



Transcriptomic Analysis Reveals the Effects of Microgravity on Rice Calli on Board the Chinese Spaceship Shenzhou 8

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Received: 12 February 2018 / Accepted: 29 May 2018 / Published online: 14 June 2018
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Abstract

Rice calli were loaded onto the stationary and rotating platforms of a biological incubator (SIMBOX) during spaceflight and ground control experiments. The calli in the SIMBOX were fixed with RNAlater in space after a 324-h spacecraft flight, as well as on the ground at the same time point in a ground control experiment. Microgravity-responsive (MR) transcripts were identified by a comparison of the spaceflight controls (F- μ g) with the 1-g ground controls (G-1g) and 1-g inflight controls (F-1g). MapMan analysis was used to classify 955 MR transcripts. These transcripts mainly belonged to the following categories: cell wall modification and metabolism, glycolysis and the tricarboxylic acid (TCA) cycle, transcription factors, protein modification and degradation, hormone metabolism and signalling, calcium regulation, receptor-like kinase activity, and transporters. Here, we focused on the effects of microgravity on the plant cell wall and discussed the relationship between the variation in the cell wall and plant cell growth under microgravity.

Keywords Microgravity · Spaceflight · Transcriptome · *Oryza sativa* · Callus cells · Cell wall · Aquaporin

Abbreviations

MR	Microgravity responsive
SIMBOX	Science in Microgravity Box
2,4-D	2,4-dichlorophenoxyacetic acid
F- μ g	Spaceflight controls
F-1g	1-g inflight controls
G-1g	1-g ground controls

Introduction

Microgravity can affect plants at the individual, organ, tissue, cell, sub-cellular, and molecular levels (Ruyters et al. 2014). Spaceflight experiments are considered to be the best method

to study the effects of altered gravity conditions on plant growth and developmental processes (Cannon et al. 2015). Spaceflight experiments have provided new insights into the effects of microgravity on plant growth and development, and new further perspectives of these effects will also be provided through the development of new research technologies (Tibbitts et al. 1989; Woodward 2007).

Microarray analysis of gene expression is one of the most powerful tools for studying gene expression under various conditions, including different gravity conditions (Martzivanou and Hampp 2003; Correll et al. 2013). Experiments have demonstrated that the plant transcriptome is altered by the influence of microgravity. In *Arabidopsis* seedlings, hundreds of genes were regulated in response to microgravity, including transcription factors and genes involved in plant hormone signalling (auxin, ethylene and abscisic acid (ABA)-responsive genes) (Correll et al. 2008, 2013). Transgenic plants have been used to investigate the effect of spaceflight stress on gene expression (Salmi and Roux 2008). We cultured rice calli in hormone-free N6 medium to study how microgravity effects gene expression. Spaceflight transcriptome data also strongly suggest that plant adaptation to spaceflight requires changes in gene expression (Jin et al. 2015).

In recent years, many studies on the effects of spaceflight on the plant transcriptome have been carried out (Paul et al. 2005, 2012, 2013; Stutte et al. 2006; Martzivanou et al.

This article belongs to the Topical Collection: Approaching the Chinese Space Station - Microgravity Research in China
Guest Editors: Jian-Fu Zhao, Shuang-Feng Wang

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12217-018-9633-6>) contains supplementary material, which is available to authorized users.

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2006; Salmi and Roux 2008; Correll et al. 2013; Zupanska et al. 2013; Fengler et al. 2015; Jin et al. 2015; Kwon et al. 2015; Johnson et al. 2017; Li et al. 2017). The results showed that the genes whose expression changed varied among different spaceflight experiments, or the trends of the change in the expression of some genes were different. The reasons for the inconsistent results may be related to differences in approaches or hardware design in the spaceflight experiments (Johnson et al. 2017). Organ-specific changes in response to spaceflight have also been demonstrated through transcriptomic analysis (Paul et al. 2013). Plant cell cultures have been used several times as materials in spaceflight experiments (Martzivanou et al. 2006; Paul et al. 2012; Zupanska et al. 2013; Fengler et al. 2015; Jin et al. 2015). The results from these experiments were also not entirely consistent due to the different durations of the experiments or different experimental procedures. However, it has been demonstrated that cell cultures can perceive and respond to spaceflight or the alteration of gravity, even though they lack the specialized cell structures normally associated with gravity perception in intact plants (Paul et al. 2012, 2013; Fengler et al. 2015, 2016; Neef et al. 2016). Some of the overlapping biochemical pathways that were differentially regulated in spaceflight have been identified (Johnson et al. 2017). Therefore, the results obtained from different tissues or different experimental processes in spaceflight experiments can reflect the effects of spaceflight from different points of view, which can provide a more comprehensive understanding of spaceflight effects.

Most of the plant calli were induced using plant hormones and were subcultured with hormones. Therefore, we also demonstrate the effects of microgravity on calli under regular culture conditions in this report to study the response of rice calli to microgravity.

We loaded rice calli cultured with 2,4-D into a Science in Microgravity Box (SIMBOX), which was then placed on board the Chinese spaceship Shenzhou 8. We subsequently

analysed gene expression in these rice calli cultured in response to microgravity. A total of 955 transcripts were identified as microgravity-responsive (MR) transcripts. The pathways in which the MR transcripts are involved were studied through MapMan analysis. The change in expression of genes related to cell wall modification and metabolism was discussed.

Results

Probe Sets Expressed in Rice Calli

The Affymetrix GeneChip[®] Rice Genome Array (Affymetrix, USA) contains 57,194 probe sets, representing 51,279 transcripts. Rice calli were harvested and used for microarray analysis. A total of 10 arrays were hybridized. The signal intensity was normalized for each replicate, and the correlation coefficients between any two replicates ranged from 0.96 to 0.98 (Table 1), indicating that the microarray analysis was highly reproducible. ‘Present’ probe sets were detected by filtering with a fraction call of 100% (for details, see the Methods). Probe sets that were detected as ‘Present’ in all biological replicates were defined as ‘expressed’ probe sets in this study. Based on these criteria, 38.87%, 38.06% and 39.84% of the 57,194 probe sets in the array were expressed under F- μ g, F-1g and G-1g, respectively (Table 2).

Gene Expression Under F-1g Versus G-1g

We compared the transcripts detected in calli grown under F-1g versus G-1g to evaluate the effect of 1-g centrifugation in space versus 1-g conditions on the ground. In total, 1728 probe sets were identified as differentially regulated (at least 2-fold, $p < 0.05$, Table 2). Among these probe sets, 707 were highly differentially regulated by F-1g compared with G-1g

Table 1 Normalized signal intensity and correlation coefficients between biological replicates in the microarray analysis

	F- μ g				F-1g			G-1g	
	1	2	3	4	1	2	3	1	2
F- μ g	2	0.97875	NA	NA	NA	NA	NA	NA	NA
	3	0.97595	0.975302	NA	NA	NA	NA	NA	NA
	4	0.974316	0.973842	0.978362	NA	NA	NA	NA	NA
F-1g	1	0.967555	0.967385	0.969279	0.969917	NA	NA	NA	NA
	2	0.969693	0.96954	0.973464	0.974536	0.97429	NA	NA	NA
	3	0.970899	0.971148	0.974144	0.974666	0.975262	0.977057	NA	NA
G-1g	1	0.973613	0.973908	0.975004	0.975069	0.971952	0.975455	0.976723	NA
	2	0.974989	0.974898	0.975786	0.975091	0.97222	0.974515	0.975938	0.979873
	3	0.973432	0.973454	0.974701	0.974088	0.972935	0.974502	0.976531	0.979688

Table 2 Percentage of expressed probe sets in ‘rice calli’

Expressed (%)	Rice calli
F- μ g	38.87%
F-1g	38.06%
G-1g	39.84%

(Fig. 1), of which more than 200 showed a fold change of more than 5 ($p < 0.05$). The differences in gene expression between F-1g and G-1g may have been caused by environmental disturbance as we discussed in a previous report (Jin et al. 2015).

Analysis of MR Transcripts

In total, 4924 probe sets were identified as differentially expressed (fold change was greater than or equal to 2; false discovery rate, $p < 0.05$).

In total, 2974 differentially expressed probe sets were identified under F- μ g vs. F-1g, whereas 1319 differentially expressed probe sets were identified only under F- μ g vs. F-1g (Table 3, Fig. 1). In total, 2268 differentially expressed probe sets were identified under F- μ g vs. G-1g, whereas 941 differentially expressed probe sets were identified only under F- μ g vs. G-1g (Fig. 1). More probe sets were up-regulated than down-regulated under F- μ g vs. F-1g and under F- μ g vs. G-1g (Table 3).

In total, 1025 differentially expressed probe sets were identified when F- μ g vs. F-1g was compared with F- μ g vs. G-1g (Fig. 1). The control in situ fixation methods employed in the centrifuge on board the spacecraft are important (Correll et al. 2013). Due to the difficulty in avoiding the environmental components of spaceflight, such as radiation and vibration, we also set up 1-g centrifugation controls on board

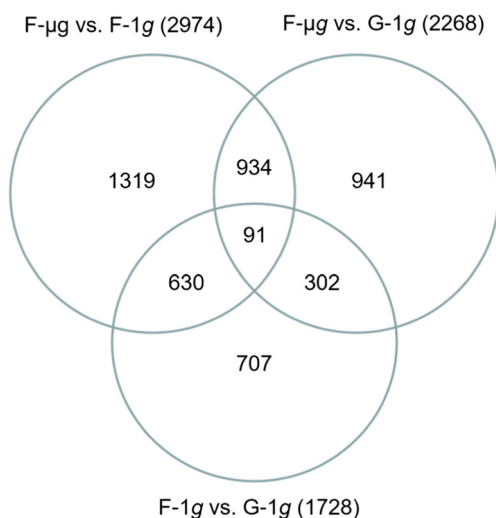


Fig. 1 Venn diagram of differentially expressed probe sets

Table 3 Number of differentially expressed probe sets in the comparison of the various experimental combinations

	Rice calli		Total
	Up	Down	
F- μ g vs. F-1g	1963	1011	2974
F- μ g vs. G-1g	1380	888	2268
F-1g vs. G-1g	798	930	1728

the spacecraft. We believe that the 1025 probe sets that were differentially expressed under F- μ g vs. G-1g and F- μ g vs. F-1g were due primarily to microgravity. Among these 1025 probe sets, 800 were up-regulated and 216 were down-regulated under microgravity. Three probe sets were up-regulated under F- μ g vs. G-1g and down-regulated under F- μ g vs. F-1g, whereas 6 probe sets were down-regulated under F- μ g vs. G-1g and up-regulated under F- μ g vs. F-1g (Fig. 2, Table S1).

Some transcripts were represented by more than one probe set. Therefore, the number of responsive probe sets was greater than the number of responsive transcripts. The 1025 probe sets identified corresponded to 955 transcripts. Among these 955 transcripts, 746 were up-regulated and 200 were down-regulated under microgravity. Three transcripts were up-regulated under F- μ g vs. G-1g and down-regulated under F- μ g vs. F-1g, while 6 transcripts were down-regulated under F- μ g vs. G-1g and up-regulated under F- μ g vs. F-1g. These 9 transcripts might respond to stress stimuli other than microgravity (Fig. 2, Table S1).

The number of probe sets that were differentially expressed in rice calli under F- μ g, F-1g and G-1g in experiments conducted in a SIMBOX on board Shenzhou 8.

Up- and down-regulated probe sets identified in rice calli under F- μ g, F-1g and G-1g in experiments conducted in a SIMBOX on board Shenzhou 8.

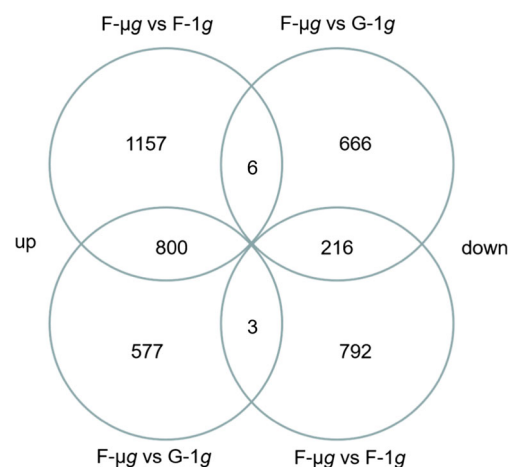


Fig. 2 Overlap of the differentially expressed probe sets

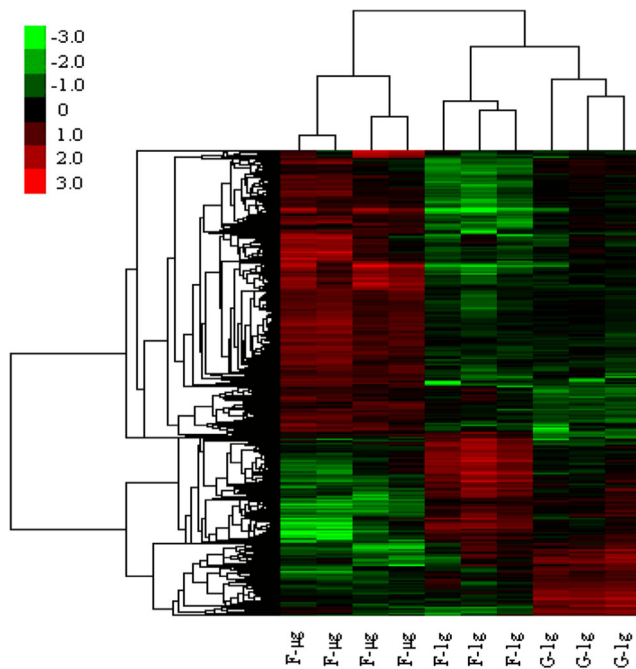


Fig. 3 Hierarchical clustering analysis of the log₂-transformed ratios of the 4924 probe sets

Hierarchical Clustering of MR Probe Sets

To evaluate the relationships of the genome-wide expression profiles associated with the different treatments, we used linkage hierarchical clustering with 4924 probe sets across 10 arrays (3 groups) (Fig. 3). The MR probe sets identified under F- μ g were clearly separated from those associated with F-1g and G-1g.

The figure shows changes in the probe sets detected in rice calli under F- μ g, F-1g and G-1g. Green represents

down-regulated probe sets, and red represents up-regulated probe sets.

Major Functions of MR Genes

Following MapMan analysis, the identified MR genes were associated with the following categories: metabolism and signalling pathways of hormones (Fig. S1), transcription factors (Fig. S2), starch, glycolysis and the electron transport chain (Fig. S3), signalling (Fig. S4), transporters (Fig. S5), protein degradation (Fig. S6) and cell wall metabolism.

Changes in the Expression of Cell Wall-Related Genes in Rice Calli Under Microgravity

The expressions of cell wall genes and cell wall-related genes change under gravitropism or microgravity (Hoson et al. 2002a, 2004; Cui et al. 2005; Strohm et al. 2012; Hu et al. 2013). In this study, we identified 8 transcripts related to cell wall formation (Table 4). Among these 8 transcripts, 3 of the down-regulated transcripts corresponded to endoglucanases (LOC_Os01g21070, LOC_Os04g57860) related to cell wall degradation and the pectinesterase-1 precursor (LOC_Os01g57854). Two endoglucanases (LOC_Os01g21070, LOC_Os04g57860) were down-regulated under F- μ g vs. both G-1g and F-1g. Five of the up-regulated transcripts corresponded to proteins involved in primary cell wall modification (xyloglucan endotransglucosylase/hydrolase protein 23 (OsXTH23, LOC_Os06g48200), beta-expansin 1a precursor (LOC_Os03g01270)), cell wall degradation (polygalacturonase (PG) (LOC_Os08g44020)), the cell wall structure (fasciclin-like arabinogalactan protein 8 (FLA8), LOC_Os01g62380) and hemicellulose synthesis through glycosyltransferases (LOC_Os03g48610). The changed levels of these cell wall loosening and cell wall precursor

Table 4 Differentially expressed transcripts of cell wall genes

Probe_Set_ID	Locus name	Annotation	Fold change	
			F- μ g vs. G-1g	F- μ g vs. F-1g
osaffx.13411.1.s1_at	LOC_Os03g48610	Protein transferase, transferring glycosyl groups	2.877	2.454
os.42475.1.s2_at	LOC_Os01g62380	Fasciclin-like arabinogalactan protein 8 precursor	2.449	3.860
os.28032.1.a1_at	LOC_Os01g21070	Endoglucanase 1 precursor	0.281	0.165
os.50125.1.s1_at	LOC_Os04g57860	Endoglucanase precursor	0.087	0.373
os.50320.1.s1_at	LOC_Os08g44020	Protein lyase	2.858	4.093
os.22839.1.s2_at	LOC_Os06g48200	Xyloglucan endotransglucosylase/hydrolase protein 23 precursor	2.296	2.146
os.9312.1.s1_at	LOC_Os03g01270	Beta-expansin 1a precursor	3.041	2.070
os.27505.1.s1_at	LOC_Os01g57854	Pectinesterase-1 precursor	0.054	0.248

synthesis-related genes under $F-\mu g$ may contribute to microgravity by affecting cell wall relaxation and extension.

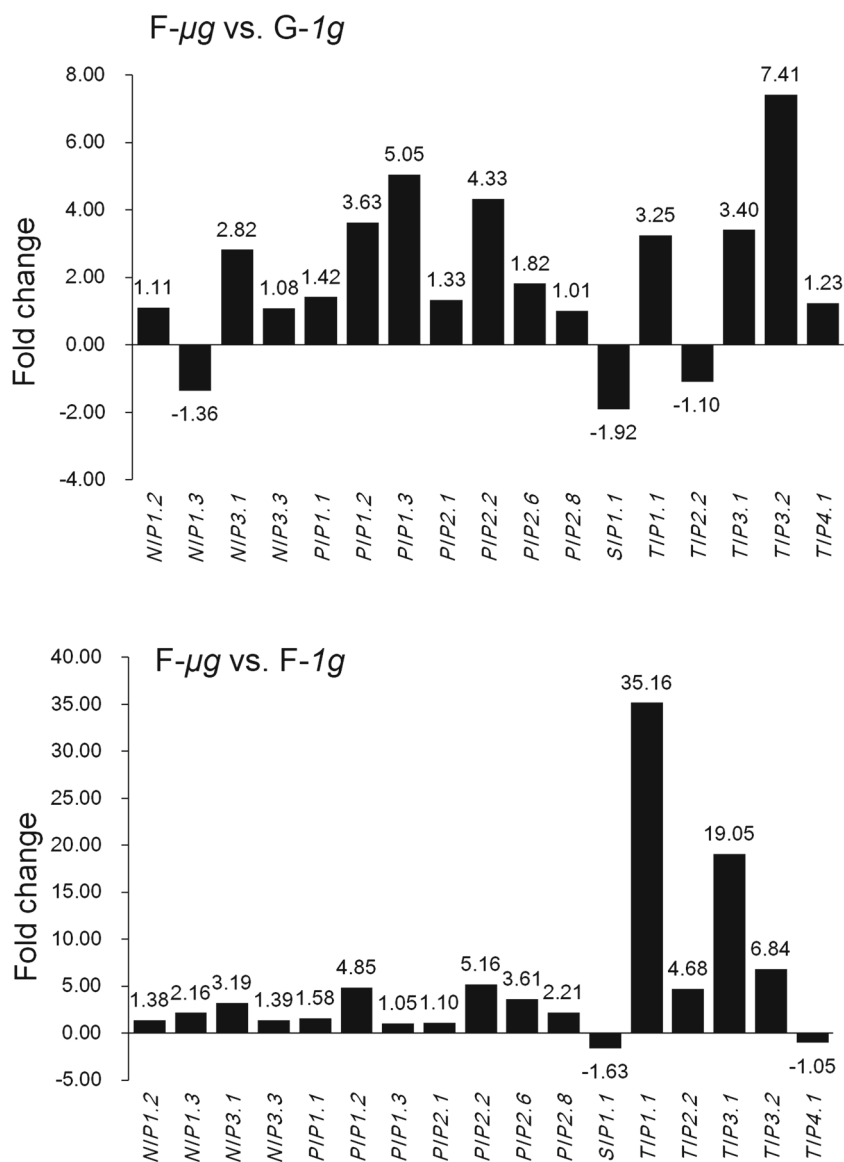
Changes in the Expression of Water Channel Genes in Rice Calli Under Microgravity

Gravity causes changes in the expression of water channel genes during plant gravitropism (Hu et al. 2007). Here, we analysed the transcripts of OsPIPs, OsTIPs and OsNIPs, i.e., genes of the aquaporin gene family (Fig. 4), most of which were up-regulated under $F-\mu g$ vs. both $G-1g$ and $F-1g$ (the fold change was greater than or equal to 2). The false discovery rate (p) of changes in the expression of three genes, PIP1.3 (LOC_Os02g57720), TIP3.2 (LOC_Os04g44570) and NIP3.1 (LOC_Os10g36924), under $F-\mu g$ vs. both $G-1g$ and $F-1g$ was lower than 0.05.

Fig. 4 Differentially expressed transcripts of OsPIPs, OsTIPs and OsNIPs in rice calli under $F-\mu g$ vs. both $G-1g$ and $F-1g$

Discussion

The rice calli used in this experiment were obtained from embryos as explants induced by 2,4-D and subcultured with 2,4-D according to the conventional method of plant tissue culture (Karthikeyan et al. 2009). To understand whether exogenous plant hormones can affect the response of cells to microgravity stimulation, we also carried out spaceflight experiments on habituated rice calli that were subcultured on hormone-free medium (Jin et al. 2015). The experimental results show that exogenous 2,4-D in the culture influenced the effects of the spaceflight experiments on the rice calli. The results indicated that for many aspects, e.g., most of the MR genes related to glycolysis and most of the MR transcript factors (TFs), the reaction trend induced under microgravity in these two types of rice calli was indeed



different, and opposite trends were observed in some cases. However, the effect of microgravity on the cell wall were consistent in both callus types (Jin et al. 2015). The results obtained from previous spaceflight experiments in which research was conducted on the transcriptome of *Arabidopsis* are inconsistent across many aspects due to the differences in experimental design and the diverse hardware used (Fengler et al. 2015; Johnson et al. 2017). In particular, analysis of individual plant organs or entire seedlings indicated organ-specific changes in response to spaceflight (Paul et al. 2013). However, cell wall remodelling and stress response results were similar in three BRIC-16 experiments (Johnson et al. 2017). Rice calli cultured with 2,4-D and hormone-free cultures represented two types of tissue with different physiological states. A transcriptome study showed that there are many differences between habituated cells and their original state (Pischke et al. 2006). The fact that cells behave differently under microgravity could be due to the following two reasons: the presence of 2,4-D may affect plant cell responses to microgravity stimulation, and habituated cells with a different physiological status may also respond differently to microgravity. We present the integrated results of these two types of callus under microgravity treatment and also emphasize and discuss the consistency of the results of the effects of microgravity on the metabolism and modification of the cell wall of rice calli.

Many differentially expressed genes were observed between the F-1g and G-1g groups, even under identical culture conditions. The major impact of nonmicrogravity-related spaceflight conditions on gene expression during the same mission (SZ-8) was demonstrated in a previous report (Fengler et al. 2015). This impact was assumed to be the result of the effects of spaceflight-related environmental conditions, including space radiation (Fengler et al. 2015). Therefore, in this paper, we chose only the genes whose expression under microgravity was different from G-1g and F-1g to conduct the MapMan analysis.

The question of how plants adapt to microgravity conditions is important. The support system of a plant is the tissue that is resistant to gravity on the ground. The plant cell wall plays a crucial role in supportive tissue. Thus, the plant cell wall is involved in the adaptation of plants to microgravity.

Cell wall genes and cell wall-related genes exhibit differences during gravitropism (Hoson et al. 2002a, 2004; Strohm et al. 2012). Two endoglucanases (LOC_Os01g21070, LOC_Os04g57860) were down-regulated under F- μ g vs. both G-1g and F-1g. Plant endoglucanases have been hypothesized to play a variety of roles in cell wall biosynthesis and modification (Libertini et al. 2004). Pectin methylesterases (PME) modulate cell-wall-matrix properties and play a role in the control of cell growth (Peaucelle et al. 2008). Pectins form a matrix around cellulose, and homogalacturonan (HG), one of the main pectic

polymers, is secreted in a highly methylesterified form and is selectively de-methylesterified by PME (Pelloux et al. 2007). After de-methylesterification, pectate can form Ca^{2+} -pectate cross-linked complexes (Grant et al. 1973). The more Ca^{2+} -pectate that is present, the denser the gel will be, and the more inextensible the cell wall is expected to be Peaucelle et al. (2012). The pectinesterase-1 precursor (LOC_Os01g57854), which was down-regulated 18-fold under F- μ g vs. F-1g and 4-fold under F- μ g vs. G-1g, may be more beneficial for cell wall extensibility in the rice calli response to microgravity.

