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Gene expression changes in Arabidopsis seedlings during short- to long-term exposure to 3-D clinorotation

Hyuncheol Soh · Chungkyun Auh · Woong-Young Soh · Kyeongsik Han · Donggiun Kim · Sukchan Lee · Yong Rhee

Abstract Seedlings of *Arabidopsis thaliana* (cv. Columbia) were used to evaluate dynamic transcriptional-level genome responses to simulated microgravity condition created by 3-D clinorotation. The DNA chip data analysis showed that the plant may respond to simulated microgravity by dynamic induction (up- and down-regulations) of the responsive genes in the genome. The qRT-PCR results on the investigated genes showed that the expression patterns of the genes (molecular response) were generally similar to the physiological response patterns detected in stress-challenged plants. Expression patterns were categorized into short or continual up- or down-regulated patterns, as well as stochastic changes from short- to long-term simulated microgravity stress. The induced genes are then assumed to establish a new molecular plasticity to the newly adjusted genome status in the basic milieu of maintaining homeostasis during the process of adaptation to simulated microgravity.

Keywords Arabidopsis · Gene expression · Microarray · Simulated microgravity · 3-D Clinorotation

Abbreviations

dCHIP	DNA-chip analyzer
qRT-PCR	Quantitative real-time transcription polymerase chain reaction
3-D clinostat	3-Dimensional clinostat
IAA	Indole-3-acetic acid

H. Soh · S. Lee (✉)
Department of Genetic Engineering,
Sungkyunkwan University, Suwon 440-746, Korea
e-mail: sukchan@skku.ac.kr

C. Auh
Division of Life Sciences,
Mokpo National University, Muan 534-729, Korea

W.-Y. Soh
Department of Biological Science,
Chonbuk National University, Jeonju 561-756, Korea

K. Han
Department of Beauty and Cosmetics, College of Health and Welfare, Woosuk University, Samnye, Chonbuk 565-701, Korea

D. Kim
Department of Biological Sciences, Silla University,
Busan 617-736, Korea

Y. Rhee (✉)
The Institute of Life Science and Technology,
Sungkyunkwan University, Suwon 440-746, Korea
e-mail: rheepd@daum.net; rheepd@skku.edu

Introduction

The growth patterns of higher plants show bi-directionality with downward orientation in the root and upward orientation in the leaves under the normal $1g$ force field of Earth. Such gravitropic growth of plants assumes the evolutionarily best-selected morphological/physiological outcomes for the photosynthetic apparatus to maximize uptake of water/nutrients (by the roots) from the soil, as well as solar photons (by the leaves). So, changes of normal gravity force can collectively constitute a novel stress which, similar to other environmental stresses, serves as a challenge to growth and development in plants. Especially, minimization or reduction of normal gravity (i.e.,

microgravity) could be created during true space flight or by machine-based simulation (clinorotation) for ground-based experiments, and considerable amounts of documentation have been accumulated about responses of plants under minimized gravity conditions.

The majority of traditional research works have focused on developmental and physiological responses of plants to minimized gravity such as morphological changes or gravitation-related hormone/calcium redistribution (Merkys and Laurinavicius 1991; Hoson 1994; Hilaire et al. 1995; Barlow 1998). On the other hand, continual progress has been also made toward a deep understanding of plant responses to minimized gravity conditions at the global genome level. For instance, microgravity effects were investigated on gene expression (at transcription level) as well as morphological and metabolic changes for dwarf wheat under true microgravity condition in space flight; the collective results showed that there were no significant alterations on such examined traits (Stutte et al. 2006). However, apart from it, the significant alterations of gene expression under minimized gravity conditions have been occasionally reported in many research works so far. An example comes from a study with the fern *Ceratopteris richardii*. The authors reported that transcriptional changes of the genes could be induced in this fern species under microgravity condition in space flight (Salmi and Roux 2008). Even though minimized gravity effect was investigated at the single cell level in this case, this work provided a promising clue to extrapolate the results to multi-cellular organisms in response to altered (minimized) gravity condition. In fact, most of interesting reports have been accumulated mainly for an easily manipulated weed plant species with small genome size, Arabidopsis. The gene expression change was reported from Arabidopsis seedlings exposed to a true microgravity environment during space flight (Paul et al. 2005). In this case, 182 genes in Arabidopsis genome showed differential expression in response to the space flight condition by more than fourfolds in comparison of ground control plants. In addition, Arabidopsis cell cultures were exposed to true microgravity on a sounding rocket and altered changes of the gene activity at transcription level were profiled via DNA chip (Martzivanou et al. 2006).

