Selection and validation of reference genes for quantitative Real-Time PCR in *Arabis alpina*

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Short title: Reference qPCR primers for *Arabis alpina*

Abstract

Arabis alpina is a perennial arctic-alpine plant and an upcoming model organism for genetics and molecular biology for the Brassicaceae family. One essential method for most molecular approaches is the analysis of gene expression by quantitative Real-Time PCR (qPCR). For the normalisation of expression data in qPCR experiments, it is essential to use reliable reference genes that are not affected under a wide range of conditions. In this study we establish a set of 15 *A. alpina* reference genes that were tested under different conditions including cold, drought, heat, salt and gibberellic acid treatments. Data analyses with geNORM, BestKeeper and NormFinder revealed the most stable reference genes for the tested conditions: *RAN3*, *HCF* and *PSB33* are most suitable for cold treatments; *UBQ10* and *TUA5* for drought; *RAN3*, *PSB33* and *EIF4a* for heat; *CAC*, *TUA5*, *ACTIN 2* and *PSB33* for salt and *PSB33* and *TUA5* for gibberellic acid treatments. *CAC* and *ACTIN 2* showed the least variation over all tested samples. In addition, we show that two reference genes are sufficient to normalize qPCR data under our treatment conditions. In future studies, these reference genes can be used for an adequate normalisation and thus help to generate high quality qPCR data in *A. alpina*.

Introduction

During the last years, *A. alpina* has been established as a new model system in the Brassicaceae family (1,2). It is native to mountains and arctic-alpine habitats (3,4) and combines several features enabling genetic and molecular studies: it is diploid, self-fertile, has a small and sequenced genome and can be transformed with *Agrobacterium tumefaciens* (1). *A. alpina* has an evolutionary distance to *A. thaliana* of about 26 to 40 million years (4,5). This facilitates functional comparisons of biological processes, as orthologous genes can be identified by sequence similarity and synteny (6).

Most molecular studies require quantitative analyses of the expression of genes of interest by quantitative Real-Time PCR. For proper comparisons of expression levels, the expression data of the genes under study are normalized using genes as a reference that show no or very little variation under different conditions. In 2009, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were published, with the aim to provide a consensus on correct performance and interpretation of qPCR experiments (Bustin et al., 2009). These guidelines should ensure that the normalisation enables the comparison of transcripts in different samples by correcting variations in yields of extraction and reverse transcription and the efficiency of amplification. A pre-requisite for any qPCR analysis are suitable primer sets for reference genes that are thoroughly tested. These need to fulfil various requirements: primers should create a specific amplicon of 80 to 200 bp, without creating primer dimers. The amplification should be carried out with close to 100 % efficiency and show a linear standard curve with a correlation of more than 0.99. In general, there should be minimal variation between replicates, indicating consistent performance of the primers.

In this study we established primer pairs for 15 reference genes that can be used for future qPCR studies in *A. alpina*: *ADENOSINE TRIPHOSPHATASE* (*ATPase*), *THIOREDOXIN*, *HIGH CHLOROPHYLL FLUORESCENCE 164* (*HCF*), *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1* (*EIF4a*), *RAN GTPASE 3* (*RAN3*), *UBIQUITIN 10* (*UBQ10*), *ACTIN 2*, *PHOTOSYSTEM B PROTEIN 33* (*PSB33*), *HISTONE H3*,

NAD(P)H PLASTOQUINONE DEHYDROGENASE COMPLEX SUBUNIT O (*NdhO*), *TUBULIN ALPHA 5* (*TUA5*), *18s RIBOSOMAL RNA* (*18srRNA*), *CLATHRIN ADAPTOR COMPLEX MEDIUM SUBUNIT* (*CAC*), *SAND family protein* (*SAND*) and *HEAT SHOCK PROTEIN 81.2/90* (*HSP81.2/90*). The primers were thoroughly tested, and reference genes were evaluated for variations in their expression under different conditions including cold, drought, heat and salt in whole seedlings and gibberellic acid (GA) treatments in leaves. Using genes specifically responding to the different treatment, we demonstrate the impact of normalization with our reference genes.

Results

Selection and validation of reference genes

To compile a set of suitable reference genes, we pursued three approaches: first, we selected *A. thaliana* genes that are known to show little variation under different conditions. Corresponding orthologs in *A. alpina* were then identified by sequence similarity and synteny. Second, we identified genes which show stable expression in *A. alpina* over an extended period of time. Third, we included a well-established reference gene from *A. alpina* from former studies. Thus, we created a set of 15 reference genes (Table 1), including nine orthologs to *A. thaliana* reference genes, five novel reference genes and *RAN3*, a known reference gene for qPCR in *A. alpina* (1). In a first step, we amplified the gene fragments and verified the amplicon by sequencing. In addition, we analysed the melting curves to exclude unspecific products and/or primer dimers (Fig S1).

69 **Table 1. Candidate reference genes, primers and amplicons.**

Name	Gene ID	Arabidopsis homolog	Primer sequence (5' to 3')		Amplicon length [bp]
ATPase	Aa_G9730	AT2G25610	F	GCCAACCTTGTATGCGGGTTA	176 **
			R	GTTGGCCATGTTGCTTGTGC	
THIOREDOXIN	Aa_G337160	AT5G03880	F	GATTGGTCGTGCCGGAAAGG	188 **
			R	TCTCGAGCAATTCCTGTCGTTTG	
HCF	Aa_G363210	AT4G37200	F	AAGGTAATGTTGTCGGGAGGCT	96
			R	CGGCTCGGGCATGAGGAAT	
EIF4a	Aa_G472320	AT3G13920	F	CCAGCTTCTCCCACCCAAGA	122
			R	GCTCGTCACGCTTCACCAAG	
RAN3	Aa_G442020	AT5G55190	F	CACAGGAAAAACCACATTCGT	174 **
			R	CCATCCCTAAGACCACCAAAT	
UBQ10	Aa_G41880	AT4G05320	F	CGTCTCCGTGGTGGTTTCTA	122
			R	AAGGCCCCAAAACACAAACG	
ACTIN 2	NA	AT3G18780	F	AGCTGTTCTCTCCCTGTACG	94 *
			R	AACCCTCGTAGATTGGCACA	
PSB33	Aa_G319470	AT1G71500	F	TGGCGACCACTGCATCTTCA	117
			R	ATCGACGGTCACGACGGAGA	
HISTONE H3	NA	AT5G65360	F	CTCACGGAGAGCGACGGTTC	97
			R	GCAACTCGCGACGAAAGCAG	
NdhO	Aa_G477070	AT1G74880	F	GCGGCGAGGTCTTGGACATT	134
			R	TCGCTCGTAAACAAGTTTCTCAGACT	
TUA5	NA	AT5G19780	F	TGTGACCCGAGGCACGGAAA	137**
			R	CCAGTAGGGCACCAGTCAACA	
18srRNA	NA	AT3G41768	F	CTCCGATCCCGAAGGCCAAC	141
			R	CCTTAACTGGCCGGGTCGTG	
CAC	Aa_G26240	AT5G46630	F	TGGACAAGACCACCAATCCA	113 *
			R	CACTCGACCGTGTTGTAACC	
SAND	Aa_G18160	AT2G28390	F	TTGCAGGATTCGCATTGAGG	178 **
			R	TCCAAAGGGACCTCCTGTTC	
HSP81.2/90	NA	AT5G56030	F	CGTCTGGTGAGGCTCTTGGT	85
			R	AGCCTACGCTCCTCAAGGTACT	

NA – not annotated (identified by nucleotide BLAST), F – forward primer, R – reverse primer, bp – base pair, * amplicon contains an intron on genomic level, **amplicon contains an intron on genomic level and at least one primer is spanning an exon-intron junction.

70 Second, we determined the primer efficiencies and correlation coefficients to demonstrate the
71 quality of the primer pairs (Table 2, Fig S2). All reference gene primers displayed an efficiency
72 between 96.42 and 107.01 % and a correlation higher than 0.99.

73

74 **Table 2. Primer efficiencies and correlation of reference gene candidates.**

Gene	Efficiency [%]	R^2
ATPase	98.33	-0.996
THIOREDOXIN	101.92	-0.992
HCF	99.98	-0.997
EIF4a	107.01	-0.992
RAN3	102.30	-0.998
UBQ10	102.52	-0.999
ACTIN 2	102.85	-0.999
PSB33	99.51	-1.000
HISTONE H3	99.14	-0.999
NdhO	94.34	-0.999
TUA5	98.32	-1.000
18srRNA	98.65	-1.000
CAC	98.07	-0.999
SAND	96.42	-0.999
HSP81.2/90	96.74	-0.999

Efficiency [%] – efficiency of the primers was calculated with $E[\%] = 100 \times (10^{\left(-\frac{1}{slope}\right)} - 1)$, slope – average Cq values (y) and log10 values of six serial dilutions (x) were used to calculate the slope of a regression line with the formula $slope = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2}$, R^2 – correlation of the Cq values calculated with the formula $\rho_{x,y} = \frac{Cov(X,Y)}{\sigma_x \times \sigma_y}$.

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76 Expression stability of reference genes after treatments

77 The expression levels of the reference genes were detected as cycle quantification (Cq) values. The
78 mean Cq values over all treatments ranged between 15.10 (*18srRNA*) and 26.68 (*SAND*). The
79 *18srRNA*, *UBQ10* and *ACTIN 2* genes showed the highest expression/lowest Cq (Fig 1). Overall, Cq
80 values for a single reference gene varied 4.30 on average, with a minimum variation of 2.82 (*UBQ10*)
81 and a maximum variation of 8.12 (*HSP81.2/90*).

82 **Fig 1. qPCR Cq values of candidate reference genes in all treatments.** The box includes all data points between
83 the 25 % quantile and the 75 % quantile. The whiskers span all values below and above, excluding outliers
84 which are displayed as dots. The median, which marks the 50 % quantile, is displayed as a line.

To determine to what extend the selected reference genes respond to different stimuli, we analysed their expression in cold, drought, heat, salt and gibberellic acid treatments. The stability was calculated using three common statistical algorithms: NormFinder (7), BestKeeper (8) and geNorm (9). These algorithms can be used to rank reference genes of a given set by their stability and determine the most stable genes for the tested conditions. In addition, geNorm provides a cut off value of 1.5, above which primer pairs are considered adequate for normalisation. All genes in all treatments, except for *HSP81.2/90* under heat stress conditions, met this criterion. Thus, all reference genes reported here can be considered suitable for a wide range of conditions. The most stable genes for cold treatments were *RAN3*, *HCF* and *PSB33*. According to the BestKeeper algorithm, *EIF4a* could be considered as a reference for cold treatments as well. However, especially geNorm advises against this gene, ranking it at position eight. After drought treatment, *UBQ10* and *TUA5* showed the least overall variation in the three methods. Here in particular, the results strongly differed between the algorithms. While *HISTONE H3* ranked first for BestKeeper, it was found to be least stable in the other two methods. On the contrary, *EIF4a* and *THIOREDOXIN* showed high stability in NormFinder and geNorm, but low values in BestKeeper. Under heat conditions, *RAN3*, *PSB33* and *EIF4a* were most stable over all algorithms and under salt conditions *CAC*, *TUA5*, *ACTIN 2* and *PSB33* performed best. Finally, the GA treatments caused least changes of the expression of *PSB33* and *TUA5*, while *THIOREDOXIN* showed stable expression only for NormFinder and geNorm. When taking all stress treatments into account, *THIOREDOXIN* and *CAC* showed the least variation. When considering all treatments, *CAC* and *ACTIN 2* showed the most stable expression (Table S2).

106 **Table 3. Ranking of gene expression stability under stress conditions and hormone stimuli.** Genes were
107 ranked using the three commonly used statistical algorithms NormFinder, BestKeeper and geNorm. The
108 stability value describes the variance (NormFinder and geNorm) or standard deviation (BestKeeper).

	Rank	NormFinder		BestKeeper		geNorm	
		Gene	**Stability**	**Gene**	**Stability**	**Gene**	**Stability**
Cold	1	*HCF*	0.04	*RAN3*	1.11	*PSB33*	0.28
	2	*RAN3*	0.06	*EIF4a*	1.13	*RAN3*	0.28
	3	*PSB33*	0.07	*HCF*	1.14	*HCF*	0.29
	4	*EIF4a*	0.10	*NdhO*	1.16	*TUA5*	0.30
	5	*ATPase*	0.11	*UBQ10*	1.17	*ATPase*	0.31
	6	*TUA5*	0.12	*TUA5*	1.18	*THIOREDOXIN*	0.31
	7	*THIOREDOXIN*	0.14	*HISTONE H3*	1.18	*CAC*	0.31
	8	*NdhO*	0.15	*PSB33*	1.18	*EIF4a*	0.32
	9	*HISTONE H3*	0.15	*SAND*	1.19	*SAND*	0.33
	10	*CAC*	0.15	*THIOREDOXIN*	1.21	*NdhO*	0.35
	11	*SAND*	0.15	*ATPase*	1.21	*HISTONE H3*	0.36
	12	*UBQ10*	0.17	*CAC*	1.23	*ACTIN 2*	0.37
	13	*ACTIN 2*	0.22	*ACTIN 2*	1.26	*UBQ10*	0.38
	14	*18srRNA*	0.28	*18srRNA*	1.34	*18srRNA*	0.45
	15	*HSP81.2/90*	0.63	*HSP81.2/90*	1.75	*HSP81.2/90*	0.92
Drought	1	*UBQ10*	0.05	*HISTONE H3*	1.11	*UBQ10*	0.19
	2	*EIF4a*	0.07	*TUA5*	1.14	*EIF4a*	0.20
	3	*THIOREDOXIN*	0.08	*NdhO*	1.18	*THIOREDOXIN*	0.21
	4	*CAC*	0.08	*HCF*	1.20	*CAC*	0.21
	5	*TUA5*	0.08	*UBQ10*	1.20	*TUA5*	0.22
	6	*SAND*	0.09	*SAND*	1.22	*ATPase*	0.22
	7	*ATPase*	0.10	*CAC*	1.22	*HCF*	0.22
	8	*HCF*	0.10	*HSP81.2/90*	1.22	*SAND*	0.22
	9	*NdhO*	0.10	*THIOREDOXIN*	1.23	*NdhO*	0.23
	10	*ACTIN 2*	0.12	*ATPase*	1.24	*ACTIN 2*	0.24
	11	*RAN3*	0.12	*18srRNA*	1.24	*RAN3*	0.24
	12	*PSB33*	0.15	*EIF4a*	1.25	*PSB33*	0.26
	13	*18srRNA*	0.17	*ACTIN 2*	1.27	*18srRNA*	0.30
	14	*HSP81.2/90*	0.19	*RAN3*	1.30	*HSP81.2/90*	0.32
	15	*HISTONE H3*	0.25	*PSB33*	1.30	*HISTONE H3*	0.39
Heat	1	*EIF4a*	0.04	*RAN3*	1.09	*EIF4a*	0.47
	2	*RAN3*	0.04	*EIF4a*	1.12	*ATPase*	0.48
	3	*PSB33*	0.04	*PSB33*	1.14	*RAN3*	0.48
	4	*HCF*	0.04	*HCF*	1.15	*THIOREDOXIN*	0.48
	5	*THIOREDOXIN*	0.08	*THIOREDOXIN*	1.18	*HISTONE H3*	0.50
	6	*ATPase*	0.09	*ATPase*	1.20	*PSB33*	0.51

	Rank	NormFinder		BestKeeper		geNorm	
		Gene	Stability	Gene	Stability	Gene	Stability
	7	*NdhO*	0.12	*NdhO*	1.20	*HCF*	0.52
	8	*HISTONE H3*	0.18	*HISTONE H3*	1.22	*ACTIN 2*	0.52
	9	*18srRNA*	0.22	*ACTIN 2*	1.28	*NdhO*	0.55
	10	*ACTIN 2*	0.24	*18srRNA*	1.28	*CAC*	0.58
	11	*CAC*	0.32	*CAC*	1.37	*TUA5*	0.60
	12	*TUA5*	0.35	*TUA5*	1.39	*18srRNA*	0.61
	13	*UBQ10*	0.40	*UBQ10*	1.47	*SAND*	0.76
	14	*SAND*	0.49	*SAND*	1.61	*UBQ10*	0.87
	15	*HSP81.2/90*	1.74	*HSP81.2/90*	5.22	*HSP81.2/90*	2.52
Salt	1	*CAC*	0.11	*TUA5*	1.16	*CAC*	0.55
	2	*THIOREDOXIN*	0.12	*CAC*	1.18	*PSB33*	0.57
	3	*ACTIN 2*	0.15	*18srRNA*	1.19	*ACTIN 2*	0.57
	4	*PSB33*	0.15	*ACTIN 2*	1.19	*TUA5*	0.57
	5	*TUA5*	0.17	*PSB33*	1.22	*THIOREDOXIN*	0.57
	6	*NdhO*	0.19	*SAND*	1.24	*HISTONE H3*	0.62
	7	*HISTONE H3*	0.22	*THIOREDOXIN*	1.24	*NdhO*	0.63
	8	*SAND*	0.27	*NdhO*	1.25	*UBQ10*	0.64
	9	*UBQ10*	0.29	*HISTONE H3*	1.25	*SAND*	0.66
	10	*HCF*	0.37	*UBQ10*	1.34	*18srRNA*	0.71
	11	*18srRNA*	0.37	*HCF*	1.4	*HCF*	0.73
	12	*ATPase*	0.41	*ATPase*	1.59	*ATPase*	0.83
	13	*HSP81.2/90*	0.69	*HSP81.2/90*	1.93	*HSP81.2/90*	1.08
	14	*EIF4a*	0.75	*EIF4a*	1.95	*EIF4a*	1.18
	15	*RAN3*	0.93	*RAN3*	2.43	*RAN3*	1.4
GA	1	*TUA5*	0.05	*SAND*	1.15	*TUA5*	0.21
	2	*THIOREDOXIN*	0.06	*RAN3*	1.15	*PSB33*	0.22
	3	*CAC*	0.07	*PSB33*	1.15	*THIOREDOXIN*	0.22
	4	*PSB33*	0.08	*TUA5*	1.16	*CAC*	0.22
	5	*NdhO*	0.09	*EIF4a*	1.17	*ACTIN 2*	0.23
	6	*ACTIN 2*	0.09	*CAC*	1.17	*NdhO*	0.23
	7	*HCF*	0.09	*ACTIN 2*	1.18	*HCF*	0.24
	8	*RAN3*	0.11	*HCF*	1.18	*RAN3*	0.24
	9	*EIF4a*	0.12	*THIOREDOXIN*	1.19	*EIF4a*	0.25
	10	*SAND*	0.13	*NdhO*	1.20	*SAND*	0.26
	11	*ATPase*	0.13	*ATPase*	1.22	*ATPase*	0.27
	12	*UBQ10*	0.15	*HISTONE H3*	1.28	*UBQ10*	0.29
	13	*HSP81.2/90*	0.16	*UBQ10*	1.32	*HSP81.2/90*	0.30
	14	*HISTONE H3*	0.20	*HSP81.2/90*	1.34	*HISTONE H3*	0.34
	15	*18srRNA*	0.35	*18srRNA*	1.37	*18srRNA*	0.53

Optimal number of reference genes

For the optimal normalisation, it is necessary to use two or more reference genes in each experiment. The optimal number of reference genes can be determined with the geNorm algorithm, which calculates the pairwise variation $V_{n/n+1}$ based on the normalisation factors NF_n and NF_{n+1}, with $n \geq 2$. If $V_{n/n+1}$ is below 0.15, n is the optimal number of reference genes. For all tested treatments, individually or combined, two reference genes are sufficient to normalise qPCR measurements (Fig 2). The use of a third reference would not improve the results significantly.

Fig 2. Optimal number of reference genes for various conditions. The geNorm algorithm was used to determine the pairwise variation (V) between the reference genes for treatments with cold, drought, heat, salt and gibberellic acid. The threshold for adequate normalisation is $V \leq 0.15$, indicated by the green dashed line.

Impact of reference genes on the normalisation of samples in abiotic stress and hormone treatments

The efficiencies of treatments with cold, drought, heat, salt and gibberellic acid were controlled by the expression analysis of specific stress response genes by qPCR using primers for *RD29A* (*RESPONSIVE TO DESICCATION 29A*, cold and drought responsive), *HSP81.2/90* (heat responsive), *TSPO* (*OUTER MEMBRANE TRYPTOPHAN-RICH SENSORY PROTEIN-RELATED*, salt responsive) and *GA3ox1* (*GIBBERELLIN 3-OXIDASE 1*, GA responsive). All primers showed efficiencies of 80.28 to 104.22 % and correlations of more than 0.99 (Table 4).

129 **Table 4. Abiotic stress and hormone responsive genes, primers and amplicons.**

Name	Gene ID	Arabidopsis homolog	Primer sequence (5' to 3')	Amplicon length [bp]	E [%]	R^2
RD29A	Aa_G396870	AT5G52310	F GCCCTTGCTTCAGGGCTAGG R TGCTCCGGTGTTTCCACTCC	89	80.28	-0.996
HSP81.2/90	NA	AT5G56030	F CGTCTGGTGAGGCTCTTGGT R AGCCTACGCTCCTCAAGGTACT	85	96.74	-0.999
TSPO	Aa_G12840	AT2G47770	F GTGGACGGTGGGTTCCACAA R CACACCACAAGCCCGGCTAA	125	104.22	-0.994
GA3ox1	Aa_G112460	AT1G15550	F TTCCGGTTACCTGTCCAACG R GCCTGAGATGGTGAAGCCTT	129	82.79	-0.999

NA – not annotated (identified by nucleotide BLAST), F – forward primer, R – reverse primer, E[%] – efficiency of the primers was calculated with $E[\%] = 100 \times (10^{\left(-\frac{1}{slope}\right)} - 1)$, slope – average Cq values (y) and log10 values of six serial dilutions (x) were used to calculate the slope of a regression line with the formula $slope = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2}$, R^2 – correlation of the Cq values calculated with the formula $\rho_{x,y} = \frac{Cov(X,Y)}{\sigma_x \times \sigma_y}$.

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131 Normalisation of the results was carried out with one or two of the most stable reference genes
132 determined in this study (Fig 3). For cold, normalisation was carried out with *RAN3* and/or *HCF*.
133 Drought samples were normalised to *UBQ10* and/or *TUA5*. G*A* and salt responsive genes were
134 normalised with *PSB33* and/or *TUA5.* Finally, the heat samples were normalised with *RAN3* and/or
135 *PSB33.* The results clearly show that all treatments were successful, leading to an increased (cold,
136 drought, heat, salt) or decreased (GA) expression of the responsive genes. Individual normalisation
137 with each reference gene led to differences in the calculated fold changes of 28.9 % (cold), 7.5 %

(drought), 3.6 % (GA), 9.3 % (heat) and 10.4 % (salt) between reference gene 1 and 2, respectively. These results clearly show the necessity to use two reference genes simultaneously.

Fig 3. Comparison of specific stress response genes normalised with different reference genes. Normalisation of the stress response genes RD29A (RESPONSIVE TO DESICCATION 29A, cold and drought responsive), HSP81.2/90 (heat responsive), TSPO (OUTER MEMBRANE TRYPTOPHAN-RICH SENSORY PROTEIN-RELATED, salt responsive) and GA3ox1 (GIBBERELLIN 3-OXIDASE 1, GA responsive) was carried out with one or two reference genes (RG1 and RG2): cold – RAN3 and HCF, drought – UBQ10 and TUA5, GA and salt – PSB33 and TUA5, heat – RAN3 and PSB33.

Material and Methods

Plant growth conditions

For the abiotic stress treatments, seeds were surface sterilised with increasing concentrations of ethanol and grown on MS (10) plates without additional sucrose. The seeds were stratified for five days. Subsequently, plants were grown under long day conditions (16h light/8h darkness) at 21±1°C and 100±20 µmol/m^2s light intensity.

The heat and cold treatments were carried out by transferring 5-7 day old seedlings to 38°C and 4°C, respectively. Samples were taken after 2 h. Drought was induced on MS plates which were exposed to liquid MS containing 20 % PEG 8000 for 24 h prior to the experiment. Samples were taken after 24 h. For salt treatments, two to three 5-7 day old seedlings were transferred from plates to liquid ½ MS (control) or liquid ½ MS containing 125 mM NaCl for 4 h under constant shaking.

For the GA treatment, plants were grown on soil under long day conditions (16h light/8h darkness) at 21±1°C and 200±20 µmol/m^2s light intensity. The treatment was started right after germination and continued twice per week. Plants were sprayed with 20 µM GA4 (Sigma Aldrich, stock solution: 100 mM GA4 in EtOH, 0.1 % Silwet L-77 Loveland industries) or mock (0.1 % EtOH, 0.1 % Silwet). Leaves were harvested 14 days after germination at Zeitgeber time (ZT) 8.

All experiments were performed in three independent biological replicates. All samples were immediately frozen in liquid nitrogen and stored at -80°C.

RNA extraction and cDNA synthesis

The frozen seedling samples were ruptured using a TissueLyser (Qiagen) and total RNA extraction was carried out with Tri-Reagent (Ambion by Life Technologies). All samples were treated with DNAseI (Thermo Fisher Scientific). The frozen leaf samples were ruptured using a TissueLyser (Qiagen), extracted with the RNAeasy Plant Mini Kit (Qiagen) and treated with DNAse (Ambion by Life Technologies). RNA integrity was controlled on a bleach gel (Aranda et al., 2012) and RNA concentration and purity was measured using a photometer (Eppendorf). cDNA was synthesised from 500 ng total RNA with oligodT primers, using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the provided protocol. The cDNA was tested for DNA contamination and integrity by PCR and subsequent gel electrophoresis. The cDNA was diluted (1:10, 1:20, 1:40, 1:80, 1:160, and 1:320) to analyse primer efficiency and determine the correlation coefficient.

Choice of candidate genes and primer design

The eight reference gene candidates *EIF4a*, *ACTIN 2*, *CAC*, *TUA5*, *HISTONE H3*, *HSP81.2/90, 18srRNA* and *SAND* were chosen due to their stable expression in other species (12–16). *PSB33*, *ATPase*, *THIOREDOXIN*, *HCF* and *NdhO* were chosen regarding their robust expression levels in Arabis in a time-course RNAseq experiment (data provided by Eva Willing, MPIPZ, Cologne) and/or due to their essential function for the plant. The *RAN3* primers were taken from Wang et al., 2009. *UBQ10* primers were kindly provided by Pan Pan Jiang.

Sequences were taken from TAIR (17), NCBI (National Centre for Biotechnology Information, www.ncbi.nlm.nih.gov) and the Genomic resources for *Arabis alpina* website (www.arabis-alpina.org; Willing et al., 2015). *In silico* sequence analysis was carried out with CLC DNA Workbench version 5.6.1. The primers were designed using GenScript Real-time PCR Primer Design (www.genscript.com)

at an optimum Tm of 60±2°C. Amplicons showed a single band of the expected size in gel electrophoresis and a single peak in the melting curve (Fig S1). The PCR products were sequenced by GATC/ Eurofins Genomics to verify specific amplification.

Quantitative real-time PCR (qPCR)

qPCRs were carried out in a QuantStudio 5 System (ABI/Life Technologies) equipped with the QuantStudio TM Design and Analysis Software version 1.4.1. The qPCRs were performed using plates (96 well, 0.2 ml) and cover foil (Opti-Seal Optical Disposable Adhesive) from BIOplastics. Reaction mixtures of 20 µl were composed from 5 µl SYBR Green (Thermo Fisher Scientific), 0.2 µl of each primer, 1 µl cDNA and 1 µl ddH_2O. Amplification was carried out with the standard settings of the QuantStudio 5 System (50°C for 2 min, 95°C for 10 sec, 40 cycles at 95°C for 15 sec and 60°C for 1 min, followed by 95°C for 15 sec and a final dissociation curve from 60°C to 95°C). For each reference gene sample, three biological and three technical replicates were analysed. The impact of normalisation on stress/hormone responsive genes was analysed for three technical replicates of one biological replicate.

Analysis of qPCR data

Efficiency calculations were carried out manually using Excel 2007. Extreme outliers were removed manually, a standard deviation of technical replicates was accepted below 0.5 Cq. Efficiency of primers was calculated in cDNA dilution series. The average Cq values (y) and log10 values of the dilutions (x) were used to calculate the slope of a regression line with the formula $slope = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2}$. The slope was then used to calculate the efficiency of the primers $E[\%] = 100 \times (10^{\left(-\frac{1}{slope}\right)} - 1)$. The correlation R^2 of the values was calculated using the formula $\rho_{x,y} = \frac{Cov(X,Y)}{\sigma_x \times \sigma_y}$. Primers for reference genes were accepted with an efficiency of 90-110 % and a correlation between -1 and -0.99. Primers for genes of interest were accepted with an efficiency of 80-120 % and a correlation between -1 and -0.99.

The stability of the reference genes within a given set of different treatments was calculated from the efficiency corrected data using the algorithms NormFinder (7), BestKeeper (8) and geNorm (9). The geNorm algorithm was also used to define the number of reference genes necessary for normalisation.

Normalisation against one reference gene was carried out using the normalisation factor, normalisation against two reference genes was carried out using the geometric mean of the normalisation factors, according to the geNorm manual (9). In accordance with this manual, standard deviations between biological replicates were calculated over the means of the single replicates, rather than the raw data.

Discussion

Gene expression analysis by qPCR is a high-throughput method, which is considered to be very sensitive and reproducible. However, the accuracy of the results strongly depends on the experimental design, adequate normalisation and exact analysis of the produced data (18). Moreover, the qPCR primers must be specific, efficient, and - in the case of reference genes - stable in the tested conditions (18). In this study, we analysed primers for one established reference gene (*RAN3*; Wang et al., 2009) and 14 novel reference genes for *Arabis alpina* in several abiotic stress and hormone treatments. *EIF4a*, *ACTIN 2*, *CAC*, *TUA5*, *HISTONE H3*, *HSP81.2/90* and *SAND* were chosen because they were already established as reference genes in other species (12–16,19,20). In addition, we considered *PSB33*, *ATPase*, *THIOREDOXIN*, *HCF* and *NdhO*. The reference genes selected here are involved in various basic cellular functions including translation, proton transport, photosynthesis, protein degradation and cytoskeletal organisation. Our data suggest that all genes reported here are appropriate reference genes for the used tissues under non-stress conditions. As *PSB33* and *HCF* are functionally related to photosynthesis, they may be more appropriate for photosynthetic tissues.

229 The reference gene primers presented here have efficiency values between 96.42 and 107.01 %.
230 Although these are very good efficiencies, it is essential to correct the qPCR results with these values
231 because of the non-linearity of the PCR amplification steps (21). Consequently, we used efficiency-
232 corrected data for identifying the most stable reference genes and for the analysis of the impact on
233 normalisation. There is currently no consensus in the community, which of the three statistical
234 algorithms – geNorm, NormFinder or BestKeeper – is most appropriate. One advantage of the
235 geNorm algorithm is that it can be used for small sample sizes (22). However, the geNorm method is
236 biased towards genes that are co-regulated (23). The algorithm takes into consideration, whether
237 genes show a similar expression pattern (24), since it is assumed that similar changes in the
238 expression of two independent genes reflect technical differences, such as the cDNA concentration,
239 rather than changes caused by a treatment. By contrast, NormFinder considers variations across
240 subgroups (7). The algorithm assumes that there is no systematic variation of the average of the
241 tested samples, which can lead to a preference for reference genes with similar systematic variation
242 (23). BestKeeper takes the standard deviation of each individual reference gene into account, which
243 is an advantage over the other methods. The disadvantage of this algorithm is the use of a
244 parametric method (Pearson correlation), which requires normally distributed data with a
245 homogenous variance (8), which is not always the case. Additionally, BestKeeper uses the raw Cq
246 values, while geNorm and Normfinder require normalised quantities. Therefore, the results obtained
247 with BestKeeper are often different from those of the other two methods.

248 It is generally recommended to use more than one reference gene to guarantee optimal
249 normalisation (18). Our data support this view. The normalised fold change values varied up to
250 28.9 % between two references. Moreover, we recommend that at least one of the reference gene
251 amplicons contains introns at the genomic level to recognize potential contaminations with genomic
252 DNA.

253 For cold treatments, we recommend *RAN3*, as the amplicon contains an intron, combined with *HCF*
254 or *PSB33*. As *HCF* and *PSB33* are photosynthesis-associated proteins located in the thylakoid

membrane, there is no obvious reason to prefer one over the other. For drought treatments, *UBQ10* and *TUA5* were the most stable transcripts, with *TUA5* also containing an intron. In heat, *RAN3* can be recommended in combination with *PSB33* or *EIF4a*. As setups for heat treatments often go along with specific light settings, it might be advisable to use *EIF4a* as a second reference here. For the salt treatment, we found that *CAC*, *TUA5* and *ACTIN 2* are suitable, intron containing reference genes, which can be combined with each other or *PSB33*. The combination *TUA5* and *PSB33* might be the most efficient choice, as it is also the best option for GA treatments.

We found that there is no single reference gene, which is the best choice for all treatments. However, all genes tested in this study, except for *HSP81.2/90* under heat conditions, meet the requirements necessary for adequate normalisation. Thus, this study provides data for the selection of suitable reference gene combinations for *Arabis alpina* in cold, drought, heat, salt and GA treatments. With this, we enable adequate normalisation in qPCR experiments under these conditions and provide novel reference genes for future experiments addressing other stresses or stimuli.

Acknowledgements

We thank Pan Pan Jiang and Eva Willing for providing us with primer sequences and expression data, respectively. We also thank Jessica Pietsch and Tamara Gigolashvili for critically reading the manuscript.

References

1. Wang R, Farrona S, Vincent C, Joecker A, Schoof H, Turck F, et al. PEP1 regulates perennial flowering in Arabis alpina. Nature [Internet]. 2009 May 15 [cited 2018 May 7];459(7245):423–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19369938

2. Willing E-M, Rawat V, Mandáková T, Maumus F, James GV, Nordström KJV, et al. Genome expansion of Arabis alpina linked with retrotransposition and reduced symmetric DNA methylation. Nat Plants [Internet]. Nature Publishing Group; 2015 Feb 2 [cited 2018 Dec 3];1(2):14023. Available from: http://www.nature.com/articles/nplants201423

3. Meusel H, JÄGER EJ, WEINERT E. Vergleichende Chorologie der Zentraleuropaeischen Flora. Jena, Germany: Fischer Verlag; 1965.

4. Koch MA, KIEFER C, EHRICH D, VOGEL J, BROCHMANN C, MUMMENHOFF K. Three times out of Asia Minor: the phylogeography of Arabis alpina L. (Brassicaceae). Mol Ecol [Internet]. Wiley/Blackwell (10.1111); 2006 Feb 23 [cited 2018 Jul 30];15(3):825–39. Available from: http://doi.wiley.com/10.1111/j.1365-294X.2005.02848.x

5. Beilstein MA, Nagalingum NS, Clements MD, Manchester SR, Mathews S. Dated molecular phylogenies indicate a Miocene origin for Arabidopsis thaliana. Proc Natl Acad Sci U S A [Internet]. 2010 Oct 26 [cited 2017 Apr 30];107(43):18724–8. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.0909766107

6. Chopra D, Wolff H, Span J, Schellmann S, Coupland G, Albani MC, et al. Analysis of TTG1 function in Arabis alpina. BMC Plant Biol [Internet]. BioMed Central; 2014 [cited 2016 Aug 3];14(1):16. Available from: http://bmcplantbiol.biomedcentral.com/articles/10.1186/1471-2229-14-16

7. Andersen CL, Jensen JL, Ørntoft TF. 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 [Internet]. 2004 Aug 1 [cited 2018 May 6];64(15):5245–50. Available from: http://cancerres.aacrjournals.org/lookup/doi/10.1158/0008-5472.CAN-04-0496

8. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol Lett [Internet]. Kluwer Academic Publishers; 2004 Mar [cited 2018 May 6];26(6):509–15. Available from: http://link.springer.com/10.1023/B:BILE.0000019559.84305.47

9. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol [Internet]. BioMed Central; 2002 Jun 18 [cited 2018 May 6];3(7):research0034.1. Available from: http://genomebiology.biomedcentral.com/articles/10.1186/gb-2002-3-7-research0034

10. Murashige T, Skoog F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant [Internet]. Wiley/Blackwell (10.1111); 1962 Jul 1 [cited 2018 May 6];15(3):473–97. Available from: http://doi.wiley.com/10.1111/j.1399-3054.1962.tb08052.x

11. Aranda PS, LaJoie DM, Jorcyk CL. Bleach gel: a simple agarose gel for analyzing RNA quality. Electrophoresis [Internet]. NIH Public Access; 2012 Jan [cited 2018 Aug 2];33(2):366–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22222980

12. Tian C, Jiang Q, Wang F, Wang G-L, Xu Z-S, Xiong A-S. Selection of Suitable Reference Genes for qPCR Normalization under Abiotic Stresses and Hormone Stimuli in Carrot Leaves. Jain M, editor. PLoS One [Internet]. Public Library of Science; 2015 Feb 6 [cited 2017 Mar 2];10(2):e0117569. Available from: http://dx.plos.org/10.1371/journal.pone.0117569

13. Wang H, Wang J, Jiang J, Chen S, Guan Z, Liao Y, et al. Reference genes for normalizing

322 transcription in diploid and tetraploid Arabidopsis. Sci Rep [Internet]. Nature Publishing
323 Group; 2015 May 27 [cited 2018 Aug 2];4(1):6781. Available from:
324 http://www.nature.com/articles/srep06781

325 14. Demidenko N V., Logacheva MD, Penin AA. Selection and Validation of Reference Genes for
326 Quantitative Real-Time PCR in Buckwheat (Fagopyrum esculentum) Based on Transcriptome
327 Sequence Data. Mariño-Ramírez L, editor. PLoS One [Internet]. Public Library of Science; 2011
328 May 12 [cited 2018 Aug 2];6(5):e19434. Available from:
329 http://dx.plos.org/10.1371/journal.pone.0019434

330 15. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R. Genome-wide identification and
331 testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol
332 [Internet]. American Society of Plant Biologists; 2005 Sep [cited 2017 Jun 9];139(1):5–17.
333 Available from: http://www.ncbi.nlm.nih.gov/pubmed/16166256

334 16. Wan Q, Chen S, Shan Z, Yang Z, Chen L, Zhang C, et al. Stability evaluation of reference genes
335 for gene expression analysis by RT-qPCR in soybean under different conditions. Li X, editor.
336 PLoS One [Internet]. 2017 Dec 13 [cited 2018 Nov 28];12(12):e0189405. Available from:
337 http://www.ncbi.nlm.nih.gov/pubmed/29236756

338 17. Berardini TZ, Reiser L, Li D, Mezheritsky Y, Muller R, Strait E, et al. The arabidopsis information
339 resource: Making and mining the “gold standard” annotated reference plant genome. genesis
340 [Internet]. 2015 Aug 1 [cited 2018 Jan 25];53(8):474–85. Available from:
341 http://doi.wiley.com/10.1002/dvg.22877

342 18. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines:
343 Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem
344 [Internet]. 2009 [cited 2017 Aug 29];55(4). Available from:
345 http://clinchem.aaccjnls.org/content/55/4/611/tab-figures-data

346 19. Wu J, Zhang H, Liu L, Li W, Wei Y, Shi S. Validation of Reference Genes for RT-qPCR Studies of

347 Gene Expression in Preharvest and Postharvest Longan Fruits under Different Experimental
348 Conditions. Front Plant Sci [Internet]. 2016 Jun 3 [cited 2018 Nov 28];7:780. Available from:
349 http://www.ncbi.nlm.nih.gov/pubmed/27375640

350 20. Najafpanah MJ, Sadeghi M, Bakhtiarizadeh MR. Reference Genes Selection for Quantitative
351 Real-Time PCR Using RankAggreg Method in Different Tissues of Capra hircus. Niemann H,
352 editor. PLoS One [Internet]. 2013 Dec 16 [cited 2018 Nov 28];8(12):e83041. Available from:
353 http://www.ncbi.nlm.nih.gov/pubmed/24358246

354 21. Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, van den Hoff MJB, et al.
355 Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data.
356 Nucleic Acids Res [Internet]. 2009 Apr 1 [cited 2018 Dec 13];37(6):e45–e45. Available from:
357 http://www.ncbi.nlm.nih.gov/pubmed/19237396

358 22. Serrano M, Moreno-Sánchez N, González C, Marcos-Carcavilla A, Van Poucke M, Calvo JH, et
359 al. Use of Maximum Likelihood-Mixed Models to select stable reference genes: a case of heat
360 stress response in sheep. BMC Mol Biol [Internet]. 2011 Aug 17 [cited 2018 Dec 9];12(1):36.
361 Available from: http://bmcmolbiol.biomedcentral.com/articles/10.1186/1471-2199-12-36

362 23. Mehdi Khanlou K, Van Bockstaele E. A critique of widely used normalization software tools
363 and an alternative method to identify reliable reference genes in red clover (Trifolium
364 pratense L.). Planta [Internet]. 2012 Nov 21 [cited 2018 Dec 9];236(5):1381–93. Available
365 from: http://www.ncbi.nlm.nih.gov/pubmed/22718310

366 24. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate
367 normalization of real-time quantitative RT-PCR data by geometric averaging of multiple
368 internal control genes. Genome Biol [Internet]. BioMed Central; 2002 Jun 18 [cited 2018 Apr
369 19];3(7):RESEARCH0034. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12184808

370

Supplementary Materials

Table S1. Raw Cq values of treatments with cold, drought, heat, salt and gibberellic acid.

