTTCCCA	GAGCCTCGTTGTCAAGAACC	126	100.7325	0.9983
ADF4	Actin-depolymerizing factor 4	Isoform_50056	GCGGTGTTTGACTTCGACTT	CGTCCAGGGTCCTCTTGAAT	145	94.8177	0.997
actin	actin	Isoform_17349	TGGTATGGAAGCTGCTGGAA	TCAGCAATACCCGGGAACAT	132	87.62201	0.991
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Isoform_15392	CCTGCTCAAGTACGACTCGA	GACGACCTGGATGTTCTTGC	100	89.1111	0.9965
60SRP	60S acidic ribosomal protein	Isoform_76159	CTTCTGAGAAGATCGCGACG	TCATCCACGCTCCTCTTCTC	103	75.22157	0.9945
ubi	polyubiquitin	Isoform_61698	ACGAACAAATCCGCAACCAA	TCAGACGACTCCACCTCAAG	137	88.70282	0.9969
SamDC	S-Adenosylmethionine decarboxylase	Isoform_38601	ATGTGCATGACTGGTCTGGA	CCACACGGCTCGAAATCAAA	158	95.62585	0.9977
elf4A	eukaryotic initiation factor 4A	Isoform_12584	CCAGCAGGTCTCTCTTGTCA	ACAAAGTTGATCGCCACACC	114	91.40804	0.9965
ARF	ADP-ribosylation factor	Isoform_38525	CCAGAACACTCAGGGCCTTA	GCAGCTCATCCTCATTCAGC	104	97.06593	0.9979
HIS1	histone H1	Isoform_51494	TACATCGAGGAGAAGCACGG	GACCTTGACGAGCTTACCCT	102	95.00621	0.9993
HIS4	histone H2B	Isoform_63718	GGCAGGAAGAAGAACAAGAAGAG	GAAGATGTCGTTGATGAAGGAGTT	135	84.31553	0.9994

Table 2. Expression stability ranking under abiotic and biotic stresses

Stability ranking under abiotic stress										
Rank	Bestkeeper		geNorm		NormFinder		deltaCt		RefFinder	
	gene	Std dev	gene	M value	gene	Stability value	gene	Average of STDEV	gene	Geomean of ranking values
1	18S	1.519843	UBC2 | β TU	0.812	UBC17	0.674	UBC17	1.4	UBC17	1.97
2	elf4A	1.925761	UBC17	0.865	UBC2	0.718	UBC2	1.41	UBC2	2
3	β TUB	2.013263	elf4A	0.921	β TUB	0.728	β TUB	1.41	β TUB	2.28
4	UBC2	2.089277	ADF4	0.987	ADF4	0.863	elf4A	1.48	elf4A	3.56
5	UBC17	2.241653	ARF	1.092	elf4A	0.886	ADF4	1.49	ADF4	5.14
6	ARF	2.28264	HIS1	1.181	ubi	1.074	ubi	1.62	ARF	6.48
7	ADF4	2.338264	ubi	1.253	ARF	1.137	ARF	1.63	18S	6.85
8	HIS1	2.374773	actin	1.328	HIS1	1.138	HIS1	1.66	ubi	7.14
10	ubi	2.730157	HIS4	1.4	HIS4	1.307	actin	1.76	HIS1	7.74
11	60SRP	2.740055	EF1α	1.465	actin	1.318	HIS4	1.77	actin	9.72
13	actin	3.043306	60SRP	1.526	60SRP	1.426	60SRP	1.87	HIS4	10.19
14	HIS4	3.305179	18S	1.596	EF1α	1.514	EF1α	1.9	60SRP	10.98
15	α TUB	3.316587	α TUB	1.696	18S	1.729	18S	2.04	EF1α	12.2
16	EF1α	3.409409			α TUB	2	α TUB	2.3	α TUB	13.74
Stability ranking under biotic stress										
Rank	Bestkeeper		geNorm		NormFinder		deltaCt		RefFinder	
	gene	Std dev	gene	M value	gene	Stability value	gene	Average of STDEV	gene	Geomean of ranking values
1	ARF	0.98	18S | UBC17	0.422	18S	0.223	18S	1.07	18S	1.68
2	actin	1.12	UBC2	0.534	elf4A	0.368	elf4A	1.1	elf4A	2.83
3	GAPDH	1.14	elf4A	0.594	UBC17	0.436	UBC17	1.14	UBC17	3.15
4	elf4A	1.23	EF1α	0.664	UBC2	0.439	UBC2	1.15	UBC2	4.56
5	HIS4	1.24	ubi	0.733	EF1α	0.556	EF1α	1.21	HIS1	6.24
6	HIS1	1.28	HIS1	0.792	HIS1	0.709	HIS1	1.26	EF1α	6.35
7	ubi	1.35	60SRP	0.838	60SRP	0.731	ubi	1.28	ARF	6.85
8	18S	1.36	β TUB	0.889	ubi	0.733	60SRP	1.28	ubi	6.96
9	UBC2	1.39	ADF4	0.928	SamDC	0.848	SamDC	1.34	actin	7.5
10	SamDC	1.5	SamDC	0.969	ADF4	0.957	β TUB	1.41	60SRP	8.56
11	UBC17	1.54	actin	1.019	β TUB	0.966	actin	1.42	SamDC	9.72
12	EF1α	1.86	ARF	1.062	actin	1.003	ADF4	1.43	GAPDH	10.03
13	60SRP	1.86	HIS4	1.158	ARF	1.111	ARF	1.45	HIS4	10.82
14	β TUB	2.2	GAPDH	1.252	HIS4	1.647	HIS4	1.9	β TUB	10.85
15	ADF4	2.3	α TUB	1.463	GAPDH	1.821	GAPDH	2.01	ADF4	11.58
16	α TUB	3.93			α TUB	2.861	α TUB	2.94	α TUB	16

Table 3. Expression stability ranking under different abiotic stresses

ABA treatment										
Rank	Bestkeeper		geNorm		NormFinder		deltaCt		RefFinder	
	gene	Std dev	gene	M value	gene	Stability value	gene	Average of STDEV	gene	Geomean of ranking values
1	18S	1.480856	UBC2 | β TU	0.501	UBC2	0.556	UBC2	1.34	UBC2	1.5
2	HIS1	1.776528	UBC17	0.65	β TUB	0.641	β TUB	1.36	β TUB	2.21
3	UBC17	2.198194	elf4A	0.76	elf4A	0.687	elf4A	1.38	elf4A	3.46
4	elf4A	2.233148	ADF4	0.851	UBC17	0.772	UBC17	1.42	UBC17	3.46
5	UBC2	2.36831	ubi	0.983	ubi	0.799	ADF4	1.48	18S	5.87
6	β TUB	2.378056	ARF	1.051	ADF4	0.811	ubi	1.48	ADF4	5.89
7	ARF	2.492222	HIS1	1.145	HIS4	1.058	ARF	1.58	HIS1	6
8	ADF4	2.817523	18S	1.213	ARF	1.077	HIS4	1.65	ubi	6.34
9	ubi	2.877639	HIS4	1.304	HIS1	1.380	HIS1	1.77	ARF	7.24
10	60SRP	3.114537	actin	1.376	actin	1.432	actin	1.85	HIS4	8.86
11	HIS4	3.184444	60SRP	1.468	60SRP	1.505	18S	1.93	actin	10.72
12	actin	3.27963	EF1α	1.578	18S	1.634	60SRP	1.94	60SRP	11.22
13	EF1α	3.879074	α TUB	1.704	EF1α	1.921	EF1α	2.23	EF1α	13
14	α TUB	3.969537			α TUB	2.222	α TUB	2.46	α TUB	14
Cold treatment										
Rank	Bestkeeper		geNorm		NormFinder		deltaCt		RefFinder	
	gene	Std dev	gene	M value	gene	Stability value	gene	Average of STDEV	gene	Geomean of ranking values
1	18S	1.577778	UBC2 | UBC1	0.407	UBC2	0.563	UBC2	1.3	UBC2	1.57
2	EF1α	2.908565	β TUB	0.665	UBC17	0.587	UBC17	1.31	UBC17	2.3
3	UBC2	2.354583	elf4A	0.819	β TUB	0.604	β TUB	1.31	β TUB	3
4	UBC17	2.35537	60SRP	0.884	60SRP	0.680	elf4A	1.34	elf4A	3.56
5	α TUB	3.851852	ubi	0.965	elf4A	0.692	60SRP	1.38	60SRP	4.73
6	β TUB	2.251389	ARF	1.014	ubi	0.855	ubi	1.44	18S	6.31
7	ADF4	2.46831	ADF4	1.06	ARF	0.919	ARF	1.46	ubi	6.64
8	actin	3.20956	EF1α	1.127	EF1α	0.946	EF1α	1.53	ARF	7.65
9	60SRP	2.352153	HIS1	1.223	ADF4	1.104	ADF4	1.58	HIS1	7.95
10	ubi	2.523843	18S	1.314	HIS1	1.410	HIS1	1.81	ADF4	8.49
11	elf4A	2.082269	HIS4	1.402	HIS4	1.492	HIS4	1.88	EF1α	8.92
12	ARF	2.533495	actin	1.487	18S	1.649	18S	1.97	HIS4	11.72
13	HIS1	2.255602	α TUB	1.635	actin	1.745	actin	2.05	actin	12.74
14	HIS4	3.51912			α TUB	2.302	α TUB	2.52	α TUB	14
Drought treatment										
Rank	Bestkeeper		geNorm		NormFinder		deltaCt		RefFinder	
	gene	Std dev	gene	M value	gene	Stability value	gene	Average of STDEV	gene	Geomean of ranking values
1	18S	1.403333	UBC17 | ADF	0.846	UBC17	0.530	UBC17	1.37	UBC17	1.68
2	β TUB	2.07706	UBC2	0.895	elf4A	0.665	elf4A	1.38	elf4A	2.83
3	UBC2	2.141134	elf4A	0.986	UBC2	0.692	UBC2	1.43	UBC2	3
4	elf4A	2.155972	β TUB	1.009	ubi	0.703	ubi	1.44	β TUB	3.98
5	HIS1	2.269259	ARF	1.056	β TUB	0.912	β TUB	1.52	ADF4	4.14
6	ARF	2.336481	ubi	1.101	ADF4	0.916	ARF	1.52	18S	5.62
7	ADF4	2.37463	HIS1	1.157	ARF	0.946	ADF4	1.53	ubi	5.79
8	UBC17	2.48	HIS4	1.211	HIS4	0.962	HIS4	1.57	ARF	6.24
9	60SRP	2.51	18S	1.284	HIS1	1.236	HIS1	1.71	HIS1	7.54
10	ubi	2.648889	actin	1.378	18S	1.442	18S	1.85	HIS4	8.92
11	HIS4	3.19662	EF1α	1.443	EF1α	1.501	EF1α	1.91	EF1α	11.94
12	α TUB	3.294167	α TUB	1.591	actin	1.656	actin	1.98	actin	11.98
13	actin	3.421574	60SRP	1.727	α TUB	2.124	α TUB	2.42	60SRP	12.54
14	EF1α	3.605926			60SRP	2.275	60SRP	2.54	α TUB	12.74
High temperature treatment										
Rank	Bestkeeper		geNorm		NormFinder		deltaCt		RefFinder	
	gene	Std dev	gene	M value	gene	Stability value	gene	Average of STDEV	gene	Geomean of ranking values
1	18S	1.741944	UBC2 | UBC1	0.393	60SRP	0.256	UBC2	1.19	UBC2	2
2	elf4A	1.819583	ADF4	0.522	UBC2	0.264	60SRP	1.22	60SRP	2.74
3	β TUB	1.916111	60SRP	0.682	UBC17	0.431	UBC17	1.23	UBC17	3
4	HIS1	2.011944	ubi	0.799	ADF4	0.738	ADF4	1.36	ADF4	4.68
5	ARF	2.079676	elf4A	0.894	β TUB	0.854	ubi	1.45	elf4A	4.74
6	ubi	2.142222	β TUB	0.946	elf4A	0.904	β TUB	1.45	β TUB	5.01
7	60SRP	2.155903	ARF	1.032	ubi	0.924	elf4A	1.46	ubi	5.69
8	UBC2	2.2525	actin	1.118	ARF	1.149	ARF	1.57	18S	6.85
9	UBC17	2.28662	HIS4	1.21	actin	1.316	actin	1.69	ARF	7.11
10	ADF4	2.345278	EF1α	1.286	HIS4	1.344	HIS4	1.74	HIS1	9.12
11	actin	2.591574	HIS1	1.358	EF1α	1.375	EF1α	1.78	actin	9.46
12	EF1α	2.956505	18S	1.487	HIS1	1.484	HIS1	1.86	HIS4	10.68
13	HIS4	3.245463	α TUB	1.613	18S	1.930	18S	2.2	EF1α	11.24
14	α TUB	3.353519			α TUB	2.127	α TUB	2.37	α TUB	14
IAA treatment										
Rank	Bestkeeper		geNorm		NormFinder		deltaCt		RefFinder	
	gene	Std dev	gene	M value	gene	Stability value	gene	Average of STDEV	gene	Geomean of ranking values
1	18S	1.503009	UBC2 | UBC1	0.52	UBC17	0.348	UBC17	1.16	UBC17	1.5
2	elf4A	1.685278	β TUB	0.602	UBC2	0.364	UBC2	1.17	UBC2	2.21
3	HIS1	1.833611	elf4A	0.738	β TUB	0.455	β TUB	1.18	β TUB	3.71
4	ARF	1.990926	18S	0.832	60SRP	0.550	60SRP	1.25	elf4A	4.28
5	UBC17	2.140833	HIS1	0.906	ubi	0.805	ubi	1.37	18S	4.95
6	UBC2	2.150903	ARF	0.956	ADF4	0.854	elf4A	1.4	HIS1	5.83
7	β TUB	2.227731	60SRP	1.005	elf4A	0.965	ADF4	1.42	60SRP	5.98
8	ubi	2.464213	ubi	1.037	HIS1	1.019	HIS1	1.45	ubi	6.51
9	ADF4	2.481319	ADF4	1.093	actin	1.177	ARF	1.53	ARF	7.09
10	60SRP	2.580787	actin	1.164	ARF	1.189	18S	1.56	ADF4	7.84
11	actin	2.934167	HIS4	1.235	HIS4	1.207	actin	1.58	actin	10.46
12	HIS4	3.09162	EF1α	1.341	18S	1.244	HIS4	1.63	HIS4	11.74
13	α TUB	3.434167	α TUB	1.523	EF1α	1.737	EF1α	2.01	EF1α	13.24
14	EF1α	3.621296			α TUB	2.451	α TUB	2.61	α TUB	13.74
Salinity treatment										
Rank	Bestkeeper		geNorm		NormFinder		deltaCt		RefFinder	
	gene	Std dev	gene	M value	gene	Stability value	gene	Average of STDEV	gene	Geomean of ranking values
1	18S	1.328102	UBC17 | ADF	0.694	UBC17	0.613	UBC17	1.42	UBC17	1.63
2	elf4A	1.814074	β TUB	0.876	ADF4	0.648	ADF4	1.43	ADF4	2.45
3	UBC2	1.886528	60SRP	1.03	β TUB	0.654	β TUB	1.43	β TUB	3.41
4	ARF	1.991574	UBC2	1.143	60SRP	0.781	60SRP	1.5	UBC2	4.4
5	β TUB	2.082639	elf4A	1.21	UBC2	1.253	UBC2	1.72	elf4A	4.56
6	HIS1	2.351898	ARF	1.273	elf4A	1.304	elf4A	1.72	60SRP	4.76
7	UBC17	2.442407	18S	1.307	ubi	1.308	ubi	1.77	18S	5.82
8	60SRP	2.569491	HIS4	1.431	HIS4	1.356	ARF	1.8	ARF	6.7
9	ADF4	2.746019	HIS1	1.521	ARF	1.382	HIS4	1.83	ubi	8.57
10	ubi	2.973889	ubi	1.586	actin	1.483	actin	1.9	HIS4	9.39
11	α TUB	3.121111	actin	1.641	HIS1	1.484	18S	1.93	HIS1	9.43
12	HIS4	3.297917	α TUB	1.696	α TUB	1.607	HIS1	1.94	actin	11.18
13	actin	3.31787	EF1α	1.744	18S	1.613	α TUB	2	α TUB	12.22
14	EF1α	3.520556			EF1α	1.708	EF1α	2.03	EF1α	14

Fig. 1. The PCR amplifcation specifcities of candidate reference genes detected by agarose gel electrophoresis. M represents the DNA size marker.

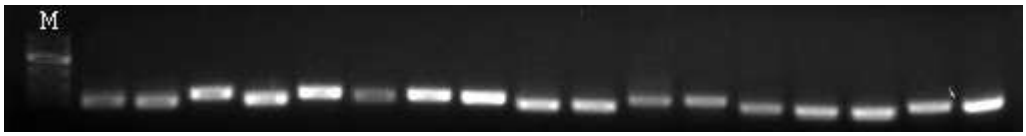


Fig. 2. Distribution of CT values of 16 candidate genes in all samples. Boxplot graph showing maximum,minimum values, medians and 25/75 percentiles.

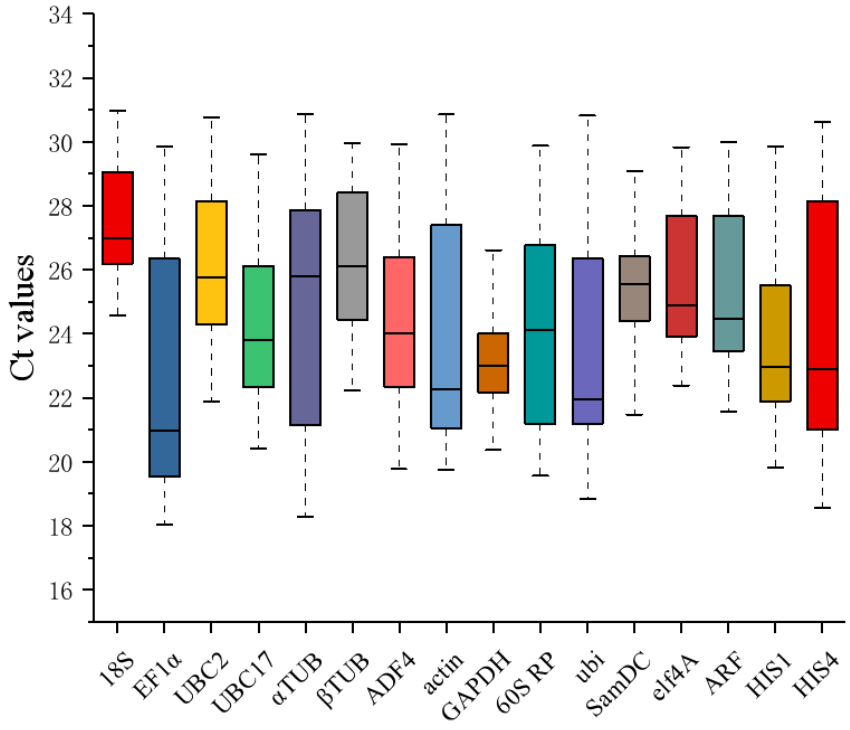
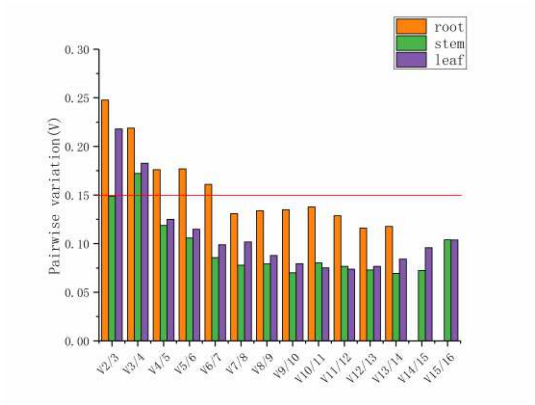


Fig. 3. (a) Pairwise variation (V) analysis of 14 candidate genes under abiotic stress in different tissues; (b) Pairwise variation (V) analysis of 14 candidate genes under different abiotic stress; (c) Pairwise variation (V) analysis of 14 candidate genes in root tissue under biotic stress; (d) Pairwise variation (V) analysis of 14 candidate genes in stem tissue under abiotic stress; (e) Pairwise variation (V) analysis of 14 candidate genes in leave tissue under abiotic stress; (f) Pairwise variation (V) analysis of 14 candidate genes under biotic stress; (g) Pairwise variation (V) analysis of 14 candidate genes under abiotic stress.


root
stem
leaf
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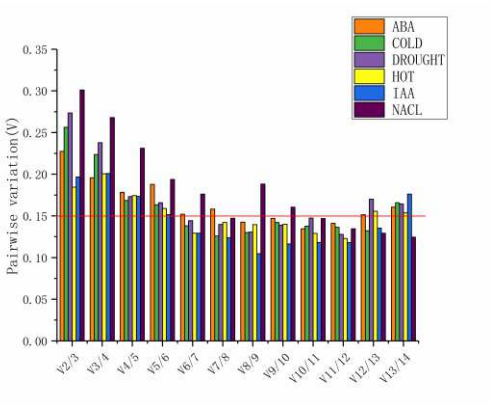
0.05
0.10
0.15
0.20
0.25
0.30
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
V8/9
V9/10
V10/11
V11/12
V12/13
V13/14
V14/15
V15/16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DROUGHT
HOT
IAA
NACL
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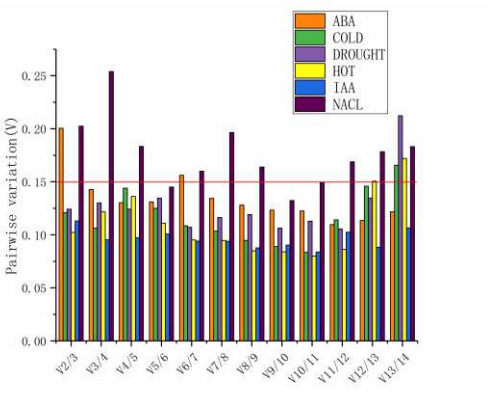
0.25
0.30
0.35
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
V8/9
V9/10
V10/11
V11/12
V12/13
V13/14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HOT
IAA
NACL
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0.25
V2/3
V3/4
V4/5
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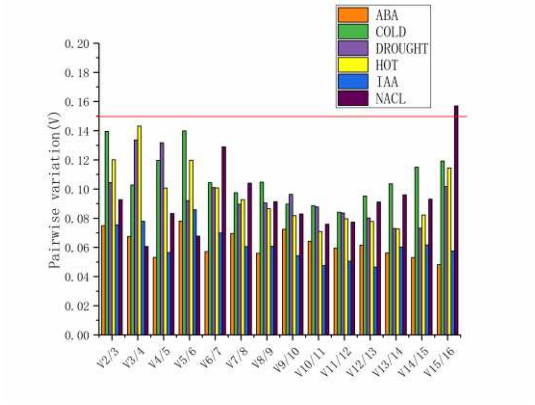
V6/7
V7/8
V8/9
V9/10
V10/11
V11/12
V12/13
V13/14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COLD
DROUGHT
HOT
IAA
NACL
Pairwise variation(V)
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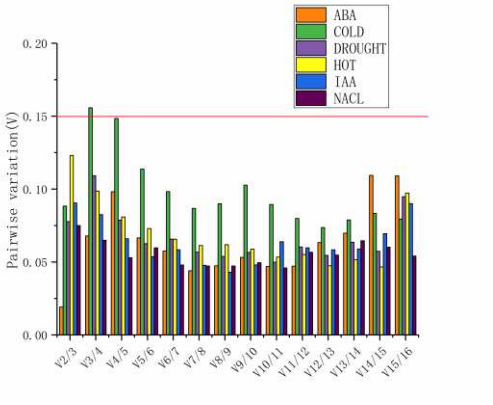
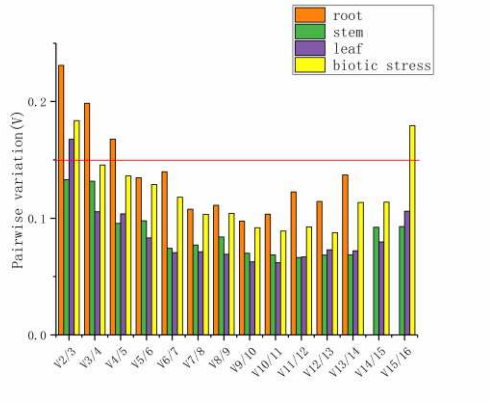
0.10
0.12
0.14
0.16
0.18
0.20
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
V8/9
V9/10
V10/11
V11/12
V12/13
V13/14
V14/15
V15/16


ABA
COLD
DROUGHT
HOT
IAA
NACL
Pairwise variation(V)
0.00
0.05
0.10
0.15
0.20
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
V8/9
V9/10
V10/11
V11/12
V12/13
V13/14
V14/15
V15/16


root
stem
leaf
biotic stress
Pairwise variation(V)
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0.1
0.2
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
V8/9
V9/10
V10/11
V11/12
V12/13
V13/14
V14/15
V15/16

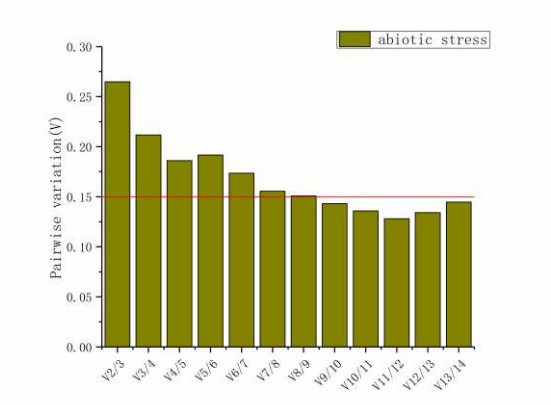
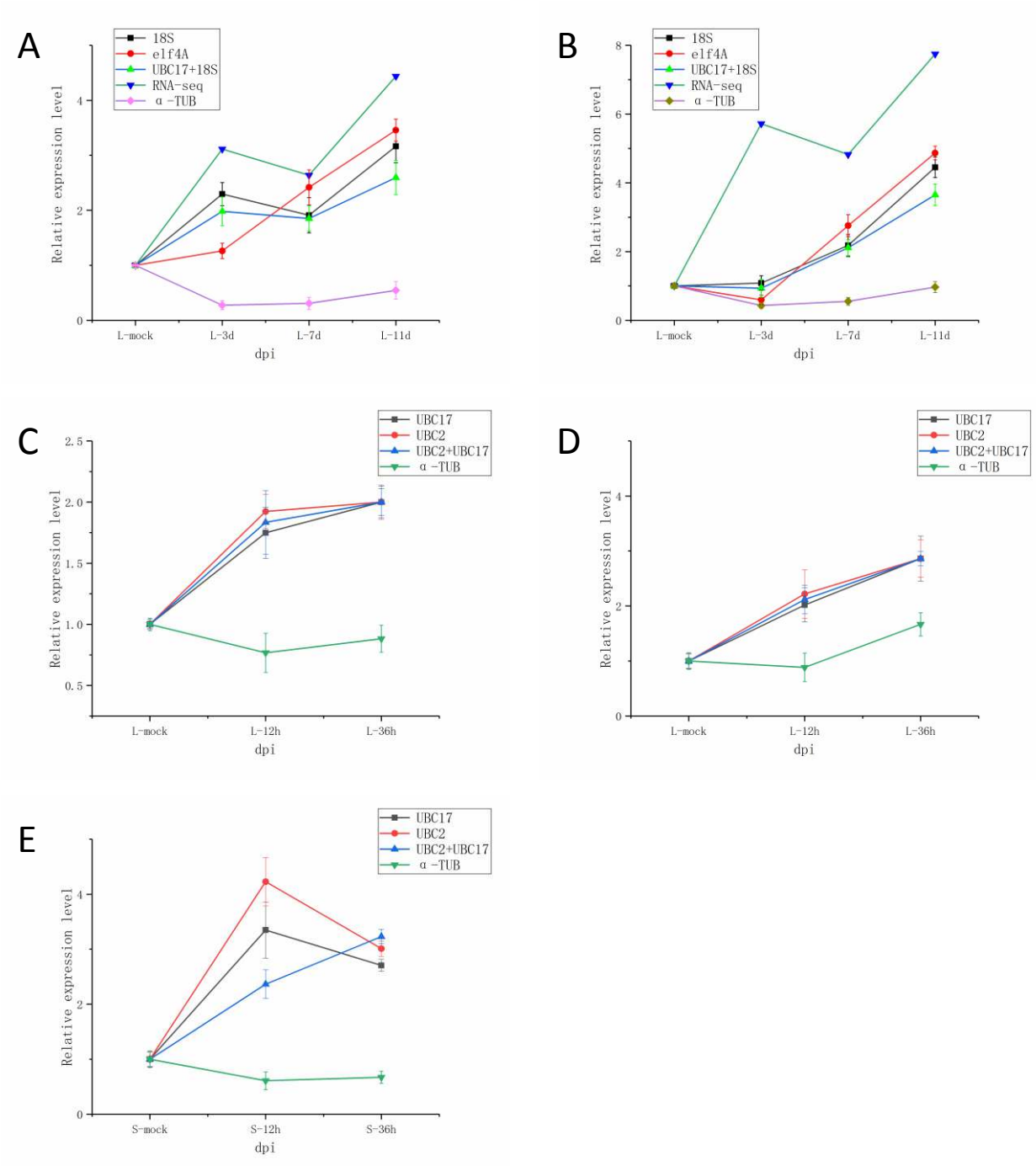

