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Research Information

Identification and evaluation of wheat reference genes for normalization of quantitative RT-PCR data during dehydration conditions

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Abstract
Gene expression analyses contribute to identification of molecular changes that occur in cells in response to internal and external stimuli. Real-time reverse transcription PCR (qRT-PCR) assay is widely used for gene expression analysis because of its fast, specific and sensitive detection of targets. At least a reference gene is needed for normalization of the expression level of target genes. Although some wheat reference genes have been identified under different experimental conditions, no reference gene was identified under dehydration stress conditions. Here, we report three reference genes, *CDCP*, *SAR* and *hnRNPQ*, suitable for normalization of gene expression under dehydration conditions in synthetic hexaploid wheat and its parental tetraploid and diploid wheat.

Introduction
Common wheat (*Triticum aestivum* L., BBAADD genome) is an allohexaploid species originated by natural hybridization between tetraploid wheat (*T. turgidum* L., BBAA genome) and *Aegilops tauschii* Coss. (DD genome) (Kihara 1944; McFadden and Sears 1944). *Ae. tauschii* can be crossed to tetraploid wheat to produce synthetic hexaploid wheat (McFadden and Sears 1944; Kihara and Lilienfeld 1949; Matsuoka and Nasuda 2004), and therefore *Ae. tauschii* is one of the potential sources for common wheat breeding. Indeed, wide variations in agronomically important traits have been observed in *Ae. tauschii* and their derived synthetic hexaploid wheat lines (Kajimura et al. 2011; Iehisa and Takumi 2012; Okamoto et al. 2012).

Transcriptome and gene expression analyses are effective for identification of molecular changes that occur in cells in response to internal and external stimuli (Mele and Hake 2003; Hazen et al. 2003). Quantitative reverse transcription PCR (qRT-PCR) assay is widely used for gene expression analysis because of its fast, specific and sensitive detection of targets (Gachon et al. 2004). To quantify the expression levels of target genes, at least one control gene (reference gene) is required for normalization to correct for variations in amount of initial samples, RNA recovery and integrity, enzymatic efficiencies of cDNA synthesis and PCR amplification, and overall transcriptional activities of the tissues or cells analyzed (Andersen et al. 2004; Chen et al. 2006). Selection of appropriate reference genes involves identifying candidates, validating the candidates under specific experimental conditions, and then revalidating the selected reference genes in each subsequent experiment (Remans et al. 2014).

Although reference genes have been identified in wheat under different experimental conditions (Paolacci et al. 2009; Long et al. 2010; Giménez et al. 2011; Tenea et al. 2011; Zhang et al. 2013; Jurczyk et al. 2014), no reference gene was identified under dehydration stress conditions. Therefore, the objective of this study was identification of reference genes for normalization of wheat gene expression under dehydration conditions in synthetic hexaploid wheat and its parental tetraploid wheat and diploid wheat relative *Ae. tauschii*.

Material and Methods

Plant materials

Two synthetic hexaploid wheat lines Ldn//PI476874 and Ldn//KU-2059, and their parental tetraploid wheat *T. turgidum* ssp. *durum* cv. Langdon (Ldn) and diploid *Ae. tauschii* PI476874 and KU-2059 accessions were used. These synthetic wheat lines were generated by interspecific hybridization of Ldn and *Ae. tauschii* (Kajimura et al. 2011).

Dehydration treatment

Seeds of hexaploid and tetraploid wheat and *Ae. tauschii* accessions were sown on soil containing plastic pots at 24°C under long day condition (16 h light and 8 h darkness). Twelve-day-old seedlings of *Ae. tauschii* and 10-d-old seedlings of hexaploid and tetraploid wheat were removed from soil. Roots were carefully washed with water and the excess of water was wiped. Dehydration treatment was performed placing the roots on dry filter paper.

Expression analysis of candidate genes

Total RNA was extracted from crown tissue of a pool of three individuals at 0, 2, 4 and 12 h after dehydration treatment using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan). The accumulation of each gene transcript was detected by qRT-PCR using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) with the gene-specific primer sets given in Table 1. The rate of amplification was monitored using THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan) according to the manufacturer’s protocol.

Data analysis

PCR efficiency of each primer pair was determined for all samples by the LinRegPCR quantitative PCR data analysis program (version 2016.1) (Ruijter et al. 2009) using raw fluorescence as input data. Using the PCR efficiency data and Cq values calculated with LightCycler 480 software (Roche), gene expression stability was analyzed with geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004).