Fig S1. Melting curves of candidate reference genes and stress/hormone responsive genes.

Fig S2. Standard curves of candidate reference genes and stress/hormone responsive genes.

Table S2. Ranking of gene expression stability under abiotic stress conditions and the combination of all treatments. Genes were ranked using the three commonly used statistical algorithms NormFinder, BestKeeper and geNorm. The stability value describes the variance (NormFinder and geNorm) or standard deviation (BestKeeper).

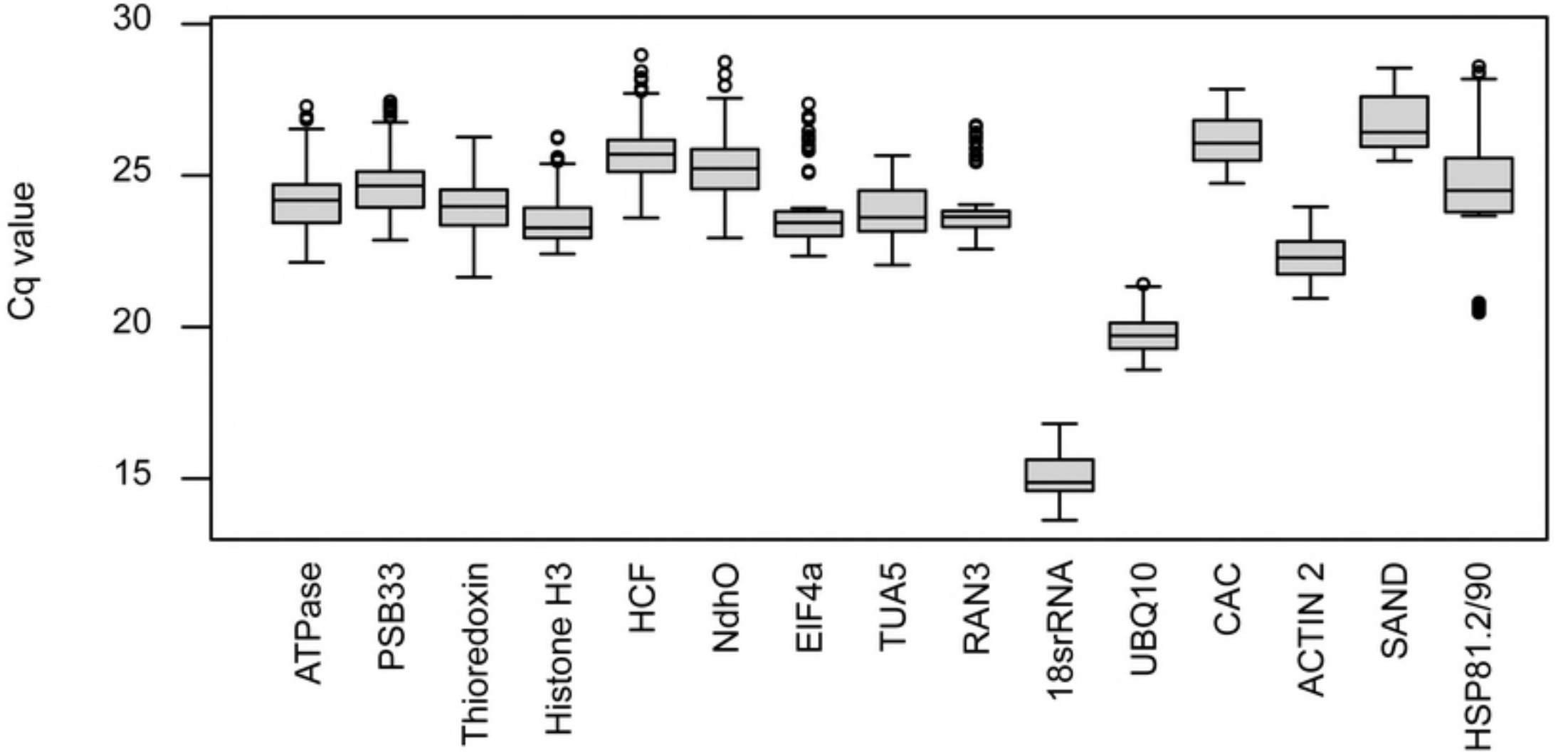
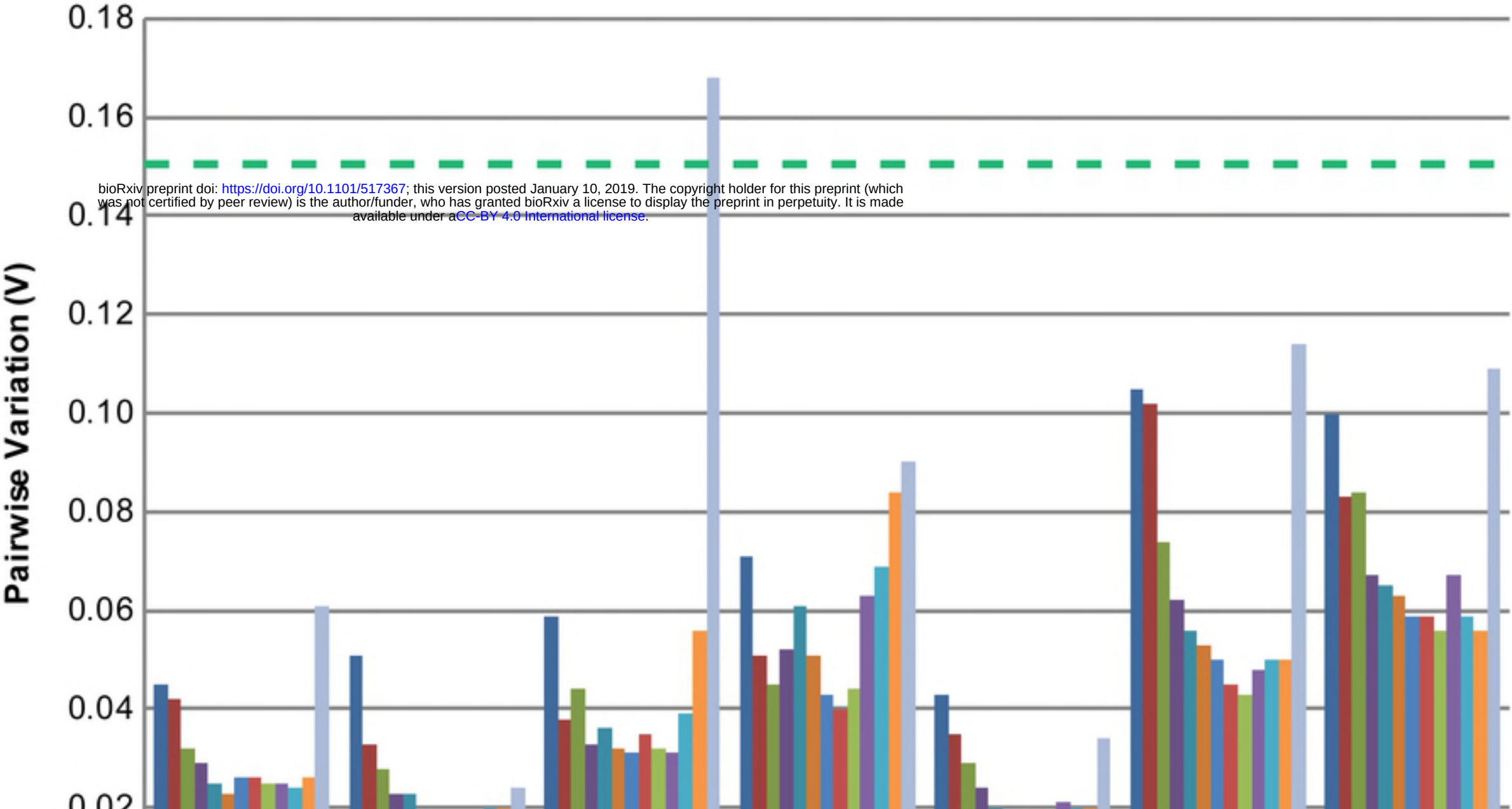
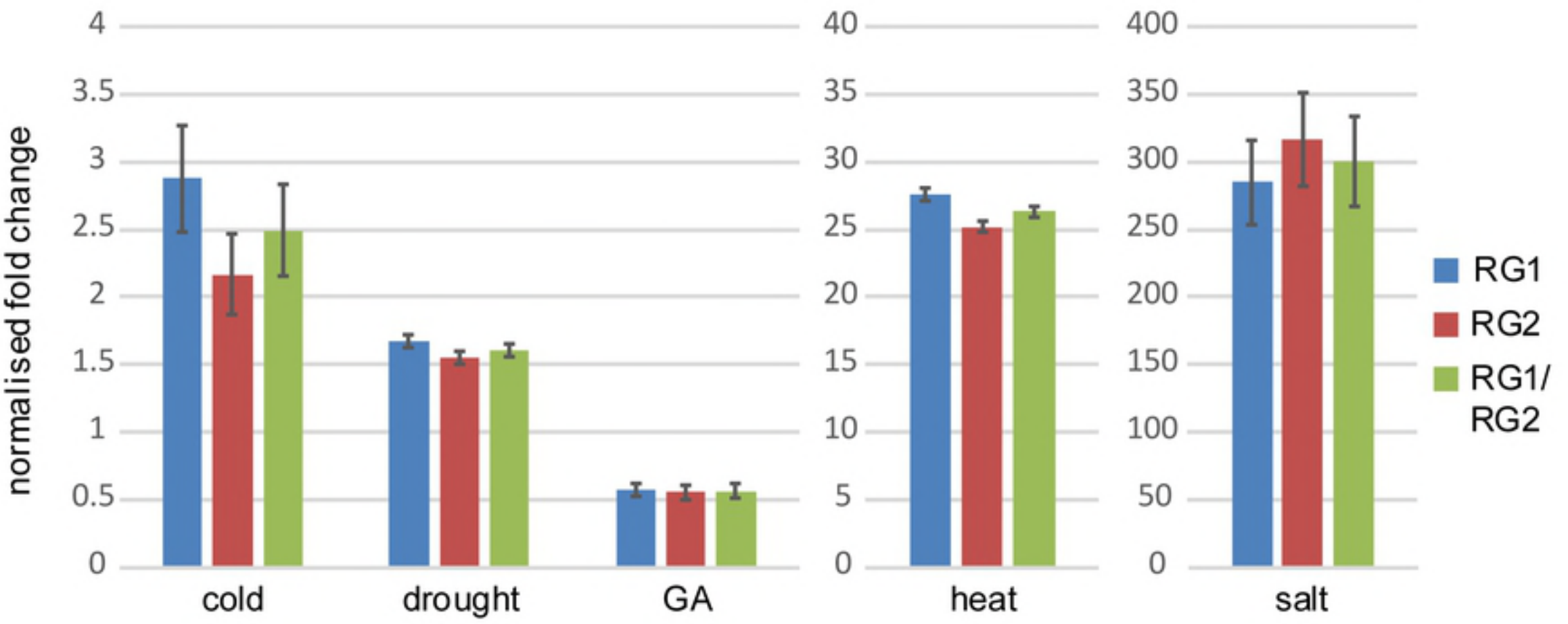

