

# Differential protein expression profiling of *Arabidopsis thaliana* callus under microgravity on board the Chinese SZ-8 spacecraft

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## Abstract

**Main conclusion** Exposure of *Arabidopsis* callus to microgravity has a significant impact on the expression of proteins involved in stress responses, carbohydrate metabolism, protein synthesis, intracellular trafficking, signaling, and cell wall biosynthesis.

Microgravity is among the main environmental stress factors that affect plant growth and development in space. Understanding how plants acclimate to space microgravity is important to develop bioregenerative life-support systems for long-term space missions. To evaluate the spaceflight-associated stress and identify molecular events important for acquired microgravity tolerance, we compared proteomic profiles of *Arabidopsis thaliana* callus grown under microgravity on board the Chinese spacecraft SZ-8 with callus grown under 1g centrifugation (1g control) in space. Alterations in the proteome induced by microgravity were analyzed by high performance liquid chromatography—electrospray ionization-tandem mass spectrometry with isobaric tags for relative and absolute quantitation labeling. Forty-five proteins showed significant ( $p < 0.05$ ) and reproducible quantitative differences in expression between the microgravity and 1g control conditions. Of these proteins, the expression level of 24 proteins was significantly up-regulated and that of 21 proteins

was significantly down-regulated. The functions of these proteins were involved in a wide range of cellular processes, including general stress responses, carbohydrate metabolism, protein synthesis/degradation, intracellular trafficking/transportation, signaling, and cell wall biosynthesis. Several proteins not previously known to be involved in the response to microgravity or gravitational stimuli, such as pathogenesis-related thaumatin-like protein, leucine-rich repeat extension-like protein, and temperature-induce lipocalin, were significantly up- or down-regulated by microgravity. The results imply that either the normal gravity-response signaling is affected by microgravity exposure or that microgravity might inappropriately induce altered responses to other environmental stresses.

**Keywords** *Arabidopsis thaliana* · Callus · Microgravity · Chinese spacecraft SZ-8 · RNAlater

## Introduction

Plants have evolved under constant gravity (1g) on Earth and hence their morphology, structure and growth behavior are coordinately adapted to the gravity condition. Removal of gravity by spaceflight causes marked stress responses on plant growth, such as induction of large-scale changes in gene expression in the spaceflight environment during relatively short-term (seconds to hours) on board parabolic flight and the STS orbiter (Paul et al. 2005). Expression of genes involved in primary metabolism (glycolysis, gluconeogenesis, and the citrate cycle), detoxification of reactive oxygen species (ROS), and heat-shock stress is affected by microgravity and hypergravity (Paul et al. 2005; Kozeko and Kordyum 2009; Hausmann et al. 2014). In addition,

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physiological changes are observed in plants exposed to relatively long-term microgravity, such as reduction in the starch content of leaves (Laurinavichius et al. 1986; Brown and Piastuch 1994) and roots (Moore et al. 1987), induction of swollen mitochondria with an electron-dense matrix and well-developed cristae (Popova 2003), and changes in chlorophyll and carotenoid contents (Meleshko et al. 1991). Determining the response and adaptation mechanism of higher plants to long-term microgravity is important for the development of bioregenerative life-support systems for space missions. However, few studies have investigated the response of plant cells at the gene or protein levels to relatively long-term exposure to microgravity. A recent study reported that relatively long-term (12 days) microgravity exposure induces changes in microsome-associated proteins of *Arabidopsis* (*Arabidopsis thaliana*) seedlings grown on board the International Space Station (Mazars et al. 2014). The effect of long-term microgravity on higher plants has begun to attract increasing attention because of the opportunity to conduct long-term experiments in space provided by the International Space Station and the increasing demand for long-duration spaceflights.

*Arabidopsis* was chosen for the present study because of its relatively small genome, which is completely sequenced (*Arabidopsis* Genome Initiative 2000). Cell cultures grow faster and are generally easier to manipulate, and owing to their uniformity, their protein profiles show better reproducibility compared with those of whole plants. In our previous studies, we showed that clinostat rotation of *Arabidopsis* callus cells and seedlings has a significant impact on the expression of proteins involved in general stress responses, metabolic pathways, gene activation/transcription, protein synthesis, and cell wall biosynthesis (Wang et al. 2006; Tan et al. 2011). In this study we designed an experiment to monitor alteration of the proteome in *Arabidopsis* callus cells subjected to relatively long-term microgravity on board the Chinese SZ-8 spacecraft using high performance liquid chromatography—electrospray ionization—tandem mass spectrometry (LC–ESI–MS/MS) both with labeling-free and isobaric tags for relative and absolute quantization (iTRAQ) labeling technology. We designed three groups of parallel experiments under the following conditions: (1) microgravity in space flight (FM); (2) 1g centrifugation in space (FC, 1g space control); and (3) 1g control on the ground (GC, ground control). A greater difference at the protein expression level was observed between the microgravity sample and the 1g ground-control sample. We assumed that this difference might be caused by both microgravity and side effects of the spaceflight condition. The goal of the present study was to examine the effect of microgravity on expression of proteins in plant cells. Thus, quantitative

analysis of differential protein expression was only carried out between the FM sample and the FC sample under the same in-flight condition on board the SZ-8 spacecraft. The importance of microgravity-responsive proteins identified in the study is discussed.

## Materials and methods

### Plant materials and experimental conditions

Callus cultures were produced from roots of 5-d old *Arabidopsis thaliana* Heynh. ecotype Columbia (Col-O) seedlings in Murashige and Skoog (1962) medium supplemented with 3 % sugar and 2 mg/L 2,4-D. The suspension cultures were grown in 250 ml conical flasks in the dark at 22–25 °C on an orbital shaker (130 rpm) and were subcultured every week. The suspension cultures were taken as stock for repeated callus formation. The cultures were subcultured on solid medium supplemented with 1 % agar (as described by Wang et al. 2006) and incubated at 23 °C. The growth rate of Calli on solid medium was monitored by weighed the fresh weight of the cells (Online Resource Fig. 1). Calli with a diameter of about 1 mm (about 11 days after subculture on the new medium) were transferred to a slide tray (made by Astrium Space Transportation, Friedrichshafen, Germany) for use in space experiments 3 days prior to flight (see Fig. 1a–c).