From substantial documentation so far, it is now obvious from the above researches that plant genome is responsive to altered (minimized) gravity condition and furthermore, its sensitivity is likely to be dependent upon their genome size. In fact, it might be quite difficult to screen out significant changes of gene activity in polyploid wheat genome with multiple copies of the genes under minimized gravity condition which could not be a potentially strong stress trigger to disturb stable genomic milieu.

In general, the living organisms should stay in the altered (zero or minimized) gravity conditions for a long period during real space flight. So, long-term exposure of plants to simulated microgravity conditions would be desirable in ground-based experiments to obtain comparable results to the information from real space flight to draw generalization about plant gene response to space environment. However, apart from one exceptional case in which the global resetting of gene expression in Arabidopsis genome was detected under a simulated microgravity environment via clinorotation for a prolonged period up to 6 days (Centis-Aubay et al. 2003), most of ground-based research works have been conducted under relatively short-term exposure of plants to minimized gravity conditions. Kittang et al. (2004) performed DNA chip experiments for profiling of the genes with altered expressions under simulated microgravity condition (1 or 24 h) and found out that about 2.2% of total 8,000 genes screened showed differential expression levels. The experiment was done for cultured Arabidopsis cells to monitor the contents of translational gene products in response to simulated microgravity condition (clinorotation) (Wang et al. 2006; Barjaktarovic et al. 2007). In this case, proteome of the exposed undifferentiated plant cells (calli) mounted a significant response to simulated microgravity conditions within a period of 10 min to 16 h. Furthermore, these earlier studies provide no information about time-course based response pattern of the plant genes from short- to long-term exposure to simulated microgravity. Actually, the information would be valuable because they provide us with important clues on the prediction of molecular tolerance or adaptation of the plant genome in real space environment.

The main objective of this study was to identify the responsive genes during a long-period (from 1 h to 6 days) exposure of Arabidopsis to simulated microgravity conditions and, subsequently, investigate time-course based gene expression patterns for them. The results are discussed in the context of long-term based plant gene responses in relation to their possible functions for tolerance and adaptation to simulated microgravity condition.

Materials and methods

Plant materials and exposure to 3-D clinorotation

All experiments were conducted using 6-day-old seedlings of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia). The seeds (Arabidopsis Biological Resource Center, The Ohio State University) were stored in a refrigerator (4°C) for 12 h prior to plating in Petri dishes for germination and initially surface-sterilized with 70% (v/v) ethanol for

5 min. After treating the seeds with a 20% commercial bleach solution, they were subsequently washed five times in sterile distilled water. The sterilized seeds were finally germinated on 1.5% (w/w) agar plates containing full-strength Murashige and Skoog nutrient medium supplemented with 3% sucrose. The seedlings were cultivated in a growth chamber with 18 h of fluorescent light (40 W m^{-2}) and a constant temperature of 24°C.

The 3-D clinostat consisted of two independently rotating frames, one rotating within the other (but in a different plane) to maximize the microgravity effect. All manipulations were carried out in accordance with the instructions of manufacturer (Watanabe Machine Design Associates, Japan; Yamashita et al. 1997) and refer to the citations (Hoson et al. 1996, 1997; Yamashita et al. 1997) for details and specifications of the machine. Under the same environmental conditions as were employed for seedling germination, the selected seedlings were exposed to simulated microgravity conditions with a 3-D clinostat, and the intact seedlings (the control) were maintained separately on the shelf during the experiments. All of the seedlings were frozen in liquid nitrogen within a few seconds immediately after harvesting. This step excluded any possibility of 1 g interference during the handling procedure for simulated microgravity-treated seedlings.

RNA preparation, probe labeling, and microarray hybridization

The harvested seedlings were immediately frozen in liquid nitrogen within a few seconds after harvesting and stored in a deep freezer at −70°C. RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and the RNA integrity was confirmed via separation on 1.2% (w/w) agarose-formaldehyde gels with ethidium bