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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	Long et al. (2010)
	AGTTGAACTTGCCCGAAACATGCC	
SAR	GAGTCTGCCCACCCATTCGTAA	Long et al. (2010)
	GACATGCCATAGGTTTCAGCGAC	
GE1,3	AGCACAGCGAAGAGAAGCAG	Long et al. (2010)
	TACCTGAGCAGACAATGGGAGAG	
EF1a	CAGATTGGCAACGGCTACG	Crismani et al. (2006)
	CGGACAGCAAAACGACCAAG	
CDCP	CAAATACGCCATCAGGGAGAACATC	Paolacci et al. (2009)
	CGCTGCCGAAACCACGAGAC	

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.

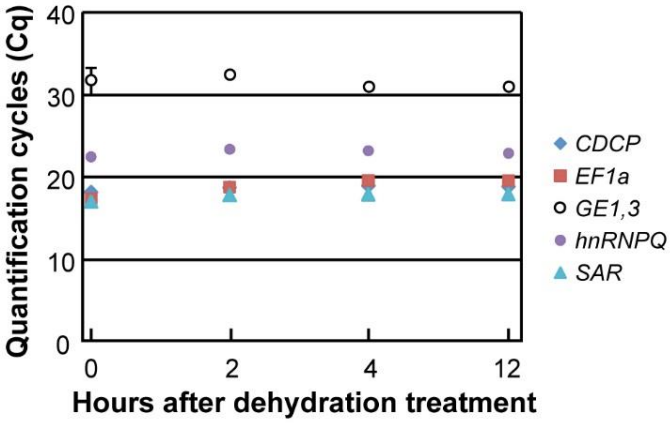

Fig. 1. Average Cq values of the five candidate reference genes in *Ae. tauschii* under dehydration condition. Average Cq values of two *Ae. tauschii* accessions PI476874 and KU-2059 at 0, 2, 4 and 12 h after dehydration treatment. *CDCP* (diamond), *EF1a* (square), *GE1,3* (open circle), *hnRNPQ* (filled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

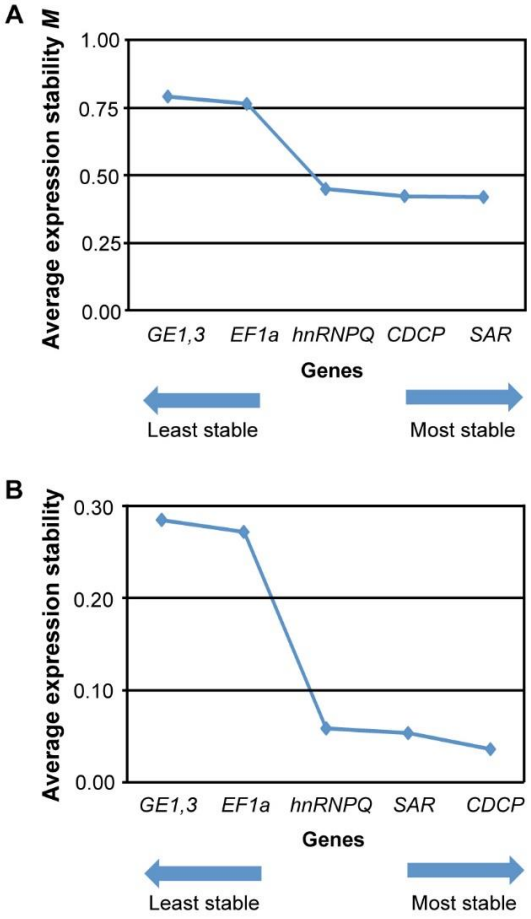

Fig. 2. Analysis of expression stability of candidate reference genes in *Ae. tauschii* under dehydration condition. Stability of gene expression was calculated using A) geNorm and B) NormFinder for *GE1,3*, *EF1a*, *hnRNPQ*, *CDCP* and *SAR* in two *Ae. tauschii* accessions PI476874 and KU-2059. In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

References

Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of realtime quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64: 5245–5250.

Brunner AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol 4: 14.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vadensompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611–622.

Chen J, Rider DA, Ruan R (2006) Identification of valid housekeeping genes and antioxidant enzyme gene expression change in the aging rat liver. J Gerontol A Biol Sci Med Sci 61: 20–27.

Crismani W, Baumann U, Sutton T, Shirley N, Webster T, Spangenberg G, Langridge P, Able JA (2006) Microarray expression analysis of meiosis and microsporogenesis in hexaploid bread wheat. BMC Genomics 7: 267.

Gachon C, Mingam A, Charrier B (2004) Real-time PCR: what relevance to plant studies? J Exp Bot 55: 1445–1454.

Giménez MJ, Pistón F, Atienza SG. Identification of suitable reference genes for normalization of qPCR data in comparative transcriptomics analyses in the Triticeae. Planta 233: 163–173.

Hazen SP, Wu Y, Kreps JA (2003) Gene expression profiling of plant responses to abiotic stress. Funct Integr Genomics 3: 105–111.

Iehisa JCM, Takumi S (2012) Variation in abscisic acid responsiveness of *Aegilops tauschii* and hexaploid wheat synthetics due to the D-genome diversity. Genes Genet Syst 87: 9–18.

Jain M, Nijhawan A, Tyagi AK, Khurana JP

(2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Commun 345: 646–651.

Jurczyk B, Pociecha E, Janeczko A, Paczyński R, Rapacz M (2014) Assessment of candidate reference genes for the expression studies with brassinosteroids in *Lolium perenne* and *Triticum aestivum*. J Plant Physiol 171: 1541–1544.

Kajimura T, Murai K, Takumi S (2011) Distinct genetic regulation of flowering time and grain-filling period based on empirical study of D-genome diversity in synthetic hexaploid wheat lines. Breed Sci 61: 130–141.

Kihara H (1944) Discovery of the DD-analyser, one of the ancestors of *Triticum vulgare*. Agr Hort 19: 889–890.

Kihara H, Lilienfeld F (1949) A new-synthesized 6x-wheat. Hereditas 35 (Suppl): 307–319.

Lland H, Hertzberg M, Marlton P (2006) Myeloid leukenmia. In: Colgan SP (Ed.), Methods and Protocols, Humana, Totowa, NJ, p. 53.

Long XY, Wang JR, Ouellet T, Rocheleau H, Wei YM, Pu ZE, Jiang QT, Lan XJ and Zheng YL (2010) Genome-wide identification and evaluation of novel internal control genes for Q-PCR based transcript normalization in wheat. Plant Mol Biol 74: 307–311.

Matsuoka Y, Nasuda S (2004) Durum wheat as a candidate for the unknown female progenitor of bread wheat: an empirical study with a highly fertile F_1 hybrid with *Aegilops tauschii* Coss. Theor Appl Genet 109: 1710–1717.

McFadden ES, Sears ER (1944) The artificial synthesis of *Triticum spelta*. Rec Genet Soc Am 13: 26–27.

Mele G, Hake S (2003) Expression profiling of plant development. Genome Biol 4: 215.

Okamoto Y, Kajimura T, Ikeda TM, Takumi S (2012) Evidence from principal component analysis for improvement of grain shape and spikelet morphology-related traits after hexaploid wheat speciation. Genes Genet Syst 87: 299–310.

Paolacci AR, Tanzarella OA, Porceddu E, Ciaffi M (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol Biol 10: 11.

Pfaffl M, Tichopad A, Prgomet C, Neuvians T (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. Biotechnol Lett 26: 509–515.

Remans T, Keunen E, Bex GJ, Smeets K, Vangronsveld J, Cuypers A (2014) Reliable gene expression analysis by reverse transcription-quantitative PCR: reporting and minimizing the uncertainty in data accuracy. Plant Cell 26: 3829–3837.

Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37: e45.

Tenea GN, Peres Bota A, Cordeiro Raposo F, Maquet A (2011) Reference genes for gene expression studies in wheat flag leaves grown under different farming conditions. BMC Res Notes 4:373.

Vandesompele J, Preter KD, Poppe B, Roy NV, Paepe AD (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: research0034.

Zhang K, Niu S, Di D, Shi L, Liu D, Cao X, Miao H, Wang X, Han C, Yu J, Li D, Zhang Y (2013) Selection of reference genes for gene expression studies in virus-infected monocots using quantitative real-time PCR. J Biotechnol 168: 7–14.

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Research Information

High-quality RNA isolation from wheat immature grains

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Grain quality is one of the most important targets in wheat breeding. Transcriptome analyses of wheat developing grains and endosperm have been performed using the microarray and RNA sequencing (RNA-seq) approaches (Wan et al. 2008, 2009; Nemeth et al. 2010; Pellny et al. 2012; Dong et al. 2015). For the RNA-seq analysis of the grain transcriptome and precise quantification of each transcript in developing grain and endosperm, the high-quality RNA is essential. For the microarray analysis, ≥7.3 RIN (RNA integrity number) value for the RNA sample quality is required according to the Agilent microarray protocol. In the previous report for the transcriptome of wheat developing grains, the total RNA samples with ≥8.0 RIN values were used for the RNA-seq analysis based on the PacBio and Illumina platforms (Dong et al. 2015). Some RNA extraction buffers containing SDS, CTAB, or TRIzol® reagent (Thermo Fisher Scientific, Waltham, Massachusetts) and several commercial kits for RNA isolation have been used to isolate total RNA from wheat grain and endosperm (Kawakami et al. 1992; Wan et al. 2008; Kang et al. 2013). However, total RNA samples from the wheat developing and immature grains are often damaged due to high content of polysaccharides and high stickiness of the solution homogenized with the RNA extraction buffer, and thus extraction of the high-quality RNA with high RIN value is quite difficult. Here, we report a protocol for the wheat grain RNA extraction using Maxwell RSC Plant RNA Kit (Promega, Madison, Wisconsin).

Two tetraploid wheat lines, *Triticum turgidum* ssp. *durum* cv. Langdon and *T. timopheevi* KU-107-1, a wild Einkorn wheat line, *T. boeoticum* KU-3620, and a wild tetraploid relative, *Aegilops cylindrica* KU-6953, were used in this study. The seeds were supplied by the National BioResource Project (NBRP)-Wheat, Japan (https://www.nbrp.jp), and plants were grown in a field at Kobe University (34˚43'N, 135˚13'E). Selfed seeds with 7 to 30 days after pollination (DPA) were ground in liquid nitrogen, and then the ground tissues were mixed with the 600 µL Homogenization buffer attached in the Promega kit or RNase-free phosphate-buffered saline (PBS) buffer (pH7.4, Thermo Fisher Scientific) including 20 µL/mL 1-thioglycerol. 200 µL Lysis buffer of the Promega kit was added to the 400 µL sample solution and mixed, and then the mixed solution was centrifuged at 14,000 g for 2 min. According to the protocol of the Maxwell RSC Plant RNA Kit, the samples were set to the Maxwell RSC Instrument (Promega) to start RNA purification. When the first seed sample amount was excessed, the magnetic beads were sometimes contaminated to the eluted RNA solution. After removing the magnetic beads, the RNA solution was purified using Plant Total RNA Extraction Miniprep System (VIOGEN, Taipei Hsien, Taiwan) as the occasion demands. The extracted total RNA was finally dissolved in 50 µL DEPC-treated water. The extracted RNA quality was estimated by NanoDrop 2000 (Thermo Fisher Scientific) and BioAnalyzer 2100 (Agilent Technology, Santa Clara, CA).

