

Proteomic analysis of temperature dependency of buckwheat seed dormancy and quality degradation

Akiko Hashiguchi · Hiroyuki Yoshioka · Setsuko Komatsu

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Abstract Buckwheat is a functional staple food, which is rich in rutin and other flavonoids with strong antioxidant potential. However, the quality of buckwheat grain is easy to degrade during storage and low temperature is better to keep the flavor and texture. We tested the hypothesis that the quality degradation is related to seed dormancy which is lost at a rate in temperature-dependent manner in most seeds. To understand physiological processes in seeds under temperature stress, which affects quality of stored buckwheat grain, a gel-free/label-free proteomic analysis was conducted. Compared to the seeds before storage, a total of 30, 76, 52, 14, and 61 proteins were affected at 5 °C for 5 months, 5 °C for 10 months, 15 °C for 5 months, 15 °C for 10 months, and 25 °C

for 5 months, respectively. Functional classification revealed that storage condition at 5 °C for 5 months resulted in different protein profile from other conditions and suitable for keeping best condition. Storage at higher temperature for longer time influenced proteins related to preparation for emergence by carbohydrate metabolism, endogenous gene expression, and protein homeostasis. Proteins such as granule-bound starch synthase and 13S seed storage protein consistently decreased in abundance during storage. These results suggest that stored buckwheat seeds experience seed dormancy release, but the processes are not clear in relation to temperature rise. Dormancy break is recognized but emergence is inhibited by abscisic acid, which was induced by temperature stress, showing the complexity of buckwheat germination control. The quality degradation of stored seeds might be due to changed starch composition and protein network.

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A. Hashiguchi
Faculty of Medicine, University of Tsukuba,
Tsukuba 305-8572, Japan

H. Yoshioka (✉)
Development Head Office, Kumamoto Flour Milling Co.
Ltd, Hanazono 1-25-1, Kumamoto 860-8625, Japan
e-mail: h-yoshioka@bears-k.co.jp

S. Komatsu (✉)
Faculty of Life and Environmental Sciences, University of
Tsukuba, Tennodai 1-1-1, Tsukuba 305-8572, Japan
e-mail: komatsu.setsuko.fu@u.tsukuba.ac.jp

Keywords Proteomic analysis · Buckwheat · Storage · Temperature stress · Seed dormancy

Abbreviations

LC	Liquid chromatography
MS	Mass spectrometry
CHO	Carbohydrate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
FBPA	Fructose-bisphosphate aldolase

HSP Heat shock protein

1 Introduction

Buckwheat is a widely grown annual crop around the world and is recognized as a type of functional food ingredient due to its potential health effects (Dietrich-Szostak and Oleszek 1999). Information on the phenolic composition, antioxidant capacity, and gluten-free character of buckwheat made the species being widely accepted as suitable food for a range of nutritional applications (Inglett et al. 2009; Torbica et al. 2012). However, it is known that buckwheat grain is easy to diminish quality in storage period. Low storage temperatures are suitable for preserving the flavor of the buckwheat flour, preventing increase of bitterness caused by storage and keeping the flour texture soft compared to higher temperature conditions (Kawakami et al. 2009a).

Seeds stop development and enter a dormant state to sustain extended periods of unfavourable conditions (Baskin and Baskin 1998). Seed dormancy is a complex trait that controls the timing of seed germination and are regulated by many environmental cues (Baskin and Baskin 1998). Factors as temperature, humidity, light, and nutrition are involved in the regulation of seed dormancy. Different signal transduction pathways including abscisic acid (ABA) and gibberellins (GAs) control seed dormancy antagonistically (Shu et al. 2016). The long-term preservation of seeds is one of the most important issues in agricultural production to guarantee food security. Therefore, monitoring the seed dormancy state by understanding the environmental signal perception and biological processes occurring in stored seeds is indispensable.

Buckwheat is known as a species of weak dormancy with risks of pre-harvest sprouting (Hara et al. 2009). Interestingly, numerous agroecotypes have been identified within buckwheat species including summer, autumn, and intermediate that allow long seeding time (Iwata et al. 2005). Recently, a new buckwheat cultivar has been developed for spring seedling as well (Hara et al. 2009). It is interesting that relatively wide range of temperature conditions can allow buckwheat germination while seed dormancy is strictly regulated in many species to ensure uniform emergence in a certain season. The question is that the

temperature- and time-dependent quality degradation in stored buckwheat grains is correlated with release of buckwheat seed dormancy which can be induced by a wide range of temperature. Here, comparison of thermal responses of buckwheat seeds was attempted with the intention to understand whether seed dormancy control is temperature-dependent in buckwheat. Gel-free/label-free proteomic technique as applied to obtain screenshot of physiology of buckwheat seeds in various conditions were used. Differences between each storage condition was analyzed, and proteins that are related to seed dormancy and factors that might be involved in impaired seed quality are discussed.

2 Material and methods

2.1 Material and treatments

Buckwheat (*Fagopyrum esculentum* Moench) cultivar Mancan grown in Chifeng, Inner Mongolia, China was purchased from Kumamoto Flour Milling Co. Ltd. (Kumamoto, Japan). After separation of seed coat, they were preserved at 5, 15, and 25 °C for 5, and 10 months and ground into flour for protein extraction. For control, seed were ground into flour and stored at −20 °C, which is referred as baseline-starting point sample. For all proteomic analyses, three independent experiments were performed as biological replicates.

2.2 Protein extraction and enrichment

The powder (100 mg) of each sample was added to a solution of 10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone, and the resulting suspension was thoroughly mixed by vortexing. The mixture was sonicated for 10 min and incubated for 1 h at −20 °C. After centrifugation at 9000×g for 20 min at 4 °C, the supernatant was removed and the remaining pellet was washed twice with 0.07% 2-mercaptoethanol in acetone. The pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and resuspended in lysis buffer containing 8 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine by vigorous vortexing for 1 h. The suspension was centrifuged at 20,000×g for 20 min and the resulting supernatant was collected as the protein extract (Komatsu et al. 2013). Extracted

proteins (100 µg) in lysis buffer were adjusted to a final volume of 100 µL, and mixed with 400 µL of methanol to each sample before addition of 100 µL of chloroform and 300 µL of water. After mixing and centrifugation at $20,000\times g$ for 10 min to achieve phase separation, the upper phase was discarded. Methanol (300 µL) was added to the lower phase and the sample was centrifuged at $20,000\times g$ for 10 min. The pellet was collected as the soluble fraction (Komatsu et al. 2013).

2.3 Protein reduction, alkylation, and digestion for proteomic analysis

Proteins were resuspended in 50 mM NH_4HCO_3 , reduced with 50 mM dithiothreitol for 30 min at 56 °C, and alkylated with 50 mM iodoacetamide for 30 min at 37 °C in the dark. Alkylated proteins were digested with trypsin and lysyl endopeptidase (Wako, Osaka, Japan) at a 1:100 enzyme/protein ratio for 16 h at 37 °C. Peptides were acidified with formic acid ($\text{pH} < 3$) and analyzed by nanoLC-MS/MS.

2.4 Measurement of protein and peptide concentrations

The method of Bradford (Bradford 1976) was used to determine the protein concentration with bovine serum albumin used as the standard. A Direct Detect Spectrometer (Millipore, Billerica, MA, USA) equipped with the Direct Detect software (version 3.0.25.0) was used to determine the peptide concentration.

2.5 Protein identification using nanoLC-MS/MS

The peptides were loaded onto the LC system (Ultimate 3000 nanoLC; Dionex, Germering, Germany) equipped with a trap column (C18 PepMap, 300 µm ID $\times$ 5 mm; Dionex) equilibrated with 0.1% formic acid and eluted with a linear acetonitrile gradient in 0.1% formic acid at a flow rate of 200 nL/min. The eluted peptides were loaded and separated on the column (C18 capillary tip column, 75 µm ID $\times$ 120 mm; Nikkyo Technos, Tokyo, Japan) with a spray voltage of 1.5 kV. The peptide ions were detected using MS system (nanospray LTQ Orbitrap Discovery MS; Thermo Fisher Scientific, San Jose, CA, USA) in the data-dependent acquisition mode

with the installed Xcalibur software (version 2.1; Thermo Fisher Scientific). Full-scan mass spectra were acquired in the MS over 400–1500 m/z with resolution of 30,000. A lock mass function was used to obtain high mass accuracy (Olsen et al. 2005). The ten most intense precursor ions were selected for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35%. Dynamic exclusion was employed within 90 s to prevent repetitive selection of peptides (Zhang et al. 2009).

2.6 MS data analysis

Identification of proteins was performed using the Mascot search engine (version 2.5.1; Matrix Science, London, UK) and the UniProt KB/Swiss-Prot database (<http://www.uniprot.org/uniprot>). Proteome Discoverer software (version 1.4.0.288; Thermo Fisher Scientific) was used to process the acquired raw data files. For the Mascot search, the carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Peptide mass tolerance was set at 10 ppm, fragment mass tolerance was set at 0.8 Da, and peptide charges were set at + 2, + 3, and + 4. An automatic decoy database search was performed as part of the search. Mascot results were filtered with the Percolator function to improve the accuracy and sensitivity of peptide identification.

2.7 Calculation of protein abundance using MS data

Abundance of the identified proteins was analyzed on the basis of exponentially modified protein abundance index (emPAI) values. Briefly, the emPAI value of each identified protein was divided by the sum of the emPAI values for all identified proteins and multiplied by 100. Protein content was estimated as the molar fraction percentage (mol%) (Ishihama et al. 2005).

2.8 Differential analysis of proteins using MS data

For differential analysis of the relative abundances of peptides and proteins between samples, the commercial label-free quantification package SIEVE (version 2.1.377; Thermo Fisher Scientific) was used. The chromatographic peaks detected by MS were aligned,

and the peptide peaks were detected as a frame on all parent ions scanned by MS/MS using 5 min of frame time width and 10 ppm of frame m/z width. Chromatographic peak areas within a frame were compared for each sample, and the ratios between samples were determined for each frame. The frames detected in the MS/MS scan were matched to the imported Mascot results. The peptide ratios between samples were determined from the variance-weighted average of the ratios in frames that matched the peptides in the MS/MS spectrum. The ratios of peptides were further integrated to determine the ratios of the corresponding proteins. In the differential analysis of protein abundance, total ion current was used for normalization. The minimum requirement for identification of a protein was two matched peptides. Significant changes in the abundance of proteins between samples were determined ($p < 0.05$).

2.9 Functional categorization

Functional categorization of identified proteins was performed using MapMan bin ontology (<http://mapman.gabipd.org/>) using Mercator Automated Sequence Annotation Pipeline (Lohse et al. 2014).

3 Results

3.1 Similarities of seed physiological status among storage conditions

To capture the changes in physiological status of stored buckwheat seeds, proteomic technique was used. Seeds were stored at 5, 15, and 25 °C for 0, 5, and 10 months, respectively, and proteins were extracted from floured seeds of seven conditions (Fig. 1a). After nanoLC-MS/MS analysis, protein abundance was compared to baseline sample of starting point. The proteins, which exhibited significantly different abundance ($p < 0.05$), were selected as proteins that characterize physiological status of buckwheat seeds at each condition. Compared to the seeds before storage, a total of 30, 76, 52, 14, and 61 proteins were affected at 5 °C for 5 months, 5 °C for 10 months, 15 °C for 5 months, 15 °C for 10 months, and 25 °C for 5 months, respectively (Supplemental Tables 1, 2, 3, 4, and 5). MS identification of proteins extracted from seeds stored at 25 °C for 10 months

were difficult by sample clogging in LS columns possibly due to carbohydrate and protein degradation during storage. Functional classification of the differentially abundant proteins was performed using MapMan bin ontology (Lohse et al. 2014). The abundance of 30 proteins identified in seeds stored at 5 °C for 5 months was functionally categorized as being predominantly involved in stress (30%), CHO metabolism (27%), and development (13%) (Fig. 1b). On the other hand, in seeds stored for longer time or in higher temperature, the dominance of glycolysis and RNA categories was noticed. The number of glycolysis-related proteins were 17, 16, and 19 proteins in 5 °C for 10 months, 15 °C for 5 months, and 25 °C for 5 months, respectively (Fig. 1b).

3.2 Acceleration of physiological change by temperature rise

To gain a better understanding of the biological processes that are altered by seed storage at various duration and temperature, proteins were grouped into those increase or decrease in abundance, compared to the starting point (Fig. 2). Limited number of increased proteins made it difficult to understand the molecular process of physiological change in buckwheat seeds (Fig. 2a), but proteins involved in stress were noted as commonly detected within increased proteins in each time point (Fig. 2a). On the other hand, there were relatively larger number of decreased proteins (Fig. 2b). Dominant functional categories were stress, CHO metabolism, and development in the condition of 5 °C for 5 months (Fig. 2b). The ranking order turned into as stress, glycolysis, and CHO metabolism, in 5 °C for 10 months (Fig. 2b). Interestingly, same categories were also observed in 15 °C for 5 months, suggesting that seeds stored in 5 °C for 10 months and 15 °C for 5 months were in similar physiological status (Fig. 2b).

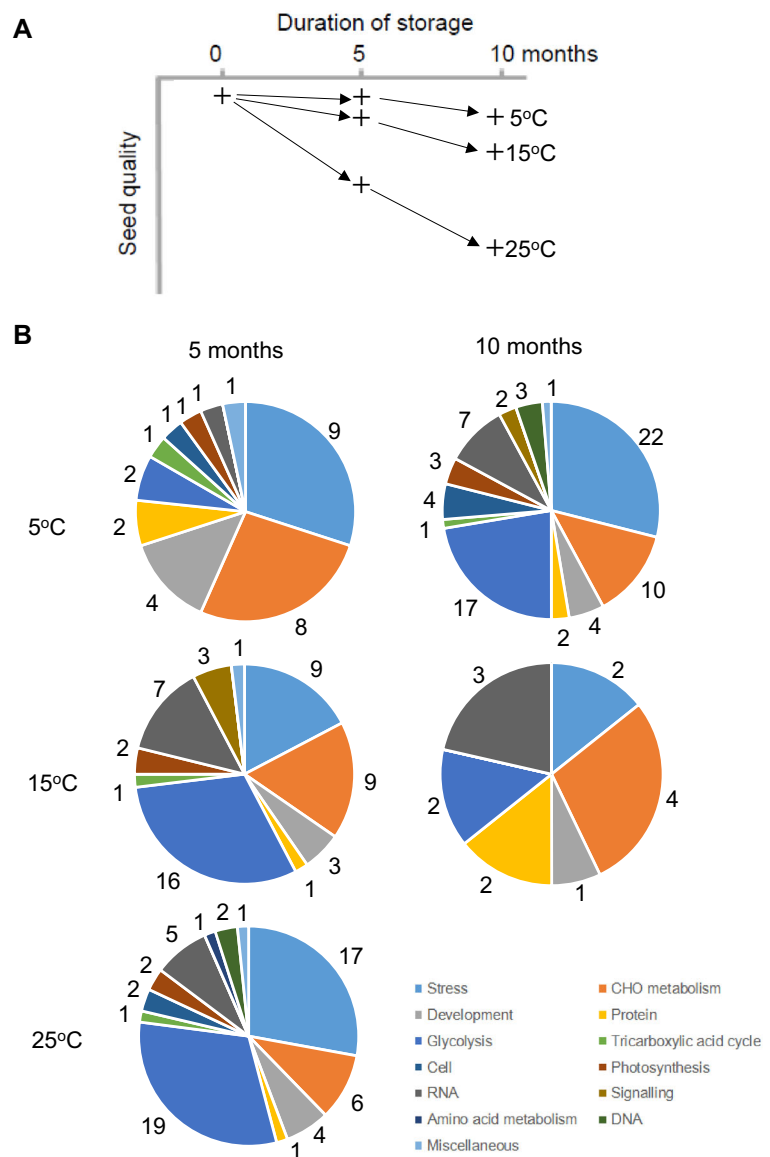
3.3 Molecular processes that progress in stored buckwheat seeds

The unique proteins in each condition were shown in Table 1. CHO metabolism and/or glycolysis-related proteins were found in all conditions whereas signal and stress-related proteins were detected in conditions of 15 °C for 5 month and 5 °C for 10 month (Table 1). Some proteins were detected under specific

Fig. 1 Proteins identified in buckwheat seeds stored in various conditions.

a Experimental design for the proteomic analysis of buckwheat seeds. Dehulled seeds were stored at 5, 15, and 25 °C for 0, 5, and 10 months. At each storage temperature and duration, seeds were grind into flour. Proteins were extracted, digested, and analyzed using nanoLC-MS/MS.

b Functional categorization of the identified proteins from buckwheat seeds stored in various conditions. Functional categorization of the identified proteins was performed using MapMan bin ontology. The numbers in the pie graph indicate the number of proteins in each category

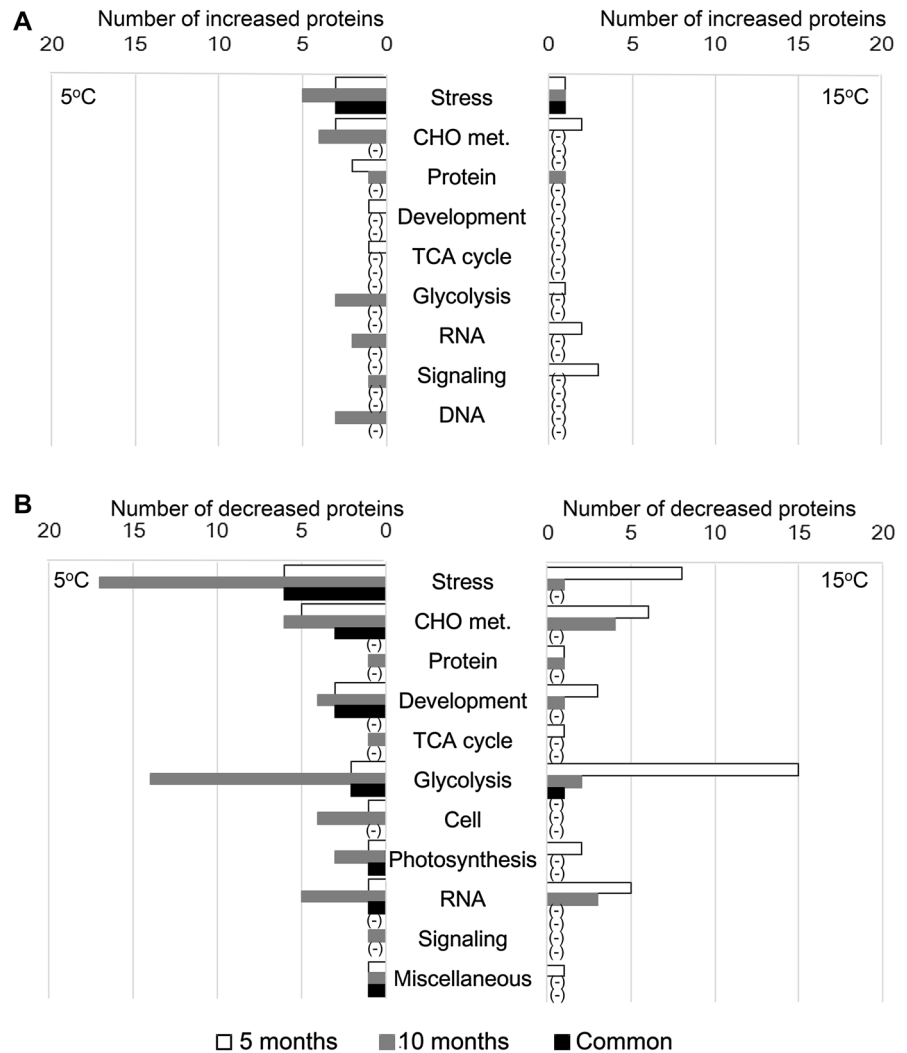


conditions, including RuBisCO large subunit binding protein in seeds stored at 15 °C for 5 month; aspartate aminotransferase in seeds stored at 25 °C for 5 month; and ABA-induced in somatic embryos 3 in seeds stored at 15 °C for 10 month (Table 1). Histones were detected in seeds stored under conditions of 25 °C for 5 month and 5 °C for 10 month (Table 1). Proteins observed in 15 °C storage conditions included chloroplastic proteins (Table 1).

To understand the degradation process in detail, proteins that showed coherent trend in abundance change throughout time course were selected

(Table 2). Sugar metabolizing enzymes were listed including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose-bisphosphate aldolase (FBPA) as well as granule-bound starch synthase. The 13S globulin, which is a major seed storage protein consisted part of proteinous component in seeds (Nair and Adachi 1999), was decreased (Table 2). The change in heat shock protein (HSP) was significant between starting point and 5 months (Table 2). In addition, the proteins identified in temperature-dependent comparison are shown, identifying granule-bound starch synthase alone (Table 3). The abundance

Fig. 2 Time-course change of protein profile of differentially abundant proteins in stored buckwheat seeds in various conditions. Proteins were grouped by abundance change trend, and functional classification was performed using MapMan bin ontology. **a** The change in functional distribution of differentially increased proteins in buckwheat seeds stored at 5 °C and 15 °C. **b** The change in functional distribution of differentially decreased proteins in buckwheat seeds stored at 5 and 15 °C. “met” means “metabolism”



of α -glucosidase significantly decreased in storage condition at 25 °C, although its level was at the same extent as in storage conditions at 5 and 15 °C (Table 3).

4 Discussion

In this study, a gel-free/label-free proteomic analysis was conducted to understand physiological processes occurring in the stored buckwheat seeds that may impact seed quality. Low temperature is suit for storing dehulled seeds used in commercial flour production (Kamiyama et al. 2015; Kawakami et al. 2009a, b), but the mechanism by which low

temperature keeps quality is not clarified. Functional classification of proteins in each storage temperature and time indicated that storage at 5 °C for 5 months alone showed distinguished protein profile from other conditions (Fig. 1). The result indicated that seeds stored in condition of 5 °C for 5 months kept better quality of buckwheat seeds than other conditions. Upper limit temperature and duration for buckwheat storage was visualized that the stored grains can be consumed within 5 months in best quality at 5 °C (Figs. 1, 2).

Comparison of specifically identified proteins in five conditions indicated that glycolysis become active in higher temperature in seeds stored for 5 months (Table 1). When the environmental conditions are

Table 1 Unique proteins in each condition

Protein ID ^a	Gene symbol	Description	M.P. ^b	Abundance ^c	S.D.	<i>p</i> -value ^d	Function ^e
5 °C-5 months							
O65314	–	ACT_SCHDU Actin OS	2	0.137	0.017	0.048	Cell
P49037	LOC543961	SUSY_SOLLC Sucrose synthase OS	2	3.948	2.251	0.015	CHO metabolism
P42036	RPS14C	RS143_ARATH 40S ribosomal protein S14_3 OS	2	2.309	0.804	0.033	Protein
P17783	MMDH	MDHM_CITLA Malate dehydrogenase_mitochondrial OS	2	1.700	0.015	0.000	TCA / org transformation
15 °C-5 months							
Q75KR1	PPDK2	PPDK2_ORYSJ Pyruvate_phosphate dikinase 2 OS	2	2.339	1.473	0.034	CHO metabolism
P12782	–	PGKH_WHEAT Phosphoglycerate kinase_chloroplastic OS	2	0.688	0.133	0.025	Glycolysis
P26301	ENO1	ENO1_MAIZE Enolase 1 OS	3	0.760	0.074	0.001	Glycolysis
P26519	GAPC	G3PC_PETCR Glyceraldehyde_3_phosphate dehydrogenase_cytosolic OS	2	0.978	0.061	0.010	Glycolysis
P08927	–	RUBB_PEA RuBisCO large subunit_binding protein subunit beta_chloroplastic OS	2	0.339	0.088	0.002	PS
P50345	–	RLA0_LUPLU 60S acidic ribosomal protein P0 OS	2	0.788	0.107	0.028	RNA
P50346	LOC547816	RLA0_SOYBN 60S acidic ribosomal protein P0 OS	2	3.068	1.148	0.027	RNA
O49995	LOC107787833	1433B_TOBAC 14_3_3_like protein B OS	2	2.057	1.021	0.027	Signalling
P29308	–	1433_SPIOL 14_3_3_like protein (Fragment) OS	3	2.108	1.019	0.016	Signalling
P93209	TFT3	14333_SOLLC 14_3_3 protein 3 OS	2	4.937	3.543	0.008	Signalling
Q02028	HSP70	HSP7S_PEA Stromal 70 kDa heat shock_related protein_chloroplastic OS	2	0.775	0.057	0.010	Stress
25 °C-5 months							
P26563	–	AATM_LUPAN Aspartate aminotransferase P2_mitochondrial (Fragment) OS	2	1.181	0.448	0.044	Amino acid metabolism
P30164	–	ACT1_PEA Actin_1 OS	3	0.583	0.077	0.033	Cell
P50566	H4-I; H4-III; H4-II	H4_CHLRE Histone H4 OS	4	1.065	0.288	0.047	DNA
P08735	GAPC1	G3PC1_MAIZE Glyceraldehyde_3_phosphate dehydrogenase 1_cytosolic OS	2	0.532	0.068	0.004	Glycolysis
P26518	GAPC	G3PC_MAGLI Glyceraldehyde_3_phosphate dehydrogenase_cytosolic OS	3	0.406	0.075	0.002	Glycolysis
P26520	GAPC	G3PC_PETHY Glyceraldehyde_3_phosphate dehydrogenase_cytosolic OS	3	0.564	0.058	0.007	Glycolysis
P34922	GAPC1	G3PC_PEA Glyceraldehyde_3_phosphate dehydrogenase_cytosolic OS	3	0.536	0.067	0.014	Glycolysis

Table 1 continued

Protein ID ^a	Gene symbol	Description	M.P. ^b	Abundance ^c	S.D.	<i>p</i> -value ^d	Function ^e
P50883	RPL12A	RL121_ARATH 60S ribosomal protein L12_1 OS	2	0.493	0.078	0.031	RNA
Q39043	MED37F	MD37F_ARATH Mediator of RNA polymerase II transcription subunit 37f OS	4	0.451	0.047	0.012	RNA
P02519	HSP17.3-B	HSP11_SOYBN 17.3 kDa class I heat shock protein OS	2	0.557	0.072	0.024	Stress
5 °C-10 months							
A2XLF2	ACT1	ACT1_ORYSI Actin_1 OS	2	0.743	0.162	0.013	Cell
P53492	ACT7	ACT7_ARATH Actin_7 OS	3	0.239	0.055	0.020	Cell
Q96482	–	ACT1_SOLLC Actin_41 (Fragment) OS	3	0.722	0.135	0.019	Cell
P49034	SUS1	SUSY_ALNGL Sucrose synthase OS	2	1.798	0.223	0.021	CHO metabolism
P55228	APS1	GLGS_ARATH Glucose_1_phosphate adenylyltransferase small subunit_chloroplastic OS	3	1.022	0.037	0.002	CHO metabolism
P85945	–	H4_PSEMZ Histone H4 (Fragments) OS	2	1.093	0.095	0.038	DNA
Q41811	H4	H43_MAIZE Histone H4.3 OS	3	1.103	0.095	0.024	DNA
Q0J8A4	GAPC1	G3PC1_ORYSJ Glyceraldehyde_3_phosphate dehydrogenase 1_cytosolic OS	3	0.570	0.040	0.019	Glycolysis
Q9LD57	PGK1	PGKH1_ARATH Phosphoglycerate kinase 1_chloroplastic OS	3	0.762	0.058	0.034	Glycolysis
Q9SM60	PGM1	PGMC_PEA Phosphoglucomutase_cytoplasmic OS	2	1.571	0.125	0.000	Glycolysis
P29197	CPN60	CH60A_ARATH Chaperonin CPN60_mitochondrial OS	2	2.448	0.900	0.042	Protein
O65731	RPS5	RS5_CICAR 40S ribosomal protein S5 (Fragment) OS	2	0.979	0.107	0.009	RNA
P41916	RAN1	RAN1_ARATH GTP_binding nuclear protein Ran_1 OS	2	0.413	0.067	0.030	Signalling
P46077	GRF4	14334_ARATH 14_3_3_like protein GF14 phi OS	2	2.443	1.119	0.022	Signalling
P24067	BIPE2	BIP2_MAIZE Luminal_binding protein 2 OS	4	0.644	0.034	0.003	Stress
P31170	HSP25.3	HS25P_ARATH 25.3 kDa heat shock protein_chloroplastic OS	2	0.423	0.102	0.014	Stress
Q943E6	HSP16.9B	HS16B_ORYSJ 16.9 kDa class I heat shock protein 2 OS	2	0.710	0.142	0.005	Stress
15 °C-10 months							
O23404	PPDK	PPDK1_ARATH Pyruvate_phosphate dikinase 1_chloroplastic OS	3	1.458	0.095	0.038	CHO metabolism
P13708	SS	SUSY_SOYBN Sucrose synthase OS	5	1.945	0.390	0.050	CHO metabolism
P22221	PPDK	PPDK_FLATR Pyruvate_phosphate dikinase_chloroplastic OS	3	1.482	0.102	0.042	CHO metabolism
Q5KTS7	CAISE3	EML_DAUCA Carrot ABA_induced in somatic embryos 3 OS	2	4.815	2.674	0.021	Development

Table 1 continued

Protein ID ^a	Gene symbol	Description	M.P. ^b	Abundance ^c	S.D.	<i>p</i> -value ^d	Function ^e
P42791	RPL18B	RL182_ARATH 60S ribosomal protein L18_2 OS	2	3.193	0.408	0.048	RNA
Q93VH9	RPS4A	RS41_ARATH 40S ribosomal protein S4_1 OS=	3	2.283	0.808	0.006	RNA

Protein abundance was calculated by SIEVE software and compared to the starting point sample. Proteins that showed significant change at were selected as differentially abundant proteins

^aProtein ID, according to the Uniprot database

^bM.P., number of matched peptides

^cRelative abundance of protein compared to starting point. The value was rounded off to three decimal points

^d*p* value, compared to starting point

^eFunction, function categorized using MapMan bin ontology

satisfied for germination, seeds degrade stored carbohydrate, produce sucrose, and initiate imbibition as well as tissue cell respiration using sucrose derived from the stored carbohydrate (Ferreira Cda et al. 2009). Here we identified chloroplast proteins including RuBisCO large subunit binding protein in 15 °C conditions. These results suggest that the stored seed might be in the process of seed dormancy break. In addition to restoring primary metabolism, seeds must synthesize and protect proteins for proper establishment of plant body after dormancy release. However, no clear correlation between storage temperature and the progress in sprouting process was obtained. Vegis (1964) proposed the idea that the range of environmental conditions permissive for seed germination extends as seed dormancy level declines. Also, a simulation model by Vleeshouwers and Bouwmeester (2001) indicated that a range of temperatures over which germination can occur changes seasonally and is widened in a certain season. Germination starts when temperatures in soil meet the range of germination-permissive temperatures of the seeds. Proteomic results suggest that environmental factors other than temperature might have strong influence on dormancy release in buckwheat seeds. We identified proteins related to protein translation and ABA-induced in somatic embryo 3 protein which belongs to small hydrophilic plant seed protein (Shiota et al. 2004). ABA is a phytohormone involved in abiotic stress responses (Gomez-Cadenas et al. 2015). ABA was shown to inhibit germination but not dormancy release

in imbibed seeds in ryegrass (Goggin et al. 2009). Taken together, temperature stress acts as environmental abiotic cue for buckwheat seeds, which are known as a species of weak dormancy, to induce seed dormancy perturbation possibly with other environmental factors. ABA acts to protect seed proteins from degradation which could be caused by the temperature stress. But seed dormancy break by abiotic stresses does not always promote germination, because ABA inhibits germination. The results presented here indicate the complexity of buckwheat seed dormancy control and suggest that further analysis on sensitivity to temperature and ABA are desired to tackle with pre-harvest sprouting of buckwheat.

To identify events that is related to seed quality degradation such as flavor and texture, proteins that showed consistent trend in abundance over time were analyzed. The change in abundance of granule-bound starch synthase might reflect the ABA-mediated sucrose mobilization (Tables 2, 3). On the basis of current data (Fig. 3) and previous knowledge on quality degradation of buckwheat seeds, it is hypothesized as follows: the impaired texture caused by longer storage time and higher temperature is mainly due to modified starch composition. Meanwhile, change in protein network influences the texture when buckwheat flour is processed into noodles (Guo et al. 2017). The change in 13S globulin, which accounts for 43% of whole seed protein in buckwheat (Nair and Adachi 1999), along with changes in protein folding chaperone RPP0A and HSP, indicate that protein

Table 2 Proteins that showed consistent trend in time-dependent manner during storage at 5 °C

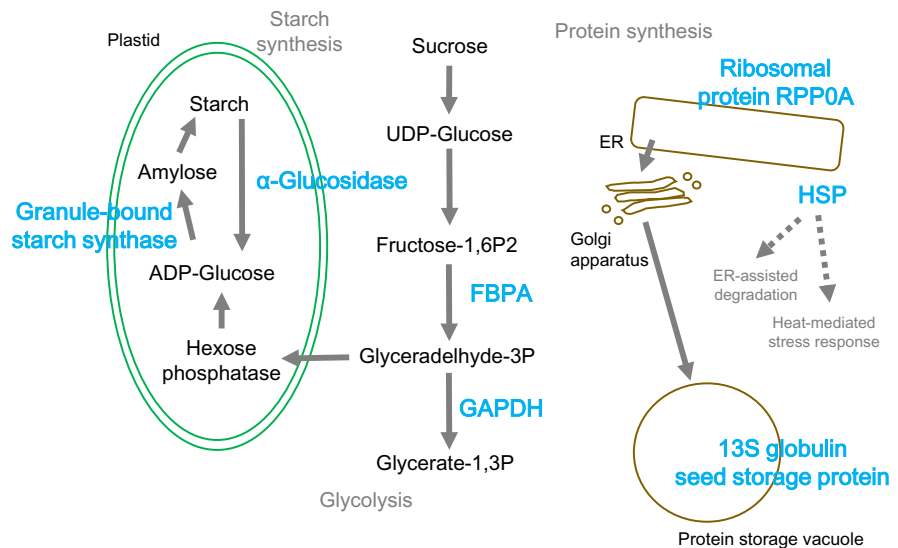
No.	Protein ID ^a	Gene symbol	Description	M.P. ^b	5 months ^c		10 months ^c		Fold ^e	<i>p</i> -value ^f	Function ^g
Downward-trend											
1	P34921	GAPDH	Glyceraldehyde_3_phosphate dehydrogenase_cytosolic	4	0.559	0.035	0.165	0.015	0.30*	0.0001	Glycolysis
2	P08440	FBPA	Fructose_bisphosphate aldolase_cytoplasmic isozyme	3	0.205	0.038	0.089	0.024	0.43*	0.0109	Photo system
3	Q43247	GAPDH3	Glyceraldehyde_3_phosphate dehydrogenase 3_cytosolic	3	0.892	0.059	0.547	0.042	0.61*	0.0012	Glycolysis
4	Q7FAH2	GAPDH2	Glyceraldehyde_3_phosphate dehydrogenase 2_cytosolic	5	0.887	0.041	0.604	0.039	0.68*	0.0010	Glycolysis
5	O23880	FA18	13S globulin seed storage protein 2	24	0.653	0.008	0.532	0.007	0.81*	0.0000	Development
6	O04893	–	Alpha_Glucosidase	2	0.680	0.047	0.559	0.034	0.82*	0.0229	Miscellaneous
7	Q43784	WAXY	Granule_bound starch synthase 1_chloroplastic/amyloplastic	2	0.967	0.035	0.820	0.063	0.85*	0.0245	CHO metabolism
8	O23878	FA02	13S globulin seed storage protein 1	28	0.617	0.008	0.541	0.007	0.88*	0.0002	Development
9	O04204	RPP0A	60S acidic ribosomal protein P0_1	2	0.938	0.049	0.845	0.031	0.90*	0.0485	RNA
10	Q943E6	HSP16.9B	16.9 kDa class I heat shock protein 2	2	0.993	0.183	0.673	0.120	0.68	0.0643	Stress

^aUniprot Accession, according to the UniProt KB/Swiss-Prot database^bM.P., number of matched peptide, the proteins with > 2 matched peptides were considered^cMonths after storage^dRatio, compared to baseline in starting point before storage by SIEVE software. Proteins that satisfy the following conditions were selected; (1) proteins that significantly changed compared to baseline at both time points of 5 month (M) and 10 M. (2) the change is larger than 10 M than 5 M^eFold change of protein abundance between 10 M compared to 5 M^f*p*-value, between 5 and 10 M. **p* < 0.05^gFunctional annotation retrieved from MapMan bin ontology

Table 3 Proteins that showed decreasing trend in temperature-dependent manner at 5 months after storage

No.	Protein ID ^a	Gene symbol	Description	M.P. ^b	5 °C		15 °C		25 °C		<i>p</i> -value ^d (5–15 °C)	<i>p</i> -value ^e (15–25 °C)
					Ratio ^c	S.D.	Ratio ^c	S.D.	Ratio ^c	S.D.		
Consistently decreasing												
1	P04713	WAXY	Granule bound starch synthase 1 chloroplastic/ amyloplastic	3	0.885	0.063	0.734	0.041	0.500	0.035	0.025*	0.007*
Decreasing at 25 °C												
2	O04893	FA02	Alpha glucosidase	2	0.648	0.070	0.777	0.077	0.380	0.079	0.099	0.003*

*Statistically significant between indicated temperature

^aUniprot Accession, according to the UniProt KB/Swiss-Prot database database^bM.P., number of matched peptide, the proteins with > 2 matched peptides were considered^cRatio, compared to baseline in starting point before storage by SIEVE software. Three conditions were separately analyzed by SIEVE and collated. Proteins that satisfy the following conditions were selected; (1) proteins that significantly changed compared to baseline at each temperature, and (2) the range of change is larger in later stage^d*p*-value, between indicated temperature^eFunctional annotation retrieved from MapMan bin ontology**Fig. 3** Schematic representation of underlying physiological change during storage of buckwheat seeds. Based on the result from proteomic analysis, proteins that showed consistent trend in abundance change along with extension of storage time as well as increased in storage temperature (Tables 2, 3). Blue letters indicate decreasing changes in protein abundance

homeostasis was disrupted during storage (Fig. 3). The decrease in 13S globulin might induce undesirable characteristics of flour prepared from stored seeds. Interestingly, 13S globulin is a prevalent allergen of buckwheat whose isoforms without specific tandem repeats are resistant to trypsin digestion (Khan et al. 2012; Sano et al. 2014). Detailed identification of 13S globulin isoforms using recently available buckwheat genome sequence will add information on the change in protein network during

by storage. Mechanisms for temperature-dependent quality degradation awaits for further analysis.

Taken together, current results indicate that buckwheat seeds in storage are not in complete quiescence but easily start germination. Temperature is not sole controlling factor of germination, and antagonistic regulation by ABA exists. In regard to seed quality degradation, starch metabolism and protein synthesis along with germination is responsive for undesirable characteristics and impaired texture of buckwheat flour made from stored buckwheat.

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Compliance with ethical standards

Conflict of interest All of the authors declare no conflict of interest.

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Supplemental Table 1. List of differentially abundant proteins identified in storage condition of 5°C for 5 months.

No.	Protein ID ^a	Gene symbol	Description	M.P. ^b	Abundance ^c	S.D.	<i>p</i> -value ^d	Function ^e
1	P51418	RPL18AB	R18A2_ARATH 60S ribosomal protein L18a_2 OS	2	5.059	2.631	0.007	protein
2	P49037	LOC543961	SUSY_SOLLC Sucrose synthase OS	2	3.948	2.251	0.015	CHO metabolism
3	P86794	-	ITR2C_FAGES Trypsin inhibitor 2c OS	4	3.091	0.943	0.025	stress
4	Q5KTS5	CAISE5	GRDH_DAUCA Glucose and ribitol dehydrogenase OS	2	2.788	0.112	0.012	CHO metabolism
5	I1JLC8	SLE2	SLE2_SOYBN Protein SLE2 OS	4	2.451	0.543	0.019	development
6	P42036	RPS14C	RS143_ARATH 40S ribosomal protein S14_3 OS	2	2.309	0.804	0.033	protein
7	P02520	HSP6834-A	HSP12_SOYBN Class I heat shock protein (Fragment) OS	2	2.133	0.174	0.024	stress
8	P17783	MMDH	MDHM_CITLA Malate dehydrogenase_ mitochondrial OS	2	1.700	0.015	0.000	TCA / org transformation
9	O82011	-	HSP11_SOLPE 17.7 kDa class I heat shock protein OS	2	1.328	0.243	0.007	stress
10	Q43134	WAXY	SSG1_SORBI Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	2	1.029	0.122	0.031	CHO metabolism
11	P04713	WAXY	SSG1_MAIZE Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	3	0.885	0.063	0.030	CHO metabolism
12	O23880	FA18	13S2_FAGES 13S globulin seed storage protein 2 OS	24	0.744	0.008	0.000	development
13	P15280	AGPS	GLGS_ORYSJ Glucose_1_phosphate adenylyltransferase small subunit_ chloroplastic/amyloplastic OS	3	0.713	0.064	0.025	major CHO metabolism
14	Q9XFM4	FAGAG1	13S3_FAGES 13S globulin seed storage protein 3 OS	26	0.706	0.007	0.000	development
15	O23878	FA02	13S1_FAGES 13S globulin seed storage protein 1 OS	26	0.657	0.007	0.000	development

16	O04893	-	AGLU_SPIOL Alpha_glucosidase OS	2	0.648	0.070	0.049	misc
17	P11143	HSP70	HSP70_MAIZE Heat shock 70 kDa protein OS	8	0.621	0.043	0.043	stress
18	P27879	HSP18.1	HSP11_MEDSA 18.1 kDa class I heat shock protein (Fragment) OS	3	0.600	0.112	0.004	stress
19	O65719	HSP70-3	HSP7C_ARATH Heat shock 70 kDa protein 3 OS	7	0.575	0.048	0.035	stress
20	P29357	SCE70	HSP7E_SPIOL Chloroplast envelope membrane 70 kDa heat shock_related protein OS	6	0.573	0.048	0.018	stress
21	P27322	HSC-2	HSP72_SOLLC Heat shock cognate 70 kDa protein 2 OS	6	0.566	0.050	0.030	stress
22	P08440	ald1	ALF_MAIZE Fructose_bisphosphate aldolase_ cytoplasmic isozyme OS	3	0.565	0.077	0.014	PS
23	P22954	MED37D	MD37D_ARATH Probable mediator of RNA polymerase II transcription subunit 37c OS	6	0.545	0.051	0.019	RNA
24	P24629	HSC-I	HSP71_SOLLC Heat shock cognate 70 kDa protein 1 OS	4	0.537	0.070	0.039	stress
25	Q43321	PGH1	ENO_ALNGL Enolase OS	3	0.534	0.022	0.002	glycolysis
26	P09842	WAXY	SSG1_HORVU Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	3	0.481	0.029	0.003	CHO metabolism
27	Q43130	PGH1	ENO_MESCR Enolase OS	2	0.472	0.028	0.006	glycolysis
28	A2Y8X2	WAXY	SSG1_ORYSI Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	2	0.461	0.029	0.003	CHO metabolism
29	O65314	-	ACT_SCHDU Actin OS	2	0.137	0.017	0.048	cell
30	P57751	UGP1	UGPA1_ARATH UTP__glucose_1_phosphate	3	0.037	0.022	0.041	CHO metabolism

Protein abundance was calculated by SIEVE software and compared to the starting point sample. Proteins that showed significant change at were selected as differentially abundant proteins.

a Protein ID, according to the Uniprot database.

b M.P., number of matched peptides.

c Relative abundance of protein compared to starting point. The value was rounded off to three decimal points.

d p-value, compared to starting point.

e Function, function categorized using MapMan bin ontology.

Supplemental Table 2. List of differentially abundant proteins identified in storage condition of 15°C for 5 months.

No.	Protein ID ^a	Gene symbol	Description	M.P. ^b	Abundance ^c	S.D.	p-value ^d	Function ^e
1	P93209	TFT3	14333_SOLLC 14_3_3 protein 3 OS	2	4.937	3.543	0.008	signalling
2	P50346	LOC547816	RLA0_SOYBN 60S acidic ribosomal protein P0 OS	2	3.068	1.148	0.027	RNA
3	Q75KR1	PPDK2	PPDK2_ORYSJ Pyruvate_ phosphate dikinase 2 OS	2	2.339	1.473	0.034	CHO metabolism
4	P86794	-	ITR2C_FAGES Trypsin inhibitor 2c OS	4	2.246	0.448	0.000	stress
5	Q5KTS5	CAISE5	GRDH_DAUCA Glucose and ribitol dehydrogenase OS	2	2.191	0.591	0.021	CHO metabolism
6	P29308	-	1433_SPIOL 14_3_3_like protein (Fragment) OS	3	2.108	1.019	0.016	signalling
7	O49995	LOC107787833	1433B_TOBAC 14_3_3_like protein B OS	2	2.057	1.021	0.027	signalling
8	A5BUU4	LOC100254883	RSSA_VITVI 40S ribosomal protein SA OS	2	1.804	0.810	0.039	RNA
9	P93804	LOC542721	PGMC1_MAIZE Phosphoglucomutase_ cytoplasmic 1 OS	2	1.002	0.159	0.013	glycolysis
10	P26519	GAPC	G3PC_PETCR Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	2	0.978	0.061	0.010	glycolysis
11	P17878	GAPC	G3PC_MESCR Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	2	0.925	0.065	0.020	glycolysis
12	Q43247	GAPC3	G3PC3_MAIZE Glyceraldehyde_3_phosphate dehydrogenase 3_ cytosolic OS	3	0.914	0.056	0.013	glycolysis
13	Q41595	-	G3PC_TAXBA Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	0.913	0.082	0.048	glycolysis