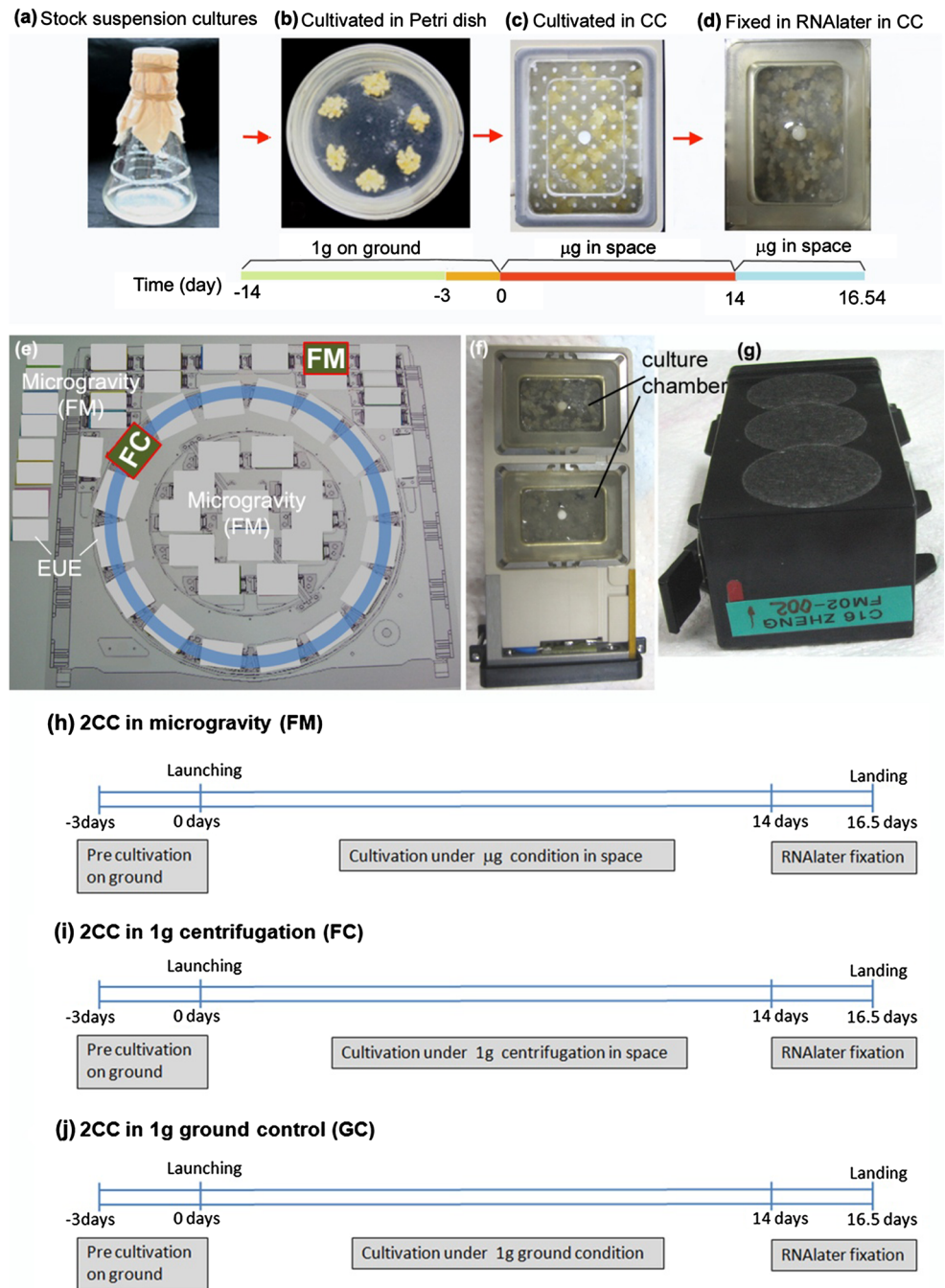
### SZ-8 flight and ground-control preparations

The culture box (SIMBOX; Fig. 1e) and the experiment-unique equipment (EUE) used for this experiment were designed and constructed by Astrium Space Transportation. The EUE included two culture chambers (CCs; each 10 ml in volume, Fig. 1f), the pump support structure, and a canal system. The chambers were closed by windows made of polysulfone. The windows had cut-outs covered by a gas-permeable membrane (so-called “biofoil”), which was glued on the windows with a special adhesive. A fixative/waste unit was connected to the CCs via tubing and tube connectors. It was mounted to the housing with four M2 × 14 mm screws. A pump was integrated into the housing. *Arabidopsis* callus cells were exposed to microgravity during space flight (FM), 1g centrifugation during space flight (FC), and 1g on the ground (GC) (Fig. 1e, h–j).

The SZ-8 spacecraft was in orbit for 16 days and 13 h (launch: 05:58, 1 November, 2011; landing: 19:00, 17 November, 2011). The calli were grown in CCs for 17 days (including 3 days preculture and 14 days under FM, FC, and GC conditions). The experiments were terminated by addition of RNAlater solution (Ambion, Austin, TX, USA) to the CCs (Fig. 1d, h–j). Hardware environmental profile

**Fig. 1** Chinese spacecraft SZ-8 experimental setup.

**a** Preparation of suspension cell cultures for space experiment. **b** Preculture in Petri dish for 11 days before take-off. Detail view of an culture chamber with samples transferred from the Petri dish 3 days before take-off (**c**), then sample grown under microgravity for 14 days and fixed by RNAlater for 2–3 days before landing (**d**). **e** Diagram of SIMBOX device used on board the Chinese spacecraft SZ-8, showing the distribution and state of experimental culture containers in SIMBOX during space flight. **f** An experimental culture container, which includes two culture chambers (CC), a pump support structure and a canal system and a fixative/waste unit connected to the CCs via tubing and tube connectors. **g** An experimental equipment (EUE), which was used as housing of the experimental culture container. Schematic view of the experiment scenario of *Arabidopsis* callus culture on board the Chinese spacecraft SZ-8 in microgravity condition (**h**), 1g centrifugation (**i**) and on ground (**j**)



monitoring began as soon as the EUE was resident in the SIMBOX device (Fig. 1e). The air temperature ranged from 15.29 to 25.04 °C, average temperature was about 23 °C, during the flight phase. The relative humidity near the incubator ranged from 20.79 to 56.35 % R.H, average about 25 % R.H, during the flight phase. The total dose and equivalent dose of radiation near the SIMBOX were 5.93–8.10 mSv and 0.37–0.51 mSv/day, respectively. The microgravity level during unified flight phase was estimated about  $10^{-3}$  to  $10^{-4}g$  for FM samples. After its return to Earth, the SIMBOX was unloaded and the CC-grown

callus was harvested approximately 10 h after landing. To minimize and average putative CC side effects, calli from two different CCs were collected, mixed together, immediately stored at 4 °C, and transported to the laboratory for analysis.

Ground-control calli were organized as described above for the space flight samples. The ground-control EUE was maintained in the control box for the duration of the shuttle mission. The control box provided an environmental profile that corresponded with the profile of the orbiter with regard to temperature and humidity. The control box profile

reflected a 24 h delay, thus the ground-control experiment was initiated 24 h after the space flight experiment. The callus in the space flight experiment and the ground control were of the same chronological age, as the ground-control calli were subcultured 1 day later than those of the space flight experiment.

#### Protein extraction and identification using LC–ESI–MS/MS with a label-free method

RNA-later-fixed callus cells of the FM, FC, and GC samples, respectively, were centrifuged for 15 min at 12,000g to remove the fixative solution and ground in liquid nitrogen to a fine powder, then resuspended in an ice-cold solution of 10 % w/v trichloroacetic acid in acetone with 0.07 % w/v DTT for at least 1 h at  $-20^{\circ}\text{C}$ , and centrifuged for 30 min at 35,000g. The pellets were rinsed twice with acetone containing 0.07 % w/v DTT for 1 h at  $-20^{\circ}\text{C}$  and then lyophilized. The resulting powder pellet was stored at  $-80^{\circ}\text{C}$  until LC–MS/MS analysis. The protein concentrations were quantified using the Bradford method (Bradford 1976).

About 180  $\mu\text{g}$  of total proteins from FM, FC and GC, respectively, were resuspended, reduced, alkylated, digested, and subjected to LC MS–MS using a label-free method as described by Mazars et al. (2014). Protein identification using MS/MS raw data was performed with the BIO-WORKS software (Thermo Finnigan, San Jose, CA, USA) searching against the *A. thaliana* EBI protein sequence database (ipi.ARATH.v3.85.REVERSED.fasta). Protein identification results were filtered with protein FDR  $\leq 0.01$ , peptide FDR  $\leq 0.01$ , and deltaCN  $> 0.1$ . LC–ESI–MS/MS analysis repeated in twice to diminish the effect of experimental variation on the results of a proteomics analysis.