Excess seed samples were related to the high stickiness of the solution homogenized with the

RNA extraction buffer. Use of the PBS buffer instead of the Homogenization buffer greatly alleviated the stickiness. However, higher RIN values were obtained in the extracted RNAs using the Homogenization buffer than in those using the PBS buffer (Table 1). Presumably, use of the Homogenization buffer tended to induce the contamination of magnetic beads in the eluted RNA solution due to the high stickiness. The beads-contaminated RNAs could be more purified using the other RNA purification kit after removing the magnetic beads. Quality checking using BioAnalyzer 2100 showed that no damage in the RNA quality was observed by the additive purification step (Fig. 1). The RNA isolation protocol enables us to extract easily the high-quality RNA with ≥8.0 RIN value from developing grains of diploid and polyploid wheat and their relatives.

Reverse transcription (RT)-PCR analysis of the wheat *Cell Division Control Protein* (*CDCP*) gene, identified as the most stably expressed gene in different tissues (Paolacci et al. 2009), was performed using the RNA samples without adjustment of their concentrations, and first-strand cDNA was synthesized from DNase I-treated RNA samples with oligo-dT primers using the high fidelity ReverTra Ace reverse

Table 1. Quality check of the RNA isolated from wheat grains

Sample Name	DAP	Sample weight (mg)	Buffer*	A260/A280 ratio**	RNA conc. (ng/μL)***	RIN value***
Langdon	20	58	PBS	2.18	435.0	7.6
Langdon	20	116	PBS	2.17	708.0	7.4
Langdon	15	48	PBS	2.16	402.0	7.4
Langdon	15	96	PBS	2.19	1,543.0	7.6
KU-107-1	30	72	PBS	2.19	690.0	7.4
KU-107-1	30	144	PBS	2.17	5,991.0	7.3
KU-107-1	20	69	PBS	2.18	330.0	7.5
KU-107-1	20	138	PBS	2.17	190.8	7.0
Langdon	20	58	HB	2.10	130.6	7.8
Langdon[a]	20	116	HB	2.22	121.0	8.7
Langdon[a]	15	48	HB	2.23	76.1	8.5
Langdon[a]	15	96	HB	2.25	87.5	8.7
KU-107-1[a]	30	72	HB	2.23	103.6	8.7
KU-107-1[a]	30	144	HB	2.27	89.1	8.5
KU-107-1[a]	20	69	HB	2.22	63.1	8.9
KU-107-1[a]	20	138	HB	2.19	61.4	7.7
KU-3620	20	51	PBS	2.17	102.0	6.9
KU-3620	20	102	PBS	2.13	307.5	6.7
KU-3620	15	33	PBS	2.19	346.0	6.6
KU-3620	15	66	PBS	2.17	156.0	6.5
KU-3620	7	17	PBS	2.19	26.0	6.7
KU-3620	7	34	PBS	2.18	127.0	6.3
KU-6953	20	51	PBS	2.17	125.0	3.9
KU-6953	20	102	PBS	2.17	1,150.0	6.8
KU-3620	20	51	HB	1.99	406.0	9.9
KU-3620[a]	20	102	HB	2.19	79.8	8.8
KU-3620	15	33	HB	2.16	259.0	9.8
KU-3620	15	66	HB	2.06	432.0	9.7
KU-3620	7	17	HB	2.17	262.0	9.4
KU-3620	7	34	HB	2.18	302.0	9.4
KU-6953	20	51	HB	2.02	100.0	9.4
KU-6953[a]	20	102	HB	2.15	47.2	9.2

*PBS, 1x PBS buffer; HB, Homogenization buffer in the Promega kit

**Estimated by NanoDrop 2000

***Estimated by BioAnalyzer 2100

[a]The total RNA was purified with the VIOGEN kit after removing the magnet beads.