Cq value
30
25
20
15
ATPase
PSB33
Thioredoxin
Histone H3
HCF
NdhO
EIF4a
TUA5
RAN3
18srRNA
UBQ10
CAC
ACTIN 2
SAND
HSP81.2/90

Figure 1



	Cold	Drought	Heat	Salt	GA	Abiotic Stress	All Treatments
V2/3	0.045	0.051	0.059	0.071	0.043	0.105	0.100
V3/4	0.042	0.033	0.038	0.051	0.035	0.102	0.083
V4/5	0.032	0.028	0.044	0.045	0.029	0.074	0.084
V5/6	0.029	0.023	0.033	0.052	0.024	0.062	0.067
V6/7	0.025	0.023	0.036	0.061	0.020	0.056	0.065
V7/8	0.023	0.019	0.032	0.051	0.019	0.053	0.063
V8/9	0.026	0.016	0.031	0.043	0.017	0.050	0.059
V9/10	0.026	0.017	0.035	0.040	0.016	0.045	0.059
V10/11	0.025	0.016	0.032	0.044	0.018	0.043	0.056
V11/12	0.025	0.015	0.031	0.063	0.021	0.048	0.067
V12/13	0.024	0.020	0.039	0.069	0.020	0.050	0.059
V13/14	0.026	0.020	0.056	0.084	0.020	0.050	0.056
V14/15	0.061	0.024	0.168	0.090	0.034	0.114	0.109

Figure 2



RG1	2.88	1.67	0.57	27.61	285.31
RG2	2.16	1.55	0.55	25.17	316.58
RG1/RG2	2.49	1.61	0.56	26.36	300.53

Figure 3