Five of the up-regulated transcripts corresponded to proteins involved in primary cell wall modification (xyloglucan endotransglucosylase/hydrolase protein 23 (OsXTH23, LOC_Os06g48200), beta-expansin 1a precursor (LOC_Os03g01270)), cell wall degradation (polygalacturonase (PG) (LOC_Os08g44020)), cell wall structure (fasciclin-like arabinogalactan protein 8 (FLA8), LOC_Os01g62380) and hemicellulose synthesis through glycosyltransferases (LOC_Os03g48610). OsXTHs, beta-expansin, PG, FLAs and glycosyltransferases have been reported to play roles in cell wall loosening and cell growth. For example, OsXTH23 (OsXTR3) plays a role in intermodal elongation in rice (Uozu et al. 2000). PG degrades α -(1 \rightarrow 4) glycosidic bonds linking galacturonopyranose units. In addition, OsXTR3 (XTH23) is up-regulated by gibberellin and BR (Uozu et al. 2000). Endogenous ethylene can induce XTH gene expression, which plays a role in tomato and apple fruit ripening by loosening the cell wall (Munoz-Bertomeu et al. 2013).

The cell wall may tend towards extensibility in an environment of microgravity, and Hoson reported cell wall loosening in rice coleoptiles under microgravity conditions in space (Hoson et al. 2004). Several MR genes related to cell wall proteins, modifications and metabolism were identified that may also be related to cell wall extensibility. The trends in the changes of cell wall metabolism were similar across groups of rice calli cultured without 2,4-D under microgravity (Jin et al. 2015). In rice calli cultured without 2,4-D under microgravity conditions, nine transcripts related to cell wall metabolism and modification were found. Two of the transcripts were down-regulated, and they were involved in secondary wall metabolism (cellulose synthase: LOC_Os02g49332, LOC_Os04g35020). Seven transcripts were up-regulated, and they were proteins associated with cell wall loosening (lipoprotein A-like double-psi beta-barrel-containing, LOC_Os04g44780), proteins related to the modification of the primary cell wall (xyloglucan endotransglycosylase LOC_Os06g48200, α expansin precursor protein 20 LOC_Os06g41700), and proteins involved in the cell structure (AGPs, LOC_Os08g38270, LOC_Os08g23180, LOC_Os06g30920, LRR, LOC_Os06g49100). Cell wall loosening and greater

cell elongation in rice coleoptiles under microgravity were also reported by Hoson et al. (2002b, 2004, 2014). The size of the callus cultures was also enhanced under microgravity compared to the 1-g controls (Fengler et al. 2015).

Due to the operation of the International Space Station, more in-depth studies on the effects of microgravity on plant growth and morphogenesis have been conducted, and the effects of microgravity are becoming more specified. As plant stem and root elongation is promoted and lateral extension is inhibited, an auto-morphogenesis phenomenon has appeared. The emergence of these changes is related to the effects of microgravity on the cell wall (Hoson et al. 2014).

In the elongation region of an organ where gravitropic bending occurs, the shape of the plant cell is maintained by the balance between cell wall rigidity and turgor pressure produced by intracellular vacuoles (Cai et al. 2016). To elongate, a cell must loosen its periphery wall by reducing cell wall rigidity and then increasing intracellular turgor pressure. The cell wall needs to be flexible to ensure the metabolism of the primary cell wall and to simultaneously allow the transport of water into the intracellular vacuoles. This equilibrium process in the elongation zone on both sides of a cell causes gravitropic bending (Cui et al. 2005, Hu et al. 2007, 2013).

According to gene chip data (<http://www.ebi.ac.uk/array-express>, E-MTAB-2518, <http://www.ncbi.nlm.nih.gov/geo>, GSE78839) obtained during a space flight experiment (Fengler et al. 2015; Jin et al. 2015), the gene expression levels of plasma membrane aquaporins (PIPs) and tonoplast aquaporins (TIPs) in *Arabidopsis thaliana* and rice cells grown under microgravity conditions were generally up-regulated. Here, we found PIP1.3 (LOC_Os02g57720), TIP3.2 (LOC_Os04g44570) and NIP3.1 (LOC_Os10g36924) were up-regulated under F- μ g vs. F-1g and F- μ g vs. G-1g (Fig. 4).

Although the gene chip results showed increased expression of some aquaporin genes under microgravity, we have no direct evidence to prove that turgor increased inside the plant cell under microgravity. However, the plant cell turgor pressure might be maintained through changes in vacuolation under microgravity (Klymchuk et al. 2003). In spite of this, we can envision the collapse of the original

balance and the rebuilding of a new balance between cell wall rigidity and cell turgor pressure caused by cell wall loosening under microgravity conditions. This may also be the cause of the growth variation of plant organs under microgravity.