14	P08477	GAPC	G3PC2_HORVU Glyceraldehyde_3_phosphate dehydrogenase 2_ cytosolic (Fragment) OS	4	0.909	0.047	0.010	glycolysis
15	Q7FAH2	GAPC2	G3PC2_ORYSJ Glyceraldehyde_3_phosphate dehydrogenase 2_ cytosolic OS	5	0.908	0.047	0.017	glycolysis
16	P25861	GAPC	G3PC_ANTMA Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	0.904	0.047	0.043	glycolysis
17	Q94A28	ACO2	ACO3M_ARATH Aconitate hydratase 3_ mitochondrial OS	2	0.901	0.107	0.003	TCA / org transformation
18	P34921	GAPC	G3PC_DIACA Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	4	0.900	0.052	0.020	glycolysis
19	P30523	AGP-S	GLGS_WHEAT Glucose_1_phosphate adenylyltransferase small subunit_ chloroplastic/amyloplastic OS	2	0.897	0.137	0.018	CHO metabolism
20	Q39769	-	G3PC_GINBI Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	0.896	0.378	0.022	glycolysis
21	O23878	FA02	13S1_FAGES 13S globulin seed storage protein 1 OS	27	0.895	0.023	0.000	development
22	Q9XFM4	FAGAG1	13S3_FAGES 13S globulin seed storage protein 3 OS	28	0.893	0.021	0.028	development
23	P26413	HSP70	HSP70_SOYBN Heat shock 70 kDa protein OS	5	0.872	0.054	0.007	stress
24	P24629	HSC-I	HSP71_SOLLC Heat shock cognate 70 kDa protein 1 OS	4	0.870	0.094	0.014	stress
25	Q9S9N1	HSP70-5	HSP7E_ARATH Heat shock 70 kDa protein 5 OS	6	0.867	0.053	0.012	stress
26	P15280	AGPS	GLGS_ORYSJ Glucose_1_phosphate adenylyltransferase small subunit_	3	0.864	0.070	0.016	major CHO metabolism

			chloroplastic/amyloplastic OS						
27	Q9C7X7	HSP70-18	HSP7N_ARATH Heat shock 70 kDa protein 18 OS	6	0.830	0.073	0.022	stress	
28	A2Y8X2	WAXY	SSG1_ORYSI Granule_bound starch synthase 1_	2	0.826	0.127	0.017	CHO	
			chloroplastic/amyloplastic OS					metabolism	
29	P11143	HSP70	HSP70_MAIZE Heat shock 70 kDa protein OS	9	0.800	0.038	0.002	stress	
30	O04204	RPP0A	RLA01_ARATH 60S acidic ribosomal protein P0_1 OS	3	0.792	0.107	0.025	RNA	
31	P50345	-	RLA0_LUPLU 60S acidic ribosomal protein P0 OS	2	0.788	0.107	0.028	RNA	
32	Q43130	PGH1	ENO_MESCR Enolase OS	4	0.786	0.072	0.004	glycolysis	
33	Q9LEI9	ENO2	ENO2_HEVBR Enolase 2 OS	4	0.785	0.085	0.002	glycolysis	
34	O04893	-	AGLU_SPIOL Alpha_glucosidase OS	2	0.777	0.077	0.030	misc	
35	Q02028	HSP70	HSP7S_PEA Stromal 70 kDa heat shock_related	2	0.775	0.057	0.010	stress	
			protein_ chloroplastic OS						
36	P22953	MED37E	MD37E_ARATH Probable mediator of RNA	9	0.765	0.043	0.003	RNA	
			polymerase II transcription subunit 37e OS						
37	O65719	HSP70-3	HSP7C_ARATH Heat shock 70 kDa protein 3 OS	8	0.764	0.047	0.004	stress	
38	P29357	SCE70	HSP7E_SPIOL Chloroplast envelope membrane 70 kDa	6	0.763	0.049	0.003	stress	
			heat shock_related protein OS						
39	P26301	ENO1	ENO1_MAIZE Enolase 1 OS	3	0.760	0.074	0.001	glycolysis	
40	P42896	LOC8288376	ENO_RICCO Enolase OS	4	0.760	0.058	0.001	glycolysis	
41	P22954	MED37D	MD37D_ARATH Probable mediator of RNA	8	0.754	0.047	0.004	RNA	
			polymerase II transcription subunit 37c OS						
42	P09842	WAXY	SSG1_HORVU Granule_bound starch synthase 1_	4	0.751	0.045	0.001	CHO	
			chloroplastic/amyloplastic OS					metabolism	

43	Q9LHA8	MED37C	MD37C_ARATH Probable mediator of RNA polymerase II transcription subunit 37c OS	7	0.751	0.051	0.005	RNA
44	P05492	ATPA	ATPAM_OENBI ATP synthase subunit alpha_mitochondrial OS	2	0.748	0.072	0.020	PS
45	Q43134	WAXY	SSG1_SORBI Granule_bound starch synthase 1_chloroplastic/amyloplastic OS	4	0.747	0.046	0.001	CHO metabolism
46	Q43784	WAXY	SSG1_MANES Granule_bound starch synthase 1_chloroplastic/amyloplastic OS	3	0.739	0.047	0.009	CHO metabolism
47	P04713	WAXY	SSG1_MAIZE Granule_bound starch synthase 1_chloroplastic/amyloplastic OS	5	0.734	0.041	0.001	CHO metabolism
48	Q43321	PGH1	ENO_ALNGL Enolase OS	5	0.720	0.060	0.045	glycolysis
49	P12782	-	PGKH_WHEAT Phosphoglycerate kinase_chloroplastic OS	2	0.688	0.133	0.025	glycolysis
50	C0Z361	CPN60B3	CPNB3_ARATH Chaperonin 60 subunit beta 3_chloroplastic OS	2	0.339	0.088	0.002	protein
51	P08927	-	RUBB_PEA RuBisCO large subunit_binding protein subunit beta_chloroplastic OS	2	0.339	0.088	0.002	PS
52	I1JLC8	SLE2	SLE2_SOYBN Protein SLE2 OS	4	0.129	0.074	0.002	development

Protein abundance was calculated by SIEVE software and compared to the starting point sample. Proteins that showed significant change at were selected as differentially abundant proteins.

a Protein ID, according to the Uniprot database.

b M.P., number of matched peptides.

c Relative abundance of protein compared to starting point. The value was rounded off to three decimal points.

d p-value, compared to starting point.

e Function, function categorized using MapMan bin ontology.

Supplemental Table 3. List of differentially abundant proteins identified in storage condition of 25°C for 5 months.

No.	Protein ID ^a	Gene Symbol	Description	M.P. ^b	Abundance ^c	S.D.	p-value ^d	Function ^e
1	Q05832	HSP18	HSP11_OXYRB 18.3 kDa class I heat shock protein OS	2	3.065	1.055	0.012	stress
2	I1JLC8	SLE2	SLE2_SOYBN Protein SLE2 OS	4	1.295	0.404	0.014	development
3	Q94A28	ACO2	ACO3M_ARATH Aconitate hydratase 3_ mitochondrial OS	2	1.189	0.109	0.030	TCA / org transformation
4	P26563	-	AATM_LUPAN Aspartate aminotransferase P2_ mitochondrial (Fragment) OS	2	1.181	0.448	0.044	amino acid metabolism
5	P50566	H4-I; H4-III; H4-II	H4_CHLRE Histone H4 OS	4	1.065	0.288	0.047	DNA
6	P08436	H4-II; H4-I	H4_VOLCA Histone H4 OS	4	1.065	0.288	0.047	DNA
7	Q41595	-	G3PC_TAXBA Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	0.916	0.062	0.008	glycolysis
8	P26413	HSP70	HSP70_SOYBN Heat shock 70 kDa protein OS	5	0.833	0.044	0.004	stress
9	Q9S9N1	HSP70-5	HSP7E_ARATH Heat shock 70 kDa protein 5 OS	6	0.826	0.044	0.005	stress
10	P17878	GAPC	G3PC_MESCR Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	2	0.804	0.105	0.027	glycolysis
11	P24629	HSC-I	HSP71_SOLLC Heat shock cognate 70 kDa protein 1 OS	4	0.760	0.083	0.022	stress
12	P08477	GAPC	G3PC2_HORVU Glyceraldehyde_3_phosphate dehydrogenase 2_ cytosolic (Fragment) OS	4	0.741	0.046	0.000	glycolysis
13	Q7FAH2	GAPC2	G3PC2_ORYSJ Glyceraldehyde_3_phosphate	5	0.741	0.046	0.000	glycolysis

			dehydrogenase 2_ cytosolic OS					
14	Q43247	GAPC3	G3PC3_MAIZE Glyceraldehyde_3_phosphate	3	0.714	0.058	0.001	glycolysis
			dehydrogenase 3_ cytosolic OS					
15	P11143	HSP70	HSP70_MAIZE Heat shock 70 kDa protein OS	8	0.710	0.035	0.002	stress
16	P34921	GAPC	G3PC_DIACA Glyceraldehyde_3_phosphate	4	0.681	0.052	0.001	glycolysis
			dehydrogenase_ cytosolic OS					
17	P09094	GAPC	G3PC_TOBAC Glyceraldehyde_3_phosphate	3	0.663	0.064	0.001	glycolysis
			dehydrogenase_ cytosolic (Fragment) OS					
18	P93804	LOC542721	PGMC1_MAIZE Phosphoglucomutase_ cytoplasmic	2	0.662	0.025	0.000	glycolysis
			1 OS					
19	P29356	-	ALF_SPIOL Fructose_bisphosphate aldolase_	2	0.652	0.045	0.020	PS
			cytoplasmic isozyme OS					
20	P25861	GAPC	G3PC_ANTMA Glyceraldehyde_3_phosphate	3	0.644	0.051	0.000	glycolysis
			dehydrogenase_ cytosolic OS					
21	P29357	SCE70	HSP7E_SPIOL Chloroplast envelope membrane 70	6	0.603	0.048	0.004	stress
			kDa heat shock_related protein OS					
22	P23343	-	ACT1_DAUCA Actin_1 OS	2	0.598	0.124	0.044	cell
23	Q42857	WAXY	SSG1_IPOBA Granule_bound starch synthase 1_	2	0.597	0.132	0.021	CHO metabolism
			chloroplastic/amyloplastic OS					
24	O65719	HSP70-3	HSP7C_ARATH Heat shock 70 kDa protein 3 OS	7	0.590	0.047	0.008	stress
25	Q9LHA8	MED37C	MD37C_ARATH Probable mediator of RNA	6	0.586	0.048	0.004	RNA
			polymerase II transcription subunit 37c OS					
26	P22953	MED37E	MD37E_ARATH Probable mediator of RNA	8	0.585	0.046	0.012	RNA