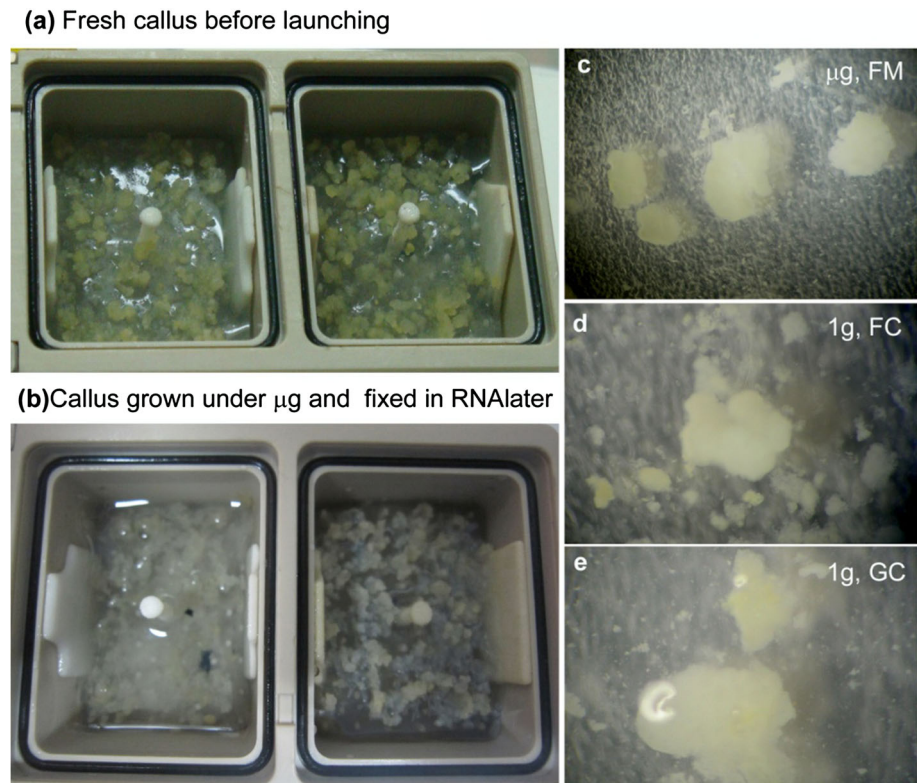
#### Protein identification using LC–ESI–MS/MS with iTRAQ labeling

Protein samples prepared as described above were reduced, alkylated, digested, and labeled with iTRAQ reagents as described previously (Wang et al. 2011). Briefly, about 300  $\mu\text{g}$  protein from FM and FC, were resuspended in 30  $\mu\text{l}$  STD buffer [4 % SDS, 1 mM DTT, 150 mM Tris HCl (pH 8.0)] in a 1:1 ratio. After incubation in boiled water for 5 min, the samples were cooled to room temperature. After addition of 200  $\mu\text{l}$  UA buffer [8 M urea, 150 mM Tris–HCl (pH 8.0)] and thorough mixing, the samples were transferred to 30 kDa Millipore ultrafiltration tubes and centrifuged for 15 min at 14,000g. A further 200  $\mu\text{l}$  UA buffer was added, the tubes were centrifuged for 15 min at 14,000g, and the supernatant was discarded. Iodoacetamide (100  $\mu\text{l}$ ; 50 mM in UA) was added to the tubes and the samples were oscillated at 600 rpm for 1 min. The samples were

incubated at room temperature for 30 min, then centrifuged again for 10 min at 14,000g. After addition of 100  $\mu\text{l}$  UA buffer, the tubes were centrifuged for 10 min at 14,000g; this procedure was repeated twice. Dissolution buffer (100  $\mu\text{l}$ ) was added and the tubes were centrifuged for 10 min at 14,000g. Next, 40  $\mu\text{l}$  trypsin buffer (3  $\mu\text{g}$  trypsin in 40  $\mu\text{l}$  dissolution buffer) was added and the tubes were oscillated at 600 rpm for 1 min, then incubated at  $37^{\circ}\text{C}$  for 16–18 h. The samples were transferred to fresh collection tubes and centrifuged for 10 min at 14,000g. The filtrate was collected for quantitative analysis of peptides at OD<sub>280</sub>. Proteins were labeled with iTRAQ tags as follows: microgravity samples-115 isobaric tag vs 1g space control-117 isobaric tag. The two labeled peptide pools were mixed together in a 1:1 ratio and stored at  $-20^{\circ}\text{C}$ . The labeled peptides were desalted with a Sep-Pak Cartridge (Waters, Milford, MA, USA), and fractionated using the Shimadzu UFLC system (Shimadzu, Japan) connected to a strong cation exchange (SCX) column (polysulfoethyl column,  $4.6 \times 100$  mm, 5  $\mu\text{m}$ , 200  $\text{\AA}$ ; PolyLC Inc., Columbia, MD, USA). Peptides were eluted with a linear gradient of 0–500 mM KCl (25 % v/v acetonitrile, 10 mM  $\text{KH}_2\text{PO}_4$ , pH 3.0) for 90 min at a flow rate of 200  $\mu\text{l}/\text{min}$ . Thirty fractions were collected. Each SCX fraction was dried, dissolved in 0.1 % formic acid, and analyzed using a Q-Exactive mass spectrometer (Thermo Finnigan, San Jose, CA, USA) and RPLC column (ZORBAX 300SB-C18 column, 5  $\mu\text{m}$ , 300  $\text{\AA}$ ,  $0.1 \times 15$  mm; Microm, Auburn, CA, USA). With an automatic sampler, the sample was injected onto a C18 cartridge (Zorbax 300SB-C18 peptide traps; Agilent Technologies, Wilmington, DE, USA), then separated with a RP-C18 column at a flow rate of 200  $\text{nl}/\text{min}$ . The HPLC conditions were as follows: 95 % buffer A (0.1 % formic acid in water) and 4 % buffer B (0.1 % formic acid in acetonitrile solution) with the following linear gradient: 110 min from 4 to 28 % B; 20 min from 28 to 40 % B; 5 min from 40 to 90 % B; and 5 min 90 % B. Samples were co-eluted on the RP HPLC and had the same m/z values (isobaric) in the MS. The first MS detected the ions as eluted from the reverse-phase column, which recorded the m/z and the relative intensity of all of the eluted ions. The survey scan also allowed individual selection of m/z values for further analysis. Peptide ions selected from the survey MS were fragmented. The second MS spectrum contained the relative amount of each tag used for quantification and the peptide fragmentation pattern used to determine the amino acid sequence. iTRAQ labeling followed by LC–ESI–MS/MS analysis was repeated in twice to diminish the effect of experimental variation on the results of a proteomics analysis.

The peptide identifications were combined using the MASCOT search engine to yield a set of Arabidopsis protein identifications with confidence values. The MASCOT

**Fig. 2** An example of *Arabidopsis* callus grew in the experimental culture container before the Chinese spacecraft SZ-8 launched (a) and after retrieval from the spaceflight (b). Note the calluses were grew for 14 days in microgravity and then fixed in RNAlater solution. Calluses grew in flight (c,  $\mu$ g, FM), 1g centrifuge (d, 1g FC) and ground (e, 1g, GC)



searches were run using the following parameters: peptide mass tolerance at 20 ppm; MS/MS tolerance set to 0.1 Da; full trypsin specificity (N- and C-terminal also applied); two missed cleavages allowed; carbamidomethyl (C), iTRAQ 4-plex (K) and iTRAQ 4-plex (N-term) were selected as fixed, and oxidation (M) as variable. The database used was *ipi.ARATH.v3.85.fasta* and the decoy database pattern was reversed sequences. The filtering parameters were set to protein false discovery rate (FDR)  $\leq 0.01$  and peptide FDR  $\leq 0.01$ . All peptides used for the calculation of protein ratios were unique to the given protein or proteins within the group, and peptide that were common to other isoforms or proteins of the same family were ignored. The average iTRAQ ratios from the double repeats were calculated for each protein. The statistical method followed that described by Mazars et al. (2014). Co-regulated proteins were annotated by GO using DAVID software v6.7 (Huang et al. 2009). GO terms with computed *p* values less than 0.05 were considered as significantly enriched.

## Results and discussion

### Experimental design and sample fixation

*Arabidopsis* callus was grown for 14 days in microgravity and 1g centrifugation condition, respectively, and then

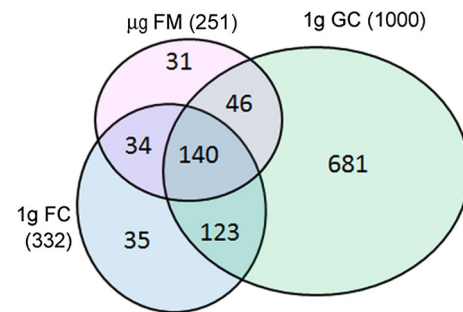
fixed in RNAlater on board the SZ-8 spacecraft (Fig. 2a, b). The average size of calli grown in microgravity in orbit (Fig. 2c) was smaller than those of 1g controls in space (Fig. 2d) and on the ground (Fig. 2e). However, all of the cultures grew well.

To halt protein expression and other cellular activities at the precise time points in space and preserve the protein profile of each sample, RNAlater was added to the CCs on day 14 on board SZ-8 and on the ground (Fig. 1d–f). RNAlater is a liquid reagent that has been proven to effectively preserve DNA and high-quality RNA and can penetrate plant tissues in less than 3 s (Kimbrough et al. 2004). RNAlater has been used successfully in spaceflight applications and has yielded high-quality RNA samples in space (Paul et al. 2005; Stutte et al. 2006; Ferl et al. 2011). To further determine if RNAlater also can be applied to preserve proteins of *Arabidopsis* calli, we compared the protein profiles of 14-day-old callus grown in EUE on the ground fixed by RNAlater and then preserved for either 30 min or 7 days at room temperature with those of non-fixed fresh samples. The proteins of callus fixed by RNAlater revealed an identical pattern at different time points on two-dimensional (2D) electrophoresis gels (Online Resource Fig. 2). Evaluation of the number and location of spots on the gels with PDQuest software showed considerable homology among the samples fixed at different time points (Online Resource Fig. 2). More than

1,700 protein spots were quantified reliably for a 2D gel. The results indicated that RNAlater could effectively preserve the proteins of *Arabidopsis* callus cultures (97 %), but affected the abundance of a small group of the identified proteins (2–3 %) in comparison with those extracted from fresh samples. To avoid this problem, samples of the two 1g controls (in space and on the ground) were also fixed in RNAlater solution at the same time point as the microgravity sample (Fig. 1d–f).

**Quantitative analysis of differentially expressed proteins in *Arabidopsis* callus cells in response to microgravity**

The samples from space flight were very limited, about 0.5 g of total callus (fresh weight) for each treatment, thus, we must treat them very carefully. As compared with iTRAQ method, label-free quantitation is easier and does not require the multi-step labeling protocols, which could lead to loss of target peptides (Abdallah et al. 2012). To get the first-hand data on the space experiment, we firstly used the label-free quantitation method. However, the label-free technique also has disadvantages in their quantification accuracy when compared iTRAQ approaches. To get a good and accuracy results, LC–ESI–MS/MS both with labeling-free and iTRAQ labeling technology were used to identify the differential expression proteins in response to microgravity. Proteins were firstly identified by LC–ESI–MS/MS with label-free analysis. When the protein confidence threshold cutoff was 1.2 with at least on peptide with 90 % confidence, 1,100 proteins in total were identified in the three experimental conditions, of which 31, 681, and 35 proteins were specific to the FM, GC, and FC conditions, respectively (Fig. 3; Online Resource Tables 1–6). This result indicated that the difference between the FM and GC samples (62.4 % of total identified proteins) was much larger than that between the FM and FC samples (3.2 % of total identified proteins). Plant cells grown on board the spacecraft might be affected by both microgravity and side effects, such as vibration during the spacecraft launch, possible space radiation, and hypoxia. The large difference in protein expression between the FM and GC samples might be caused by both microgravity and side effects of the space flight condition (Online Resource Tables 1 and 2), while the difference between the FC and the GC samples might be indicated the influence of the side effects by the space flight (Online Resource Tables 5 and 6). The environments experienced by the FM and FC samples during the space flight mainly differed in microgravity only, whereas the difference between the FM and GC conditions, including a combination of microgravity and other physical factors experienced during space flight, is more complicated (Fig. 4; Online Resource Fig. 3). To



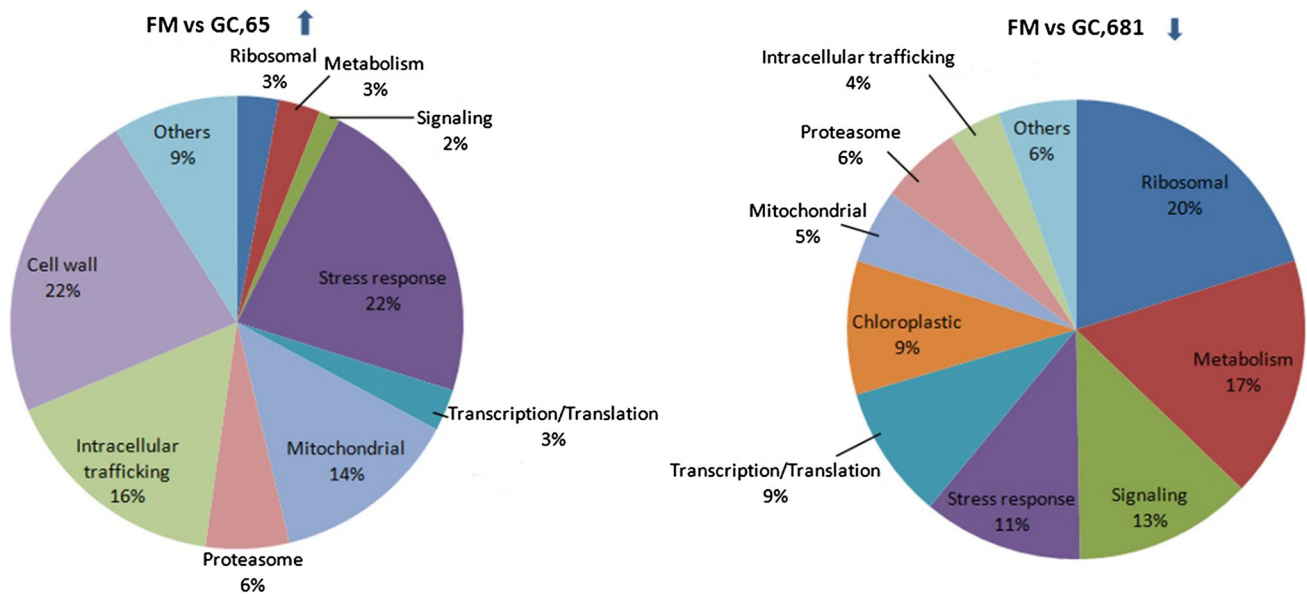
**Fig. 3** Venn diagram of proteomic data.  $\mu$ g FM, Fight samples in microgravity on board the SZ-8 Chinese spacecraft; 1g FC, Fight samples in 1g centrifuge on board the SZ-8 Chinese spacecraft; 1g GC, 1g ground control

focus on the effect of microgravity on protein expression, we mainly focused on the differential expression of proteins in the FM and FC samples in the following quantitative proteomic analysis.

We used iTRAQ LC–ESI–MS/MS to evaluate the relative quantity of proteins from FM and FC samples. Eighty-nine proteins from 748 peptides were observed to be differentially expressed between the FM and FC samples ( $p < 0.05$ ). Of these 89 proteins, 45 proteins exhibited 1.5-fold increase or decrease in abundance, consisting of 24 up-regulated proteins (Table 1) and 21 down-regulated proteins (Table 2). On the basis of their functional properties, both up- and down-regulated proteins were involved in stress response, followed by proteins involved in metabolism, signaling, protein synthesis/degradation, cell wall biosynthesis, transporters, and other functions (Fig. 5).

#### Stress response-related proteins

Proteins involved in stress response, including glutamate dehydrogenase (GDH) 1 and 2, Jacalin lectin family protein 33 and 34, glucose-regulated protein (GRP 94), and a putative ubiquinol–cytochrome *c* reductase hinge protein, were increased by about 2.25-, 2.20-, 1.68-, 1.65-, 3.72-, and 2.38-fold, respectively (Table 1). Plants have evolved a number of mechanisms to cope with biotic and abiotic stresses. Oxidative stress is a key underlying component of most abiotic stresses. Gravity is considered to be a physical stimulus and gravity sensing in plant cells involves signals related to oxidative stress (Barjaktarovic et al. 2007, 2009). The primary role of GDH is glutamate oxidation and ensuring availability of sufficient carbon skeletons for effective functioning of the tricarboxylic acid cycle under conditions where carbon is limited (Robinson et al. 1991). Up-regulation of GDH with increase in amounts of ROS has been observed in tobacco and grape tissues under salt stress (Skopelitis et al. 2006). GDH is suggested to



**Fig. 4** Distribution according their putative functions of the 65 proteins up-regulated and 681 proteins down-regulated in microgravity (percentage of the total) in comparison with that under 1g control

on ground. Co-regulated proteins were annotated by GO using DAVID software, GO terms with computed *p* values less than 0.05 were considered as significantly enriched

represent a stress-monitoring protein (Dubois et al. 2003). In the present study, two isoforms of GDH exhibited apparently up-regulated expression after exposure to relatively long-term (14 days) microgravity, which suggested microgravity caused possible carbon starvation to the callus cells. In plants, Jacalin lectin family proteins, which bind to simple sugars and/or complex carbohydrate, have attracted increasing attention because of their association with growth and development-related endogenous roles and adaptation to biotic and abiotic stress environments, such as high salinity, drought, cold, and strong light (Bae et al. 2003; Yong et al. 2003; Abebe et al. 2005; Subramanyam et al. 2006). Increased expression of Jacalin lectin family proteins in *Arabidopsis* callus under microgravity indicated that these proteins might be involved in specific endogenous protein–carbohydrate interactions in the regulation of adaptation of the cells to the microgravity condition. Heat shock proteins (HSPs), which were induced only in response to prolonged microgravity or simulated microgravity conditions, might play a role in maintaining cytoskeleton architecture and cell shape signaling (Zupanska et al. 2013). Increased abundance of the HSP90-like protein GRP94 suggested this protein might play a role in the adaptation of *Arabidopsis* callus cells to microgravity by reorganizing organelles as well as the cytoskeleton. Increased expression of the ubiquinol–cytochrome *c* oxidoreductase complex of mitochondria, which is involved in both respiration and protein processing, suggested that cells might adjust metabolic activity to adapt to the microgravity stress environment.

By contrast, pathogenesis-related thaumatin-like protein, L-ascorbate oxidase, peroxidase 34, peroxidase 37, curculin-like (mannose-binding) lectin family protein, acid phosphatase class B family protein, and germin-like protein 5 were decreased in abundance (0.25-, 0.60-, 0.21-, 0.19-, 0.31-, 0.59- and 0.16-fold, respectively) in the FM samples relative to the FC samples (Table 2). Peroxidase 34 and 37, L-ascorbate oxidase, and acid phosphatase class B family protein, which are involved in general stress responses, are reportedly up-regulated under many stress conditions. However, these proteins were all down-regulated in *Arabidopsis* callus cells after growth for 14 days under microgravity in comparison with the 1g control in space (Table 2), which suggested that the general defense response of the cells might decrease under microgravity exposure. This idea is indirectly supported by observation that the expression of a group of pathogen-response proteins, such as pathogenesis-related (PR) thaumatin-like protein, curculin-like (mannose-binding) lectin family protein, and germin-like protein 5, was obviously down-regulated in FM samples in comparison with that in FC samples (Table 2). This result is also consistent with the suggestion that plants grown under microgravity are less resistant to pathogens relative to ground controls (Leach et al. 2001; Ryba-White et al. 2001; Foster et al. 2014). The underlying causes for this reduced tolerance to pathogens are not known. Down-regulation of the group of general stress response proteins and pathogen-responsive proteins under microgravity in the current study indicated that the activation of plant defense response components might be impaired under microgravity.

**Table 1** Proteins that showed up-regulated expression in *Arabidopsis thaliana* callus cells grown in microgravity compared with that of callus cells grown under 1g centrifugation on board the SZ-8 spacecraft

Ref. no. <sup>a</sup>	Identity/accession number <sup>b</sup>	Peptides identified <sup>c</sup>	Coverage <sup>d</sup> (%)	Mr(kDa)/PI <sup>e</sup>	µg/1g <sup>f</sup>
<b>Stress</b>					
36	Glutamate dehydrogenase 2 [Q94F14]	10/3	12.28	24.8/7.77	2.25
61	Glutamate dehydrogenase 1 [AT5G18170.1]	4/2	3.41	44.5/6.39	2.20
39	Jacalin lectin family protein 33 [AT3G16460.1]	7/3	3.97	72.5/5.31	1.68
42	Jacalin lectin family protein 34 [AT3G16450.1]	5/3	8.67	32.0/5.06	1.65
77	HSP90-like protein GRP 94 [AT4G24190.1]	2/2	2.92	94.2/4.94	3.72
87	Putative mitochondrial ubiquinol–cytochrome <i>c</i> reductase hinge protein [AT1G15120.2]	2/2	13.25	19.0/5.68	2.38
<b>Metabolic</b>					
18	Pyruvate decarboxylase 1 [AT4G33070.1]	23/4	7.08	66.2/5.7	2.52
40	Pyruvate decarboxylase-2 [AT5G54960.1]	6/3	5.11	65.8/5.63	3.51
79	Phosphoenolpyruvate carboxykinase(ATP) [AT4G37870.1]	2/2	2.83	73.4/6.61	2.82
71	Malate dehydrogenase 1, mitochondrial [AT1G53240.1]	3/2	5.57	35.8/8.54	3.04
<b>Protein synthesis/degradation</b>					
14	Putative mitochondrial-processing peptidase subunit beta-1 [AT3G02090.1]	7/5	8.66	59.2/6.3	3.41
26	Putative mitochondrial-processing peptidase subunit alpha-1 [AT1G51980.1]	5/4	7.75	54.4/5.94	3.60
27	Chaperonin CPN60, mitochondrial [AT3G23990.1]	4/4	7.11	61.3/5.66	1.96
21	60S ribosomal protein L4-2 [AT5G02870.1]	9/4	10.81	44.7/10.31	1.98
<b>Transporter</b>					
41	Mitochondrial outer membrane protein porin 1[AT3G01280.1]	6/3	7.25	29.4/8.77	2.07
62	Voltage-dependent anion channel 2 [AT5G67500.1]	4/2	8.33	29.6/8.88	5.83
<b>Signaling</b>					
66	Temperature-induced lipocalin TIL 1 [AT5G58070.1]	3/2	10.22	21.4/5.98	2.52
75	Jasmonate-inducible protein[O04315]	2/2	3.26	46.6/8.2	1.70
56	Pleckstrin homology domain-containing protein [AT4G23895.3]	6/2	5.35	52.0/5.88	3.30
83	Protein disulfide isomerase [AT1G21750.2]	2/2	5.34	54.2/4.97	2.10
<b>Cell wall biosynthesis</b>					
25	Putative aldose 1-epimerase [AT3G47800.1]	5/4	8.10	39.7/9.12	1.89
<b>Others</b>					
5	Linoleate 9S-lipoxygenase [AT1G55020.1]	8/6	8.15	98.0/5.34	3.35
64	AT3G16420 protein [AT3G16450.1]	4/2	6.88	29.8/5.44	1.68
77	Endoplasmic homolog [AT4G24190.1]	2/2	2.92	94.2/4.94	3.72