abiotic stress
Pairwise variation(V)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
V8/9
V9/10
V10/11
V11/12
V12/13
V13/14

Fig. 4. (a) Relative expression level of *PsaCPK3* under BYDV-GAV infection in leave tissue and corresponding transcriptome data RNA-seq; (b) relative expression level of *PsaHSP70-3* under BYDV-GAV infection in leave tissue; (c) relative expression level of *PsaHSP70-3* under salinity stress in leave tissue;(d) relative expression level of *PsaCPK3* under salinity stress in leave tissue;(e) relative expression level of *PsaHSP70-3* under salinity stress in stem tissue.



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Figures

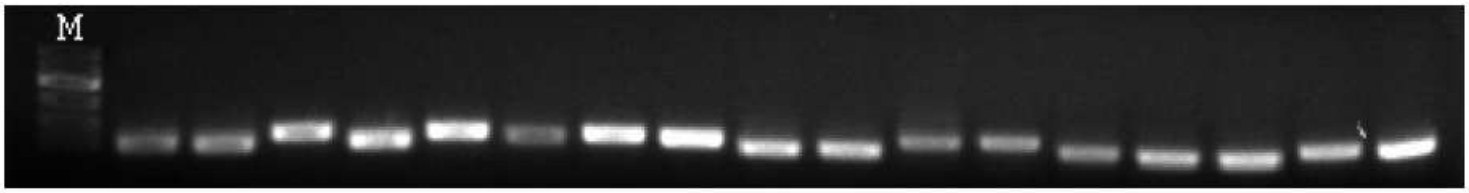

Figure 1

Fig. 1. The PCR amplifcation specifcities of candidate reference genes detected by agarose gel electrophoresis. M represents the DNA size marker.