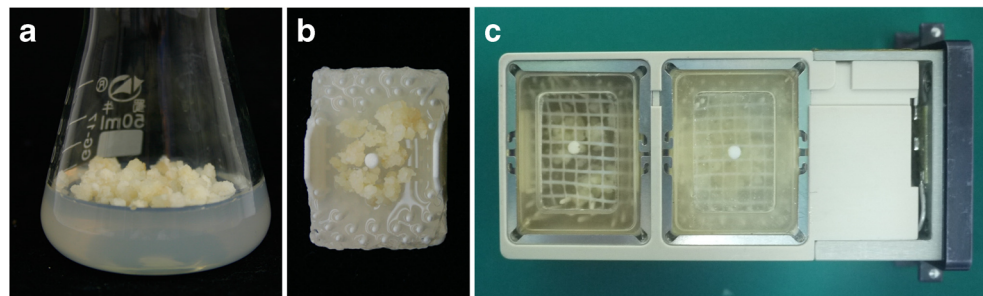
The growth of terrestrial plants that evolved from the sea is guided by gravity under a constant direction and magnitude on Earth. Plants developed a set of gravity resistance mechanisms during evolution, but they lose the stable and reliable signal of gravity under microgravity conditions when they are subjected to orbital flight in space. Thus, plant growth and morphogenesis will be modified under space conditions. These changes can be explained by the effects of microgravity on plant cell wall extensibility, aquaporin gene expression, and the establishment of a new balance between cell wall rigidity and cell turgor pressure under microgravity conditions. The loosening of the cell wall may be regarded as the adaptation of plants under microgravity due to the disuse of the support system. The involvement of cortical microtubules in growth stimulation in space has been demonstrated (Hoson et al. 2004). The cortical microtubules likely lead to cell wall changes through signal transduction processes involved in plant gravity resistance (Hoson et al. 2014). All of these factors together create the effects of microgravity on cell growth.

Materials and Methods

Materials

Rice calli were generated from embryos of *Oryza sativa* under sterile conditions. Calli were placed on N6 medium (containing 2 mg/L 2,4-D, 1% agar) and were grown at 23 ± 2 °C in the dark. Rice calli were cultured on a specimen slide with N6 medium containing 2 mg/L 2,4-D; a 0.2-g sample of cells was used on each specimen slide (Fig. 5a). Several hours before launch, specimen slides carrying rice calli subjected to 4 days of pre-culture were transferred to the housing chamber (Fig. 5b). Then, the experiment-unique equipment (EUE) was loaded into the culture box (SIMBOX) aboard Shenzhou 8 (Jin et al. 2015).

Fig. 5 The rice calli used in the Shenzhou 8 experiment. **a** Twenty-day-old rice calli. **b** Rice calli pre-cultured on a slide. **c** Rice calli transferred into the chamber



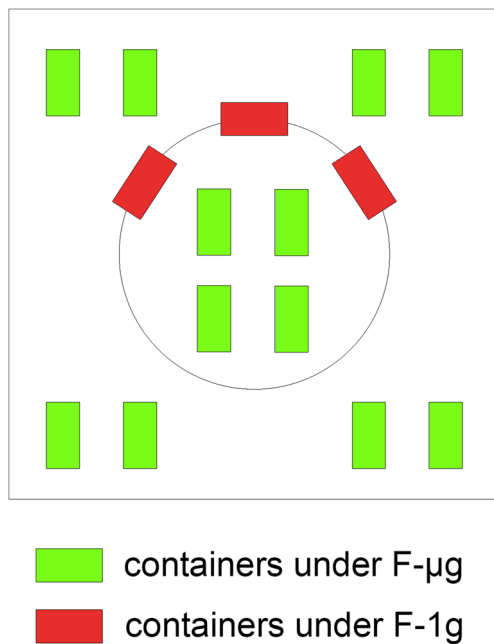


Fig. 6 Diagram of the distribution and conditions of the experimental culture containers in the SIMBOX: two containers under the $F-\mu g$ condition and one container under the $F-1g$ state, as 1- g inflight controls

Hardware

The experimental facility was designed and constructed by Astrium Space Transportation (ASTRIUM) in Germany. The EUE containing the culture chamber and fixation unit has been described in detail in a previous report (Jin et al. 2015).