Results and Discussions

Expression level of the candidate reference genes in Ae. tauschii

Housekeeping genes, which are involved in basic cellular processes such as cell structure maintenance or primary metabolism has been widely used as reference genes for qRT-PCR. In wheat, housekeeping genes such as 18S rRNA, actin, alpha-tubulin have been traditionally used (Paolacci et al. 2009). However, it was previously found that the expression levels of many other genes are more stable than those of the traditionally used reference genes (Paolacci et al. 2009; Long et al. 2010). Based on the previous studies (Paolacci et al. 2009; Long et al. 2010), we selected five genes for *Cell Division Control Protein* (*CDCP*), *Elongation Factor 1-alpha* (*EF1a*), *Heterogeneous Nuclear Ribonucleoprotein Q* (*hnRNPQ*), *Scaffold-associated regions DNA binding protein* (*SAR*) and *Glucan endo-1,3-beta-glucosidase 4 precursor* (*GE1,3*) as candidates. First, the

Table 1. List of primers used in this study

Gene	Primer sequence (5’ to 3’)	Reference
hnRNPQ	TCACCTTCGCCAAGCTCAGAACTA AGTTGAACTTGCCCGAAACATGCC	Long et al. (2010)
SAR	GAGTCTGCCCACCCATTCGTAA GACATGCCATAGGTTTCAGCGAC	Long et al. (2010)
GE1,3	AGCACAGCGAAGAGAAGCAG TACCTGAGCAGACAATGGGAGAG	Long et al. (2010)
EF1a	CAGATTGGCAACGGCTACG CGGACAGCAAAACGACCAAG	Crismani et al. (2006)
CDCP	CAAATACGCCATCAGGGAGAACATC CGCTGCCGAAACCACGAGAC	Paolacci et al. (2009)

expression levels of these genes were evaluated in the two *Ae. tauschii* accessions (PI476874 and KU-2059) under dehydration condition. The mean quantification cycle (Cq) of the two accessions for *GE1,3* was around 30 at different time points of dehydration treatment (Fig. 1). In contrast, the rest of genes showed a mean Cq value of around 20 indicating that the expression level of *GE1,3* was low. Previous study has suggested that the expression levels of reference genes should not be very low (Cq > 30) or very high (Cq < 15) (Lland et al., 2006).

The mean amplification efficiency of each primer set was calculated using amplification curves of these samples. The amplification efficiency ranged from 1.62 for *GE1,3* to 2.00 for *hnRNPQ* (Table 2).

Expression stability of candidate genes in Ae. tauschii

To determine the expression stability of candidate reference genes, we used three algorithms widely used for this purpose; geNorm, NormFinder and BestKeeper. geNorm determines pairwise variation for every gene respect to all other genes as the standard deviation of the logarithmically transformed expression ratios (*M* value). Thus, genes with the lowest *M* values have the most stable expression (Vandesompele et al. 2002). *GE1,3* and *EF1a* presented higher *M* value, and the other three genes lower values (Fig. 2A), indicating that *hnRNPQ*, *CDCP* and *SAR* showed more stable expression among the samples in this experimental conditions.

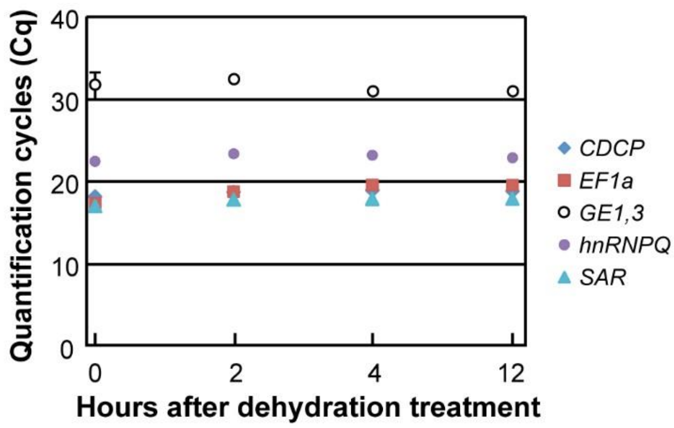

Fig. 1. Average Cq values of the five candidate reference genes in *Ae. tauschii* under dehydration condition. Average Cq values of two *Ae. tauschii* accessions PI476874 and KU-2059 at 0, 2, 4 and 12 h after dehydration treatment. *CDCP* (diamond), *EF1a* (square), *GE1,3* (open circle), *hnRNPQ* (filled circle) and *SAR* (triangle) were analyzed.

Table 2. Amplification efficiencies of the primer sets analyzed in *Ae. tauschii*

Gene	Efficiency
CDCP	1.88
EF1a	1.89
GE1,3	1.62
hnRNPQ	2.00
SAR	1.97

NormFinder uses model-based and inter- and intra-group expression variation to determine the stability of each gene (Andersen et al. 2004). This algorithm also showed similar result to that of geNorm (Fig. 2B). *GE1,3* and *EF1a* were the least stable genes, and the other three were the most stable genes.

BestKeeper evaluates gene expression stability for each candidate reference gene based mainly on standard deviation (S. D.) of Cq values and Pearson's coefficient of correlation (*r*). This correlation coefficient is calculated between each candidate reference gene and the BestKeeper index, which in turn is calculated combining all highly correlated candidate reference genes. The genes with smaller S. D. and higher *r* values are considered to be suitable as reference gene (Pfaffl et al. 2004). In concordance with the results obtained in geNorm and NormFinder, the analysis with BestKeeper also indicated that *hnRNPQ*, *CDCP* and *SAR* were the most suitable genes (Table 3).

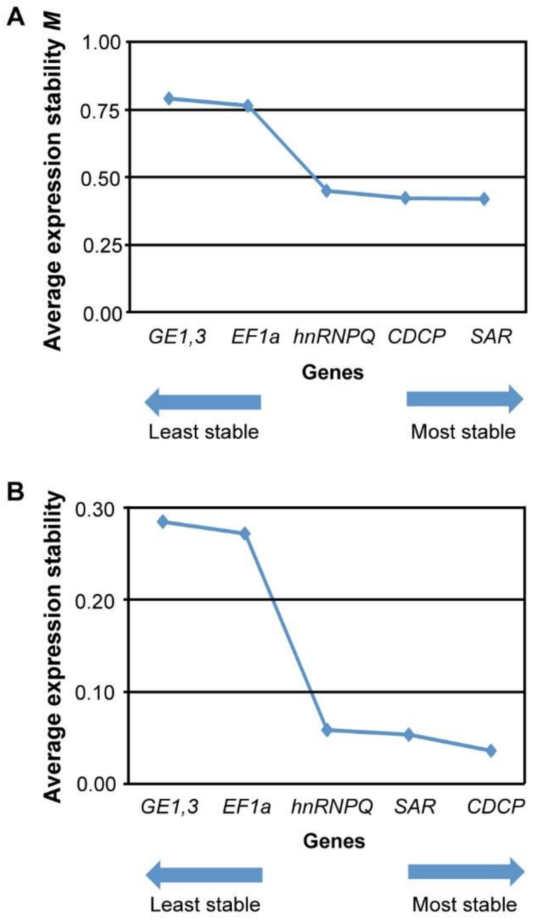

Fig. 2. Analysis of expression stability of candidate reference genes in *Ae. tauschii* under dehydration condition. Stability of gene expression was calculated using A) geNorm and B) NormFinder for *GE1,3*, *EF1a*, *hnRNPQ*, *CDCP* and *SAR* in two *Ae. tauschii* accessions PI476874 and KU-2059. In both algorithms, lower values indicate higher expression stability.

Table 3. Analysis of expression stability using BestKeeper

	CDCP	***hnRNPQ***	***SAR***	***EF1a***	***GE1,3***
S. D.	0.25	0.26	0.31	0.75	0.84
r	0.94	0.89	0.97	0.84	-0.07

Standard deviation (S. D.) of the Cq values and the Pearson's coefficient of correlation (*r*) between each gene and the BestKeeper index are presented.

Evaluation of selected reference genes in hexaploid, tetraploid and diploid wheat

To evaluate expression stabilities of the *hnRNPQ*, *CDCP* and *SAR* genes in hexaploid, tetraploid and diploid wheat under dehydration conditions, two synthetic hexaploid wheat lines, Ldn//PI476874 and Ldn//KU-2059, and their parental accessions were used. Among the three genes analyzed, *hnRNPQ* showed the highest stability with geNorm and NormFinder (Table 4). BestKeeper also showed the same result, and *hnRNPQ* represented the lowest S. D. of Cqs and the highest *r* value. The stability values were similar between *SAR* and *CDCP*, but *SAR* gave better stability when analyzed with BestKeeper. Although *hnRNPQ* performed better when used hexaploid, tetraploid and diploid wheat, these three genes presented similar performance in *Ae. tauschii* under dehydration conditions. *CDCP* has been identified as the most stable gene under different growth conditions (different tissues, developmental stages and temperature stress) (Paolacci et al. 2009), and *SAR* and *hnRNPQ* were identified among more stable genes under biotic and abiotic stress conditions (NaCl, ABA, PEA, yellow rust and low temperature stress treatments) (Long et al. 2010). It is recommended to use multiple reference genes for normalization of qRT-PCR data (Bustin et al. 2009; Remans et al. 2014). Therefore, these three genes could be used for normalization of genes involved in dehydration responses of wheat at the different ploidy levels. There is no universal reference gene in which the expression is stable under different conditions and tissues (Brunner et al. 2004; Jain et al. 2006). Therefore, it is important to evaluate the stability of the candidate reference genes under each experimental condition.

Table 4. Analysis of expression stability in hexaploid, tetraploid and diploid wheat

Gene	geNorm	NormFinder	BestKeeper*
hnRNPQ	0.52	0.14	0.39 (0.85)
SAR	0.73	0.36	0.46 (0.53)
CDCP	0.73	0.36	0.60 (0.71)

*The number outside the parenthesis is S. D. and Pearson’s correlation coefficient (*r*) are in the parenthesis.

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