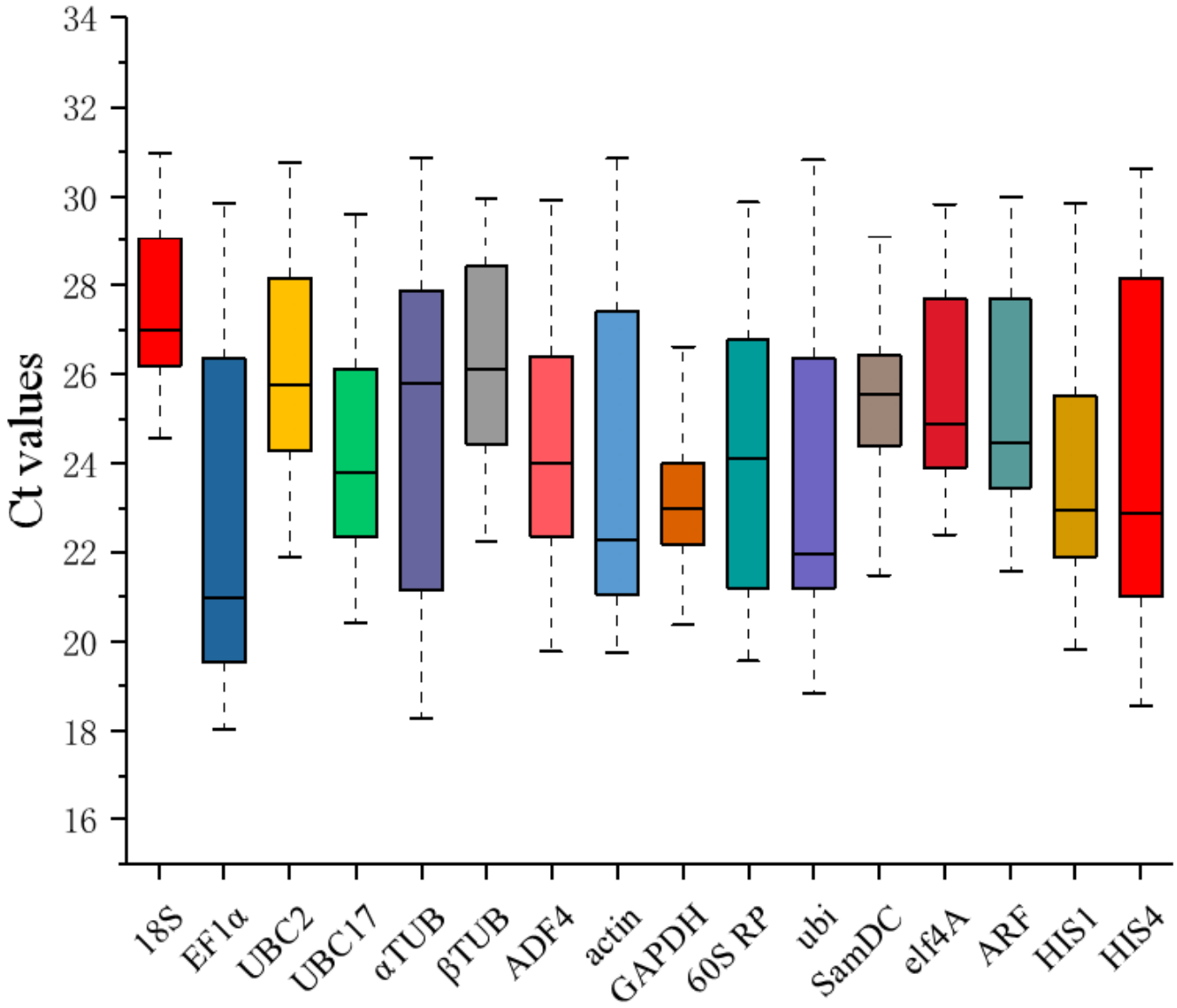

Figure 2

Fig. 2. Distribution of CT values of 16 candidate genes in all samples. Boxplot graph showing maximum, minimum values, medians and 25/75 percentiles.