Gravity Treatment

Similar to most of the research on space biology, we set up $G-1g$ as a control. The effects of spaceflight were determined by comparing 1- g ground controls ($G-1g$) with the spaceflight samples ($F-\mu g$). In this experiment, a 1- g centrifuge was included on board. A comparison between $F-\mu g$ and

$F-1g$ samples allowed us to further distinguish between the effects of spaceflight and true microgravity on living systems. The effects of nonmicrogravity were evaluated by comparing $F-1g$ and $G-1g$ samples. Therefore, three treatments were used in our experiment, designated as $F-\mu g$, $F-1g$, and $G-1g$. The $F-\mu g$ and $F-1g$ sites are presented in Fig. 6. The time schedule for the microgravity treatment is shown in Fig. 7. The time from launch until the onset of microgravity was approximately 10 min, and the time from launch until the centrifuge was switched on was approximately 36 min, which is similar to the experiment conducted by Fengler et al. (2015). Calli were fixed with RNAlater (ABI, US) after approximately 324 h of flight. Following the recovery of the sample module after 17 days of flight, the RNAlater-treated samples were transferred to Eppendorf cups and stored at a temperature lower than $-20\text{ }^{\circ}\text{C}$ until RNA extraction. Because of the limited hardware capacities, we had only 2 EUEs for $F-\mu g$, 1 EUE for $F-1g$, and 2 EUEs for $G-1g$. Two biological replicates were taken from each EUE of $F-\mu g$ due to scarce availability of spaceflight specimens, and a total of 4 biological replicates of $F-\mu g$ were obtained. Three biological replicates were taken from the EUE of $F-1g$, and 3 biological replicates were randomly selected from the 2 EUEs of $G-1g$.

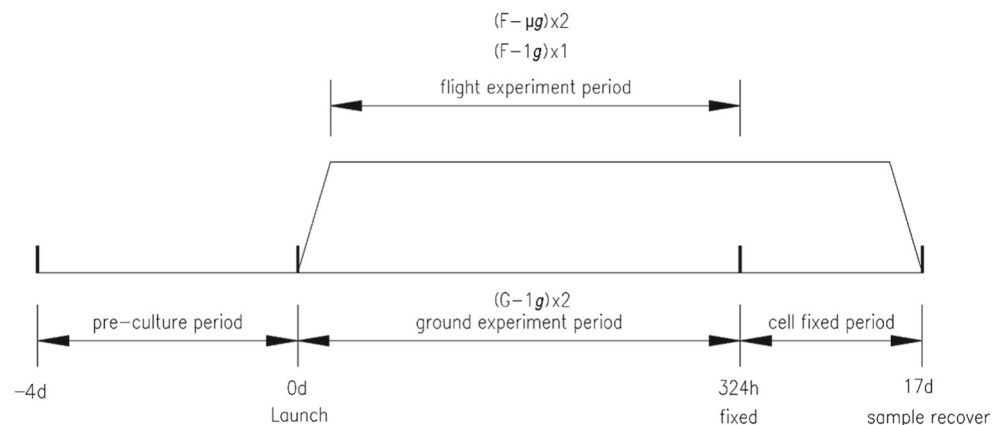
RNA Isolation

Total RNA was extracted using the QIAGEN RNAeasy Mini Kit (Qiagen, CA) according to the manufacturer's instructions. The quality and integrity of the isolated RNA were assessed using an Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA).

Affymetrix Microarray Hybridization

Affymetrix microarray hybridization was performed by the Shanghai Bio Corporation. We have explained the details of this procedure in a previous report (Jin et al. 2015).

Fig. 7 Time schedule for the rice calli cultured on board the Chinese spacecraft SZ-8 under three conditions



Array Data Analysis, Probe Set Filtering and Data Analysis

Three groups of samples were used for microarray analysis. Two biological replicates (two replicates for each biological replicate) were performed for F- μ g, and three replicates were performed for F-1g and G-1g. MR-unique differential expression probe sets were selected using the procedures we described in a previous report (Jin et al. 2015). Microarray data are available in the NCBI database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78839>) under accession number GSE78839.

We primarily employed MapMan to analyse the pathways of MR genes (Thimm et al. 2004; Usadel et al. 2005). MapMan is available at <http://gabi.rzpd.de/projects/MapMan/>.

Affymetrix Rice Array 1.12. Cluster online [ShanghaiBio Corporation, www.ebioservice.com] was employed to perform hierarchical clustering analysis using the Euclidean distance and the average linkage method to create probe sets and sample trees. JAVA Treeview was employed to view cluster images (Page 1996).

Acknowledgements The Technology and Engineering Center for Space Utilization (Chinese Academy of Sciences) is acknowledged for project coordination. The spaceflight equipment was developed by the Shanghai Institute of Technical Physics (Chinese Academy of Sciences) Microarray analysis was conducted by Shanghai Biochip Co., Ltd., and Xue Li (Institute of Plant Physiology and Ecology, SIBS, CAS) helped with the data analysis.

Author Contributions Conception, design, experimentation, data analysis and manuscript writing: Jing Jin Experimentation: Haiying Chen. Conception, design and final approval of manuscript: Weiming Cai.

Funding Information This work was supported by the National Natural Science Foundation of China (Grant Nos. U1738107, 31570859, 31500236 and 31600684), Strategic Priority Research Program of the Chinese Academy of Sciences (Grant Nos. XDA04020202-15, XDA04020415), and the China Manned Space Flight Technology Project.

Compliance with Ethical Standards

Conflict of interests All authors reviewed the manuscript and declared no competing financial interests.

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