Proteins were identified using iTRAQ- LC–ESI–MS/MS raw data

<sup>a</sup> Index of identified proteins

<sup>b</sup> Accession number in the NCBI, SWISS-Prot, or PIR databases

<sup>c</sup> The value before the slash represents the number of peptides identified containing overlaps; the number following the slash represents the number of peptides excluding overlaps

<sup>d</sup> The mass of peptides identified here as a percentage of the total mass of the protein

<sup>e</sup> Expected molecular weight (Mr) and isoelectric point (pI), which were calculated from the complete sequences

<sup>f</sup> µg/1g, ratio of amount of identified protein of the microgravity samples and 1g space control. Functional classification analysis use the DAVID bioinformatics tools v 6.7 (<http://david.abcc.ncifcrf.gov>)

### Metabolism-related proteins

Two isoforms of pyruvate decarboxylase (PDC1 and PDC2), a key enzyme of the fermentative pathway under low-oxygen conditions, were up-regulated (2.52- and

3.51-fold, respectively) in the FM condition (Table 1). Several previous space studies indicated that the exchange of gases between plant cells and the atmosphere is limited by diffusion processes and under microgravity is much slower than in a normal gravity environment (Porterfield



**Table 2** Proteins that showed down-regulated expression in *Arabidopsis thaliana* callus cells grown in microgravity compared with that of callus cells grown under 1g centrifugation on board the SZ-8 spacecraft using iTRAQ- LC-ESI-MS/MS raw data

Ref no <sup>a</sup>	Identity/accession number <sup>b</sup>	Peptides identified <sup>c</sup>	Coverage <sup>d</sup> (%)	Mr(kDa)/PI <sup>e</sup>	μg/1g <sup>f</sup>
<b>Stress</b>					
4	Pathogenesis-related thaumatin-like protein [AT2G28790.1]	15/6	26.51	27.0/7.61	0.25
50	Plant L-ascorbate oxidase [AT5G21105]	3/3	5.71	60.8/8.65	0.60
76	Peroxidase 34 [AT3G49120.1]	2/2	5.10	38.8/7.57	0.21
44	Peroxidase 37 [AT4G08770.1]	5/3	10.12	38.2/7.55	0.19
2	Curculin-like (mannose-binding) lectin family protein [AT1G11580.1]	59/11	22.90	49.1/7.82	0.31
70	Acid phosphatase class B family protein [AT1G04040]	3/2	8.49	31.1/9.22	0.59
57	Germin-like protein 5 [AT1G09560.1]	6/2	10.96	22.9/8.57	0.16
<b>Metabolic</b>					
20	Cell wall-bound invertase CWINV1 [AT3G13790.1]	11/4	7.71	66.3/9.14	0.31
48	Cytoplasmic invertase CINV1 [AT1G35580.1]	3/3	5.44	62.8/6.37	0.58
<b>Protein synthesis/degradation</b>					
19	Cysteine proteinase inhibitor 4 [AT4G16500.1]	16/4	35.90	12.6/9.16	0.64
<b>Transporter</b>					
53	Uncharacterized mscS family protein [AT1G78610.1]	7/2	1.99	96.6/8.73	0.35
59	Aquaporin PIP2-7 [AT4G35100.1]	5/2	11.07	29.7/8.97	0.63
<b>Cell wall biosynthesis</b>					
85	Non-specific lipid-transfer protein 2 [AT2G38530.1]	2/2	16.10	11.9/9.32	0.44
15	Leucine-rich repeat extensin-like protein 3 [AT4G13340.1]	27/4	5.13	82.2/6.49	0.37
58	Isoform 1 of leucine-rich repeat extensin-like protein 5 [AT4G18670.1]	5/2	2.45	90.8/6.24	0.35
16	Leucine-rich repeat extensin-like protein 4 [AT3G24480]	26/4	7.89	54.7/6.46	0.39
<b>Others</b>					
6	Unknown protein [AT3G23450.1]	7/6	14.16	34.8/10.81	0.27
11	Possible apospory-associated like protein [AT4G25900.1]	9/5	13.07	37.5/9.83	0.36
67	Ribulose biphosphate carboxylase large chain [ATCG00490.1]	3/2	4.80	53.0/5.88	0.39
69	T10O24.12 [Q9XIK2]	3/2	1.17	73.9/7.93	0.66
72	AT4g39730/T19P19_120	3/2	12.09	19.9/8.45	0.57

<sup>a</sup> Index of identified proteins

<sup>b</sup> Accession number in the NCBI, SWISS-Prot, or PIR databases

<sup>c</sup> The value before the slash represents the number of peptides identified containing overlaps; the value following the slash represents the number of peptides excluding overlaps

<sup>d</sup> The mass of peptides identified here as a percentage of the total mass of the protein

<sup>e</sup> Expected molecular weight (Mr) and isoelectric point (pI) which were calculated from the complete sequences

<sup>f</sup> μg/1g, ratio of amount of identified protein of the microgravity samples and 1g space control. Functional classification analysis use the DAVID bioinformatics tools v 6.7 (<http://david.abcc.ncifcrf.gov>)

2002). Increased expression of PDCs in FM samples suggested that the cells might be subjected to a low-oxygen-like environment. Phosphoenolpyruvate carboxykinase (PEPCK) and mitochondrial malate dehydrogenase (MDH) 1 were also up-regulated (2.82- and 3.04-fold, respectively) in the FM condition (Table 1). PEPCK is an enzyme involved in malate metabolism and gluconeogenesis, and is at an interface between the metabolism of amino acids, organic acids, sugars, lipids, and some

secondary metabolites (Leegood and Walker 2003). Mitochondrial MDH1 normally uses NADH to reduce oxaloacetate to malate, which is then exported to the cytosol. Up-regulation of these two enzymes indicated that the adjustment of the central metabolism and redox homeostasis between organelle compartments could be important for adaptation of callus cells to microgravity. Invertases (β-fructofuranosidase; EC 3.2.1.26) catalyze the hydrolysis of sucrose to glucose and fructose, and are

classified on the basis of cellular location (cytoplasmic, vacuolar, and cell wall bound). In the present study, expression of a cell-wall bound invertase (CWINV)1 and a cytoplasmic invertase (CINV)1 were apparently down-regulated (0.31- and 0.58-fold, respectively) when callus cells were exposed to microgravity (Table 2). The route of sucrose catabolism has important implications for energy conservation and carbon allocation in non-photosynthetic cells. Previous studies have shown that microgravity conditions in space, randomization of the gravity vector by clinorotation, and creation of hypergravity conditions by centrifugation all affect the sugar concentration and the metabolic fluxes of plant cells (Obenland and Brown 1994; Hampp et al. 1997; Martzivanous and Hampp 2003; Wang et al. 2006; Tan et al. 2011). Down-regulation of CWINV1 and CINV1 under microgravity suggested that regulation of sucrose metabolism might be an important strategy for callus cell adaptation to long-term microgravity exposure.