			polymerase II transcription subunit 37e OS					
27	P42896	LOC8288376	ENO_RICCO Enolase OS	2	0.585	0.049	0.005	glycolysis
28	P30164	-	ACT1_PEA Actin_1 OS	3	0.583	0.077	0.033	cell
29	P22954	MED37D	MD37D_ARATH Probable mediator of RNA	7	0.581	0.046	0.006	RNA
			polymerase II transcription subunit 37c OS					
30	Q43130	PGH1	ENO_MESCR Enolase OS	3	0.568	0.052	0.009	glycolysis
31	P05492	ATPA	ATPAM_OENBI ATP synthase subunit alpha_	2	0.566	0.055	0.005	PS
			mitochondrial OS					
32	P26520	GAPC	G3PC_PETHY Glyceraldehyde_3_phosphate	3	0.564	0.058	0.007	glycolysis
			dehydrogenase_ cytosolic OS					
33	O82011	-	HSP11_SOLPE 17.7 kDa class I heat shock protein	3	0.561	0.072	0.004	stress
			OS					
34	P27322	HSC-2	HSP72_SOLLC Heat shock cognate 70 kDa protein 2	6	0.560	0.049	0.004	stress
			OS					
35	P02519	HSP17.3-B	HSP11_SOYBN 17.3 kDa class I heat shock protein	2	0.557	0.072	0.024	stress
			OS					
36	Q943E7	HSP16.9C	HS16C_ORYSJ 16.9 kDa class I heat shock protein 3	2	0.557	0.072	0.024	stress
			OS					
37	P31673	HSP17.4	HS174_ORYSJ 17.4 kDa class I heat shock protein	2	0.553	0.072	0.005	stress
			OS					
38	P27879	HSP18.1	HSP11_MEDSA 18.1 kDa class I heat shock protein	4	0.538	0.064	0.001	stress
			(Fragment) OS					
39	P25858	GAPC1	G3PC1_ARATH Glyceraldehyde_3_phosphate	4	0.538	0.058	0.004	glycolysis

			dehydrogenase GAPC1_ cytosolic OS					
40	P34922	GAPC1	G3PC_PEA Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	0.536	0.067	0.014	glycolysis
41	P27880	HSP18.2	HSP12_MEDSA 18.2 kDa class I heat shock protein OS	3	0.535	0.064	0.009	stress
42	P08735	GAPC1	G3PC1_MAIZE Glyceraldehyde_3_phosphate dehydrogenase 1_ cytosolic OS	2	0.532	0.068	0.004	glycolysis
43	P04796	GAPC	G3PC_SINAL Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	0.532	0.059	0.002	glycolysis
44	P09842	WAXY	SSG1_HORVU Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	3	0.531	0.031	0.000	CHO metabolism
45	Q9ZW31	HSP17.6B	HS17B_ARATH 17.6 kDa class I heat shock protein 2 OS	2	0.529	0.064	0.014	stress
46	Q9LEI9	ENO2	ENO2_HEVBR Enolase 2 OS	2	0.527	0.060	0.019	glycolysis
47	P04713	WAXY	SSG1_MAIZE Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	3	0.500	0.035	0.000	CHO metabolism
48	Q43134	WAXY	SSG1_SORBI Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	2	0.493	0.036	0.000	CHO metabolism
49	P50883	RPL12A	RL121_ARATH 60S ribosomal protein L12_1 OS	2	0.493	0.078	0.031	RNA
50	Q43784	WAXY	SSG1_MANES Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	2	0.491	0.036	0.000	CHO metabolism
51	O23878	FA02	13S1_FAGES 13S globulin seed storage protein 1 OS	32	0.453	0.011	0.000	development
52	Q03684	BIP4	BIP4_TOBAC Luminal_binding protein 4 OS	5	0.451	0.047	0.004	stress

53	Q39043	MED37F	MD37F_ARATH Mediator of RNA polymerase II transcription subunit 37f OS	4	0.451	0.047	0.012	RNA
54	O23880	FA18	13S2_FAGES 13S globulin seed storage protein 2 OS	25	0.435	0.014	0.000	development
55	Q9XFM4	FAGAG1	13S3_FAGES 13S globulin seed storage protein 3 OS	31	0.431	0.014	0.000	development
56	P26518	GAPC	G3PC_MAGLI Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	0.406	0.075	0.002	glycolysis
57	Q42434	HSC70	BIP_SPIOL Luminal_binding protein OS	3	0.403	0.051	0.011	stress
58	Q43321	PGH1	ENO_ALNGL Enolase OS	6	0.390	0.033	0.001	glycolysis
59	O04893	-	AGLU_SPIOL Alpha_glucosidase OS	2	0.380	0.079	0.001	misc
60	C0Z361	CPN60B3	CPNB3_ARATH Chaperonin 60 subunit beta 3_ chloroplastic OS	2	0.375	0.047	0.005	protein
61	P49040	SUS1	SUSY1_ARATH Sucrose synthase 1 OS	2	0.368	0.116	0.024	CHO metabolism

Protein abundance was calculated by SIEVE software and compared to the starting point sample. Proteins that showed significant change at were selected as differentially abundant proteins.

a Protein ID, according to the Uniprot database.

b M.P., number of matched peptides.

c Relative abundance of protein compared to starting point. The value was rounded off to three decimal points.

d p-value, compared to starting point.

e Function, function categorized using MapMan bin ontology.

Supplemental Table 4. List of differentially abundant proteins identified in storage condition of 5°C for 10 months.

No.	Protein ID ^a	Gene Symbol	Description	M.P. ^b	Abundance ^c	S.D.	<i>p</i> -value ^d	Function ^e
1	P29197	CPN60	CH60A_ARATH Chaperonin CPN60_ mitochondrial OS	2	2.448	0.900	0.042	protein
2	P46077	GRF4	14334_ARATH 14_3_3_like protein GF14 phi OS	2	2.443	1.119	0.022	signalling
3	P49398	RPS4	RS4_ORYSJ 40S ribosomal protein S4 OS	2	2.441	2.121	0.009	RNA
4	P49034	SUS1	SUSY_ALNGL Sucrose synthase OS	2	1.798	0.223	0.021	CHO metabolism
5	A5BUU4	LOC100254883	RSSA_VITVI 40S ribosomal protein SA OS	2	1.646	0.703	0.046	RNA
6	Q9SM60	PGM1	PGMC_PEA Phosphoglucosyltransferase_ cytoplasmic OS	2	1.571	0.125	0.000	glycolysis
7	P86794	-	ITR2C_FAGES Trypsin inhibitor 2c OS	3	1.424	0.241	0.002	stress
8	P08436	H4-II; H4-I	H4_VOLCA Histone H4 OS	2	1.315	0.140	0.025	DNA
9	P34924	GAPC	G3PC_PINSY Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	2	1.194	0.214	0.024	glycolysis
10	P30523	AGP-S	GLGS_WHEAT Glucose_1_phosphate adenylyltransferase small subunit_ chloroplastic/amyloplastic OS	2	1.168	0.116	0.003	CHO metabolism
11	Q41595	-	G3PC_TAXBA Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	1.141	0.121	0.004	glycolysis
12	Q41811	H4	H43_MAIZE Histone H4.3 OS	3	1.103	0.095	0.024	DNA
13	P49040	SUS1	SUSY1_ARATH Sucrose synthase 1 OS	2	1.093	0.133	0.020	CHO metabolism
14	P85945	-	H4_PSEMZ Histone H4 (Fragments) OS	2	1.093	0.095	0.038	DNA
15	P02520	HSP6834-A	HSP12_SOYBN Class I heat shock protein (Fragment) OS	2	1.064	0.055	0.019	stress
16	O82011	-	HSP11_SOLPE 17.7 kDa class I heat shock protein OS	3	1.047	0.056	0.000	stress

17	Q943E7	HSP16.9C	HS16C_ORYSJ 16.9 kDa class I heat shock protein 3 OS	2	1.045	0.056	0.006	stress
18	P31673	HSP17.4	HS174_ORYSJ 17.4 kDa class I heat shock protein OS	2	1.041	0.056	0.014	stress
19	P55228	APS1	GLGS_ARATH Glucose_1_phosphate adenylyltransferase small subunit_ chloroplastic OS	3	1.022	0.037	0.002	CHO metabolism
20	Q42857	WAXY	SSG1_IPOBA Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	2	0.988	0.034	0.000	CHO metabolism
21	Q94A28	ACO2	ACO3M_ARATH Aconitate hydratase 3_ mitochondrial OS	2	0.979	0.101	0.003	TCA / org transformation
22	O65731	RPS5	RS5_CICAR 40S ribosomal protein S5 (Fragment) OS	2	0.979	0.107	0.009	RNA
23	P27879	HSP18.1	HSP11_MEDSA 18.1 kDa class I heat shock protein (Fragment) OS	4	0.967	0.042	0.000	stress
24	P27880	HSP18.2	HSP12_MEDSA 18.2 kDa class I heat shock protein OS	3	0.966	0.042	0.000	stress
25	Q9ZW31	HSP17.6B	HS17B_ARATH 17.6 kDa class I heat shock protein 2 OS	2	0.962	0.042	0.007	stress
26	P04713	WAXY	SSG1_MAIZE Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	3	0.954	0.030	0.000	CHO metabolism
27	Q43134	WAXY	SSG1_SORBI Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	2	0.841	0.063	0.000	CHO metabolism
28	Q43784	WAXY	SSG1_MANES Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	2	0.822	0.063	0.000	CHO metabolism
29	P09842	WAXY	SSG1_HORVU Granule_bound starch synthase 1_ chloroplastic/amyloplastic OS	3	0.804	0.051	0.000	CHO metabolism
30	Q9LD57	PGK1	PGKH1_ARATH Phosphoglycerate kinase 1_ chloroplastic OS	3	0.762	0.058	0.034	glycolysis