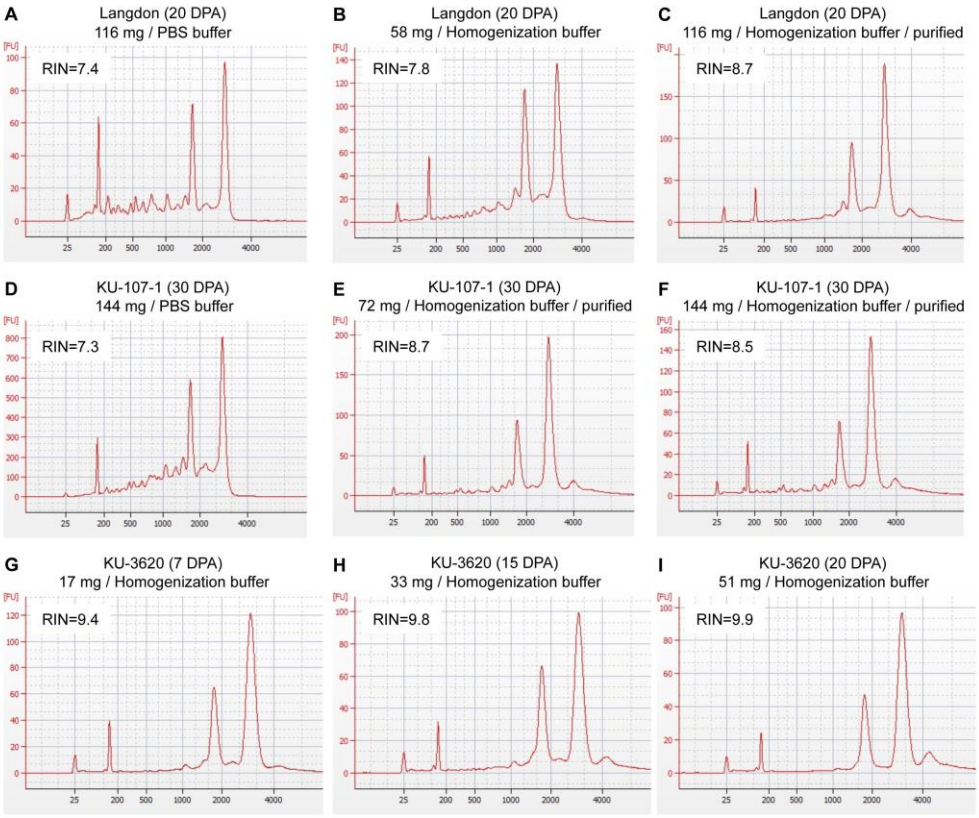

Figure 1. Quality check data of the isolated RNA samples by BioAnalyzer 2100.

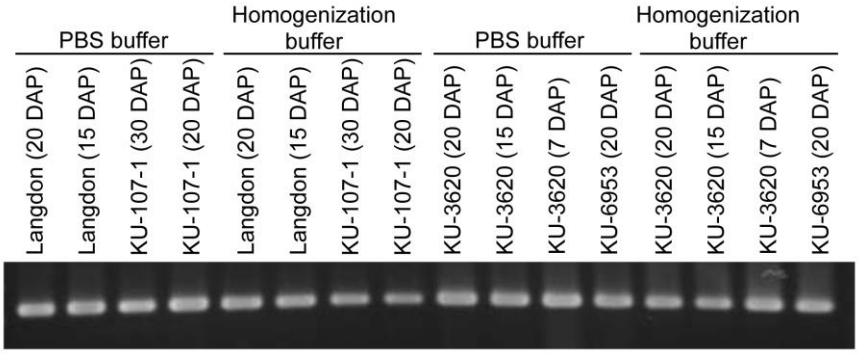

Figure 2. RT-PCR analysis of wheat *CDCP* gene expression using the RNA samples from grains.

transcriptase (Toyobo, Osaka, Japan). The gene-specific primer set for *CDCP*, which has been used as an internal control for wheat quantitative RT-PCR analyses (Paolacci et al. 2009; Rikiishi and Maekawa 2014; Iehisa and Takumi 2017), was 5'-CAAATACGCCATCAGGGAGAACATC-3' and 5'-CGCTGCCGAAACCACGAGAC-3'. The PCR condition was 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 20 s, 58°C for 30 s, and 68°C for 45 s, and then 1 cycle of 68°C for 1 min. The RT-PCR products were separated by electrophoresis through a 1.5% agarose gel and stained with ethidium bromide. The RT-PCR fragments for *CDCP* were clearly amplified in all of the RNA samples (Fig. 2). The high-quality RNA isolated from immature grains using the protocol conducted here can be applied to any transcriptome analysis and precise quantification of each transcript.

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References

Dong L, Liu H, Zhang J, Yang S, Kong G, Chu JSC, Chen N, Wang D (2015) Single-molecule real-time transcript sequencing facilitates common wheat genome annotation and grain transcriptome research. BMC Genomics 16: 1039.

Iehisa JCM, Takumi S (2017) Identification and evaluation of wheat reference genes for normalization of quantitative RT-PCR data during dehydration conditions. Wheat Inform Serv 124: eWIS124.1.

Kang GZ, Xu W, Liu GQ, Guo TC (2013) Comprehensive analysis of the transcription of starch synthesis genes and the transcription factor RSR1 in wheat (*Triticum aestivum*). Genome 56: 115-122.

Kawakami N, Miyake Y, Noda K (1992) Differential changes in levels of mRNAs during maturation of wheat seeds that are susceptible and resistant to preharvest-sprouting. Plant Cell Physiol 33: 511-517.

Nemeth C, Freeman J, Jones HD, Sparks C, Pellny TK, Wilkinson MD, Dunwell J, Andersson AAM, Aman P, Guillon F, Saulnier L, Mitchell RAC, Shewry PR (2010) Down-regulation of the *CSLF6* gene results in decreased (1,3;1,4)-ß-D-glucan in endosperm of wheat. Plant Physiol 152: 1209-1218.

Paolacci AR, Tanzarella OA, Porceddu E, Ciaffi M (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol Biol 10: 11.

Pellny TK, Lovegrove A, Freeman J, Tosi P, Love CG, Paul Knox J, Shewry PR, Mitchell AC (2012) Cell walls of developing wheat starchy endosperm: comparison of composition and RNA-seq transcriptome. Plant Physiol 158: 612-627.

Rikiishi K, Maekawa M (2014) Seed maturation regulators are related to the control of seed dormancy in wheat (*Triticum aestivum* L.). PLoS ONE 9: e107618.

Wan Y, Poole RL, Huttly AK, Toscano-Underwood C, Feeney K, Welham S, Gooding MJ, Mills C, Edwards KJ, Shewry PR, Mitchell RAC (2008) Transcriptome analysis of grain development in hexaploid wheat. BMC Genomics 9: 121.

Wan Y, Underwood C, Toole G, Skeggs P, Zhu T, Leverington M, Griffiths S, Wheeler T, Gooding M, Poole RL, Edwards KJ, Gezan S, Welham S, Snape J, Clare Mills EN, Mitchell RAC, Shewry PR (2009) A novel transcriptomic approach to identify candidate genes for grain quality traits in wheat. Plant Biotechnol J 7: 401-410.

Wheat Inf. Serv. 124: eWIS 124.3, 2017. www.shigen.nig.ac.jp/ewis

Others

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Journal articles:

Payne PI, Holt LM, Law CN (1981) Structural and genetical studies on the high molecular weight subunits of wheat glutenin. Theor Appl Genet 60:229-236.

Book chapters:

Peacock WJ, Dennis ES, Gerlach WJ (1981) Molecular aspects of wheat evolution:

repeated DNA sequences. In: Evans LT and Peacock WJ (eds.) Wheat Science - Today and Tomorrow. Cambridge Univ. Press, Cambridge, UK, pp. 41-60.

Books:

Knott DR (1989) The Wheat Rusts - Breeding for Rust Resistance. Springer-Verlag, New York, USA.

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Nomenclature

Nomenclature of genes and chromosomes should follow the 'Catalogue of gene symbols for wheat' (McIntosh et al.: 10th Int. Wheat Genet. Symp. 2003).

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