#### Protein synthesis/degradation-related proteins

Four up-regulated proteins [putative mitochondrial-processing peptidase (MPP) subunit alpha-1 and beta-1, mitochondrial chaperonin CPN60, and 60S ribosomal protein L4-2; 3.41-, 3.6-, 1.96- and 1.98-fold, respectively] and one down-regulated protein (cysteine proteinase inhibitor 4; 0.64-fold) under microgravity are involved in protein synthesis and degradation (Tables 1, 2). MPP is an ATP-independent protease involved in cleavage of the targeting peptide from the translocating precursor protein for the majority of mitochondrial matrix proteins. CPN60, which is a HSP chaperone, plays a role in protein folding, assembly, translocation, and degradation in many normal cellular processes, as well as stabilization of proteins and membranes, and can assist in protein refolding under stress conditions. Up-regulation of mitochondrial chaperonin CPN60 under microgravity might play a role in protecting callus cells against microgravity stress by re-establishing normal protein conformation in mitochondria. Cysteine proteinase inhibitor 4 is one of many protease inhibitors, which are an important element of the plant defense response to insect predation and are regulated by a wounding signal transduction pathway. The reason for down-regulation of this protein under microgravity is unknown.

#### Cellular trafficking and transporter-related proteins

Cellular trafficking and transportation determine the activity of cellular status and signaling, which ensure that the cell responds correctly and adapts to the surrounding environment. In the current study, two transporters

(mitochondrial outer membrane protein porin 1 and voltage-dependent anion channel 2) exhibited up-regulated expression (2.07- and 5.83-fold, respectively) and two proteins (uncharacterized MscS family protein and aquaporin PIP2) were down-regulated (0.35- and 0.63-fold, respectively) under the FM condition (Tables 1, 2). Porin 1 is a voltage-dependent anion-selective channel (VDAC) protein in the mitochondrial outer membrane. VDACS are suggested to function in intracellular signaling, cell life, and death in animal cells (Shoshan-Barmatz et al. 2006). In plant cells, VDACS in the plasmalemma are suggested to provide a mechanism for the control of salt efflux from guard cells and are a major component of the tRNA import machinery in mitochondria (Keller et al. 1989; Salinas et al. 2006). The increased expression of VDAC proteins under microgravity suggested that these proteins could participate in regulation of mitochondrial metabolism by blocking the flux of anionic metabolites, leading to changing cellular conditions induced by microgravity. Two proteins down-regulated by microgravity are an uncharacterized MscS family protein and aquaporin PIP2-7. These proteins are reported to act as a biological emergency release valve and water channel, respectively. To our knowledge, no previous study has reported the involvement of these two proteins in response to altered gravity. Why these proteins are down-regulated under microgravity remains to be elucidated.

#### Signaling-related proteins

Four up-regulated proteins [temperature-induced lipocalin (TIL) 1, jasmonate-inducible protein, pleckstrin homology (PH) domain-containing protein, and protein disulfide isomerase (PDI); 2.52-, 1.7-, 3.3-, and 2.1-fold, respectively] are predicted to play roles in signaling (Table 1). The signaling pathways mediated by these proteins could regulate a variety of defense responses. TILs are reported to play important roles in tolerance to oxidative stress. The PH domain at the N-terminus of the putative Dbl domain is a characteristic of human SWAP70 proteins, which are critical for binding to phosphatidylinositol 3,4,5-triphosphate (PIP3) (Stevenson-Paulik et al. 2003). The PH domain-containing protein might function as a Rac/Rop GEF to control actin-dependent morphogenesis (Basu et al. 2008). The increase in PDI expression under microgravity suggests that reduced gravity also affects the functioning of the secretory apparatus. PDI affects many cellular functions, including storage protein folding, receptor activity, cell–cell interactions, gene expression, and actin filament polymerization. Increase in expression of jasmonate-inducible protein under microgravity indicated the involvement of jasmonate in adaptation of callus cells to long-term microgravity exposure.

## Cell wall-related proteins

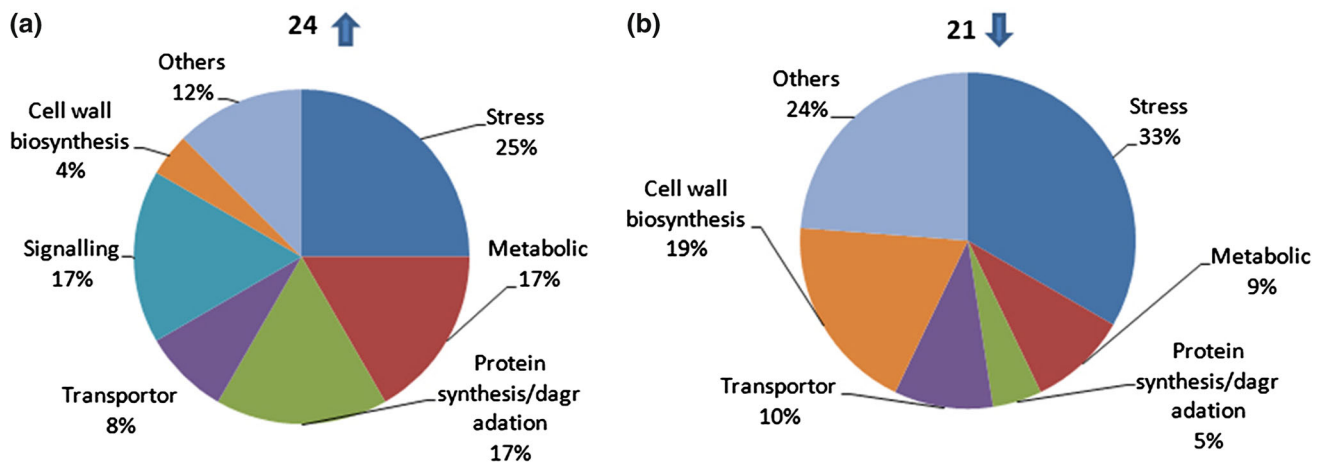
One up-regulated protein [putative aldose 1-epimerase (AEP); 1.89-fold] and four down-regulated proteins [non-specific lipid-transfer protein (LTP) 2, leucine-rich repeat extensin (LRRE)-like protein 3, LRRE-like protein 4, and isoform 1 of LRRE-like protein 5; 0.44-, 0.37-, 0.39- and 0.35-fold, respectively] in the FM condition are involved in cell wall growth and modification (Table 1). Increase in putative AEP expression suggests that microgravity might affect glycoprotein modification in the cell wall. AEP limits lactose assimilation and influences cellulose formation in *Hypocrea jecorina* (Fekete et al. 2008). In *Arabidopsis*, a glycoprotein modified with a terminal *N*-acetylglucosamine and localized at the nuclear rim showed sequence similarity to AEP (Heese-Peck and Raikhel 1998). Non-specific LTP in *Arabidopsis* provides enhanced resistance to a trichothecene mycotoxin by reducing oxidative stress, but why microgravity inhibits expression of this protein is unknown. LRRE proteins play a role in the regulation of plant cell wall expansion. One proposed function of LRRE is to reinforce the polysaccharide structure of the wall through cross-linking among polysaccharides and/or other cell wall components (Carpita and Gibeau 1993). LRRE1 is reported to be required for root hair morphogenesis in *Arabidopsis* and the function of LRRE1 might be involved in the control of polarized growth or cell wall formation and assembly (Baumberger et al. 2001). LRREs are potentially involved in the regulation of cell wall expansion in response to signaling (Baumberger et al. 2003). LRRE3, LRRE4, and isoform 1 of LRRE-like protein 5 were all down-regulated by microgravity, which indicated that reduced gravity might affect cell expansion. This result is consistent with a previous observation on rapeseed callus grown in space (Skagen and Iversen 2000) and our observation of smaller-sized callus under the FM condition in comparison with calli grown under the FC and GC conditions (Fig. 2c–e).

## Conclusions

Previous authors have hypothesized that the action of microgravity might depend on the duration of exposure to microgravity (Claassen and Spooner 1994; Matía et al. 2010). The difference in responses of plants grown in space for a short term (e.g. seconds or hours) and a long term (e.g. several days or months) is exhibited at various levels. With exposure to short-term microgravity, plant cells first exhibit an abiotic stress response, such as dramatically increasing production of ROS and other radicals (Babbick et al. 2007), whereas under long-term microgravity exposure plants show a strong capacity to withstand an

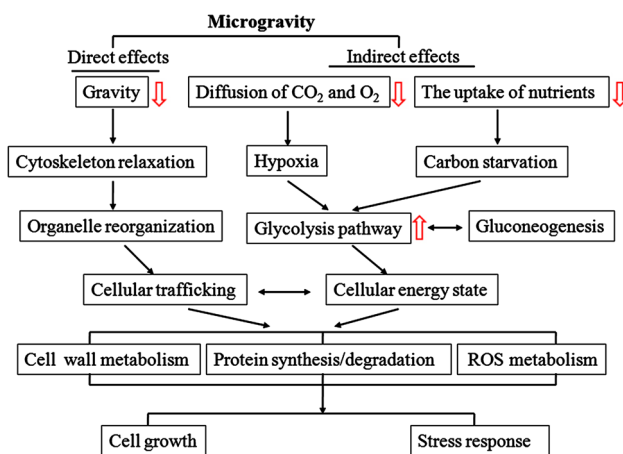
unfavorable condition by establishing metabolic changes and adaptation to oxidative stress. In the present study, callus of *A. thaliana* was cultured on board the Chinese spacecraft SZ-8 under microgravity (FM) and 1g centrifugation (FC) in space. Callus was grown for 14 days and then fixed in-flight with RNAlater. Proteins from the FM and the FC samples were analyzed by means of iTRAQ LC–ESI–MS/MS. The 45 identified proteins that responded to microgravity are involved in a wide range of cellular processes, including general stress responses, carbohydrate metabolism, protein synthesis/degradation, intracellular trafficking/transportation, signaling, and cell wall biosynthesis (Fig. 5). Changes in the gene expression of proteins such as GDH, HSP90, Jacalin lectin family proteins, MDH, and peroxidase have been previously observed under altered gravitational conditions (Martzivanous and Hampp 2003; Kimbrough et al. 2004; Kamada et al. 2005; Kozeko and Kordyum 2009). Several proteins, such as pathogenesis-related thaumatin-like protein, LRRE-like protein, and temperature-induced lipocalin, which were not known previously to be involved in the response to microgravity or gravitational stimuli, were significantly up- or down-regulated in *Arabidopsis* callus grown for 14 days under microgravity on board SZ-8. This suggested the possible involvement of these proteins in adaptation to long-term microgravity. The functional properties of these proteins imply that either the normal gravity-response signaling is affected by spaceflight or that spaceflight might inappropriately induce altered responses to environmental stresses, such as pathogen, temperature and hormone responses, other than microgravity.

Effects of microgravity on plant cells in space could include into aspects: direct and indirect effects (Fig. 6). The direct effect is a consequence of the decrease of gravity. Organelles (nucleus, plastids, and mitochondria) in the cells driven by their weight sediment and impact on the cytoskeleton networks on the 1g condition, while the weight of the organelles will disappear under microgravity condition and these organelles could exhibit no sedimentation in the cell, which should cause relaxation of cytoskeleton and reorganization of organelles, thus altering the cellular trafficking and transporting (Volkman et al. 1991; Mazars et al. 2014). The indirect effects of microgravity is known to modify the dynamics of fluids and small particles, thus altering the diffusion of gases (e.g. carbon dioxide and oxygen) and the uptake of water and nutrients (Wolff et al. 2013). Plant cells grown in such an environment might exhibit carbon starvation and hypoxia. Although we currently do not know to what extent cell metabolism during culture is affected by limited nutrient or gas diffusion under microgravity, indications of plant cell hypoxia in space experiments are frequently reported (Paul et al. 2001; Stout et al. 2001). In the present study, up-



**Fig. 5** Distribution according their putative functions of 24 proteins up-regulated (a) and 21 proteins down-regulated (b) in microgravity (percentage of the total) in comparison with that in 1g centrifuge in

space. Co-regulated proteins were annotated by GO using DAVID software, GO terms with computed *p* values less than 0.05 were considered as significantly enriched



**Fig. 6** Schematic diagram illustrating possible effect of microgravity on the *Arabidopsis* callus cells according to the data of differential expression of proteins in the FM sample in comparison with that in the FC and GC samples, respectively. Effects of microgravity on plant cells could include two different aspects: direct and indirect. (1) Direct effects: organelles (nucleus, plastids, and mitochondria) in the cells could sediment on the cytoskeleton networks driven by their weight under the 1g gravity condition. Lack of sedimentation under microgravity condition should cause relaxation of cytoskeleton and reorganization of organelles, thus altering the cellular trafficking and transporting. (2) Indirect effects: microgravity may induce change of the diffusion of gases (e.g. carbon dioxide and oxygen) and the uptake nutrients and water from the culture medium. Plant cells grown under such condition might exhibit carbon starvation and hypoxia. To cope with these hypoxia and carbon starvation, plant cells could enhance the glycolysis activation, which work more efficiently under low-oxygen condition. Alterations cellular trafficking and cellular energy state directly affect cell wall metabolism, protein synthesis/degradation and ROS, which determine cell growth rate and stress response

this hypoxic stress, *Arabidopsis* callus could enhance the expression of certain key enzymes, such as GDH1 and GDH2, to maintain effective functioning of the carbohydrate metabolic pathway and cellular energy state during the adaptation to microgravity. The impact of microgravity on cellular trafficking and cellular energy state might modify the plant cell wall metabolism, protein synthesis/degradation and ROS metabolism and thus determines the cell growth and response to environment.

The proteins analyzed in the current study only provide a snapshot of the status at the protein level of cells undergoing adaptation to microgravity exposure. The differences between FM (microgravity) and FC (1g space) are much smaller than those between FC and GC (1g ground) (Fig. 3). Many differential expression proteins (34 up-regulated and 535 down-regulated polypeptides) found in the FC in comparison with the GC samples were not microgravity related (Online Resource Table 5 and Table 6; Online Resource Fig. 3) and could cause by other physical factors experienced during space flight, such as, possible space radiation, vibration and noise from spacecraft, and the alteration of convective air movement. In addition, the number of the down-regulated proteins in the FC sample in comparison with the GC sample was much more than those of the up-regulated proteins (Online Resource Table 5 and Table 6; Online Resource Fig. 3). This result indicated that physical factors not microgravity-related in space flight could cause very strong stress responses of the *Arabidopsis* callus cells and resulted in the degradation of a large amount of proteins in the cells. Future experiments with cell cultures should include suitable sensors to monitor these physical environmental factors inflight and to distinguish differences in physiological response, which are microgravity-related or not

regulation of two isoforms of a fermentative key enzyme, PDC1 and PDC2, might also be indicative of hypoxia in *Arabidopsis* callus cells under microgravity. To cope with

microgravity-related. This is important for designing an optimized experiment for space mission to discover the molecular basis of plant adaptation to space environment and the real influence of microgravity on plant growth and development.

**Author contribution** HZ conceived and designed the research. YZ and LW conducted the experiments. JX analyzed the data. HZ wrote the manuscript. All authors read and approved the manuscript.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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