31	O04204	RPP0A	RLA01_ARATH 60S acidic ribosomal protein P0_1 OS	2	0.758	0.036	0.006	RNA
32	P26413	HSP70	HSP70_SOYBN Heat shock 70 kDa protein OS	5	0.756	0.034	0.012	stress
33	Q9S9N1	HSP70-5	HSP7E_ARATH Heat shock 70 kDa protein 5 OS	6	0.752	0.033	0.014	stress
34	I1JLC8	SLE2	SLE2_SOYBN Protein SLE2 OS	4	0.746	0.215	0.001	development
35	A2XLF2	ACT1	ACT1_ORYSI Actin_1 OS	2	0.743	0.162	0.013	cell
36	P11143	HSP70	HSP70_MAIZE Heat shock 70 kDa protein OS	8	0.739	0.032	0.009	stress
37	Q05832	HSP18	HSP11_OXYRB 18.3 kDa class I heat shock protein OS	3	0.727	0.142	0.000	stress
38	Q96482	-	ACT1_SOLLC Actin_41 (Fragment) OS	3	0.722	0.135	0.019	cell
39	P42896	LOC8288376	ENO_RICCO Enolase OS	2	0.716	0.029	0.010	glycolysis
40	Q943E6	HSP16.9B	HS16B_ORYSJ 16.9 kDa class I heat shock protein 2 OS	2	0.710	0.142	0.005	stress
41	Q9LHA8	MED37C	MD37C_ARATH Probable mediator of RNA polymerase II transcription subunit 37c OS	6	0.703	0.037	0.011	RNA
42	P24629	HSC-I	HSP71_SOLLC Heat shock cognate 70 kDa protein 1 OS	4	0.698	0.040	0.013	stress
43	P29357	SCE70	HSP7E_SPIOL Chloroplast envelope membrane 70 kDa heat shock_related protein OS	6	0.685	0.037	0.005	stress
44	O65719	HSP70-3	HSP7C_ARATH Heat shock 70 kDa protein 3 OS	7	0.684	0.037	0.011	stress
45	P22954	MED37D	MD37D_ARATH Probable mediator of RNA polymerase II transcription subunit 37c OS	7	0.676	0.030	0.003	RNA
46	P27322	HSC-2	HSP72_SOLLC Heat shock cognate 70 kDa protein 2 OS	6	0.674	0.038	0.006	stress
47	Q9C7X7	HSP70-18	HSP7N_ARATH Heat shock 70 kDa protein 18 OS	6	0.670	0.031	0.008	stress
48	Q43130	PGH1	ENO_MESCR Enolase OS	2	0.665	0.026	0.000	glycolysis
49	P05492	ATPA	ATPAM_OENBI ATP synthase subunit alpha_mitochondrial OS	2	0.664	0.047	0.019	PS

50	P22953	MED37E	MD37E_ARATH Probable mediator of RNA polymerase II transcription subunit 37e OS	8	0.664	0.030	0.003	RNA
51	P29356	-	ALF_SPIOL Fructose_bisphosphate aldolase_ cytoplasmic isozyme OS	2	0.663	0.031	0.001	PS
52	Q03684	BIP4	BIP4_TOBAC Luminal_binding protein 4 OS	5	0.644	0.034	0.001	stress
53	P24067	BIPE2	BIP2_MAIZE Luminal_binding protein 2 OS	4	0.644	0.034	0.003	stress
54	Q42434	HSC70	BIP_SPIOL Luminal_binding protein OS	3	0.618	0.054	0.012	stress
55	Q43321	PGH1	ENO_ALNGL Enolase OS	4	0.595	0.023	0.000	glycolysis
56	Q7FAH2	GAPC2	G3PC2_ORYSJ Glyceraldehyde_3_phosphate dehydrogenase 2_ cytosolic OS	5	0.592	0.040	0.003	glycolysis
57	P08477	GAPC	G3PC2_HORVU Glyceraldehyde_3_phosphate dehydrogenase 2_ cytosolic (Fragment) OS	4	0.589	0.040	0.002	glycolysis
58	Q0J8A4	GAPC1	G3PC1_ORYSJ Glyceraldehyde_3_phosphate dehydrogenase 1_ cytosolic OS	3	0.570	0.040	0.019	glycolysis
59	O23880	FA18	13S2_FAGES 13S globulin seed storage protein 2 OS	24	0.566	0.008	0.000	development
60	O04893	-	AGLU_SPIOL Alpha_glucosidase OS	2	0.560	0.034	0.000	misc
61	Q43247	GAPC3	G3PC3_MAIZE Glyceraldehyde_3_phosphate dehydrogenase 3_ cytosolic OS	3	0.547	0.042	0.010	glycolysis
62	O23878	FA02	13S1_FAGES 13S globulin seed storage protein 1 OS	27	0.544	0.007	0.000	development
63	Q9XFM4	FAGAG1	13S3_FAGES 13S globulin seed storage protein 3 OS	27	0.533	0.007	0.000	development
64	P09094	GAPC	G3PC_TOBAC Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic (Fragment) OS	3	0.490	0.043	0.016	glycolysis
65	P31170	HSP25.3	HS25P_ARATH 25.3 kDa heat shock protein_	2	0.423	0.102	0.014	stress

			chloroplastic OS						
66	P41916	RAN1	RAN1_ARATH GTP_binding nuclear protein Ran_1 OS	2	0.413	0.067	0.030	signalling	
67	Q39769	-	G3PC_GINBI Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	0.277	0.112	0.006	glycolysis	
68	P53492	ACT7	ACT7_ARATH Actin_7 OS	3	0.239	0.055	0.020	cell	
69	P23343	-	ACT1_DAUCA Actin_1 OS	2	0.197	0.058	0.043	cell	
70	C0Z361	CPN60B3	CPNB3_ARATH Chaperonin 60 subunit beta 3_ chloroplastic OS	2	0.175	0.023	0.002	protein	
71	P34921	GAPC	G3PC_DIACA Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	4	0.165	0.015	0.002	glycolysis	
72	P25858	GAPC1	G3PC1_ARATH Glyceraldehyde_3_phosphate dehydrogenase GAPC1_ cytosolic OS	4	0.163	0.015	0.035	glycolysis	
73	P04796	GAPC	G3PC_SINAL Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	0.162	0.015	0.021	glycolysis	
74	P17878	GAPC	G3PC_MESCR Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	2	0.111	0.027	0.005	glycolysis	
75	P08440	ald1	ALF_MAIZE Fructose_bisphosphate aldolase_ cytoplasmic isozyme OS	3	0.091	0.024	0.000	PS	
76	P57751	UGP1	UGPA1_ARATH UTP__glucose_1_phosphate uridylyltransferase 1 OS	4	0.079	0.039	0.046	CHO metabolism	

Protein abundance was calculated by SIEVE software and compared to the starting point sample. Proteins that showed significant change at were selected as differentially abundant proteins.

a Protein ID, according to the Uniprot database.

b M.P., number of matched peptides.

c Relative abundance of protein compared to starting point. The value was rounded off to three decimal points.

d p-value, compared to starting point.

e Function, function categorized using MapMan bin ontology.

Supplemental Table 5. List of differentially abundant proteins identified in storage condition of 15°C for 10 months.

No.	Protein ID ^a	Gene symbol	Description	M.P. ^b	Abundance ^c	S.D.	p-value ^d	Function ^e
1	P51418	RPL18AB	R18A2_ARATH 60S ribosomal protein L18a_2 OS	2	6.688	3.576	0.007	protein
2	Q5KTS7	CAISE3	EML_DAUCA Carrot ABA_induced in somatic embryos 3 OS	2	4.815	2.674	0.021	development
3	P42791	RPL18B	RL182_ARATH 60S ribosomal protein L18_2 OS	2	3.193	0.408	0.048	RNA
4	Q93VH9	RPS4A	RS41_ARATH 40S ribosomal protein S4_1 OS	3	2.283	0.808	0.006	RNA
5	P02520	HSP6834-A	HSP12_SOYBN Class I heat shock protein (Fragment) OS	2	2.151	0.184	0.029	stress
6	P49398	RPS4	RS4_ORYSJ 40S ribosomal protein S4 OS	4	2.129	0.683	0.012	RNA
7	P34924	GAPC	G3PC_PINSY Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	2	1.982	0.381	0.036	glycolysis
8	P13708	SS	SUSY_SOYBN Sucrose synthase OS	5	1.945	0.390	0.050	CHO metabolism
9	P49040	SUS1	SUSY1_ARATH Sucrose synthase 1 OS	3	1.751	0.219	0.022	CHO metabolism
10	P22221	PPDK	PPDK_FLATR Pyruvate_ phosphate dikinase_ chloroplastic OS	3	1.482	0.102	0.042	CHO metabolism
11	O23404	PPDK	PPDK1_ARATH Pyruvate_ phosphate dikinase 1_ chloroplastic OS	3	1.458	0.095	0.038	CHO metabolism
12	Q41595	-	G3PC_TAXBA Glyceraldehyde_3_phosphate dehydrogenase_ cytosolic OS	3	1.096	0.037	0.041	glycolysis
13	P86794	-	ITR2C_FAGES Trypsin inhibitor 2c OS	4	0.739	0.072	0.009	stress
14	C0Z361	CPN60B3	CPNB3_ARATH Chaperonin 60 subunit beta 3_ chloroplastic	2	0.333	0.060	0.013	protein

Protein abundance was calculated by SIEVE software and compared to the starting point sample. Proteins that showed significant change at were selected as differentially abundant proteins.

a Protein ID, according to the Uniprot database.

b M.P., number of matched peptides.

c Relative abundance of protein compared to starting point. The value was rounded off to three decimal points.

d p-value, compared to starting point.

e Function, function categorized using MapMan bin ontology.