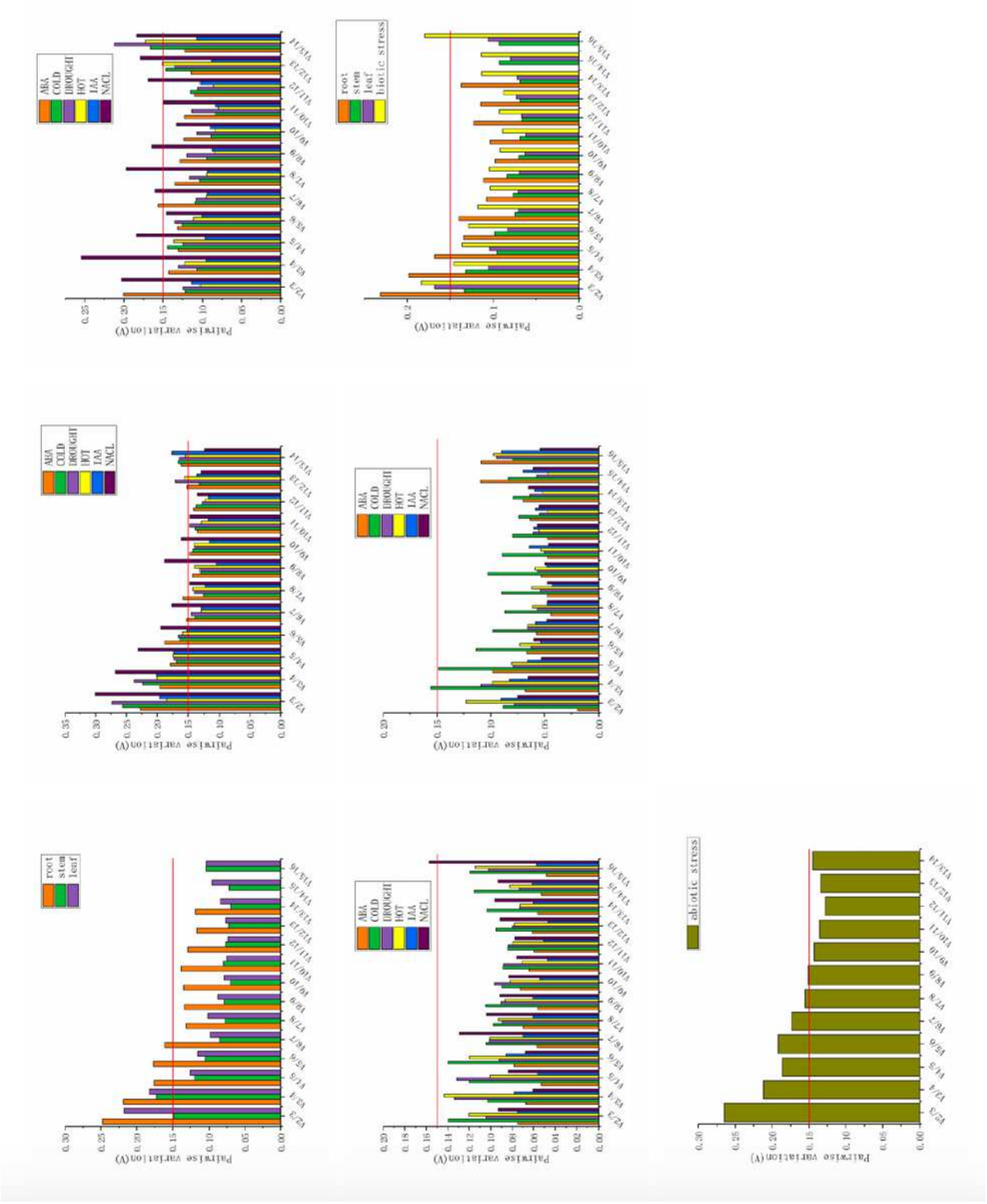

Figure 3

Fig. 3. (a) Pairwise variation (V) analysis of 14 candidate genes under abiotic stress in different tissues; (b) Pairwise variation (V) analysis of 14 candidate genes under different abiotic stress; (c) Pairwise variation (V) analysis of 14 candidate genes in root tissue under biotic stress; (d) Pairwise variation (V)

analysis of 14 candidate genes in stem tissue under abiotic stress; (e) Pairwise variation (V) analysis of 14 candidate genes in leave tissue under abiotic stress; (f) Pairwise variation (V) analysis of 14 candidate genes under biotic stress; (g) Pairwise variation (V) analysis of 14 candidate genes under abiotic stress.

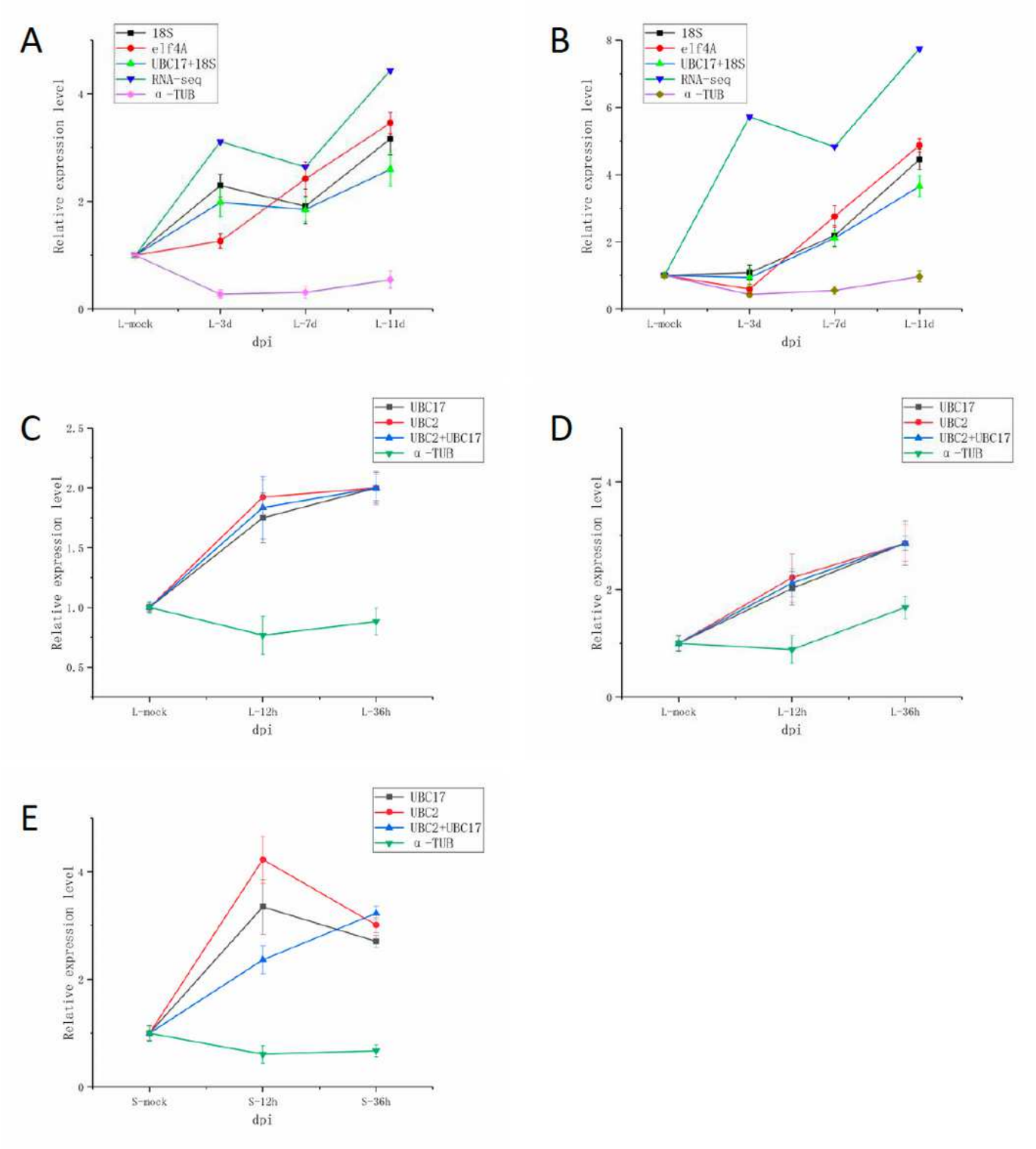

Figure 4

Fig. 4. (a) Relative expression level of PsaCPK 3 under BYDV GAV infection in leave tissue and corresponding transcriptome data RNA seq; (b) relative expression level of Psa HSP70 3 under BYDV GAV

infection in leave tissue; (c) relative expression level of Psa HSP70 3 under salinity stress in leave tissue; (d) relative expression level of PsaCPK 3 under salinity stress in leave tissue;(e) relative expression level of Psa HSP70 3 under salinity stress in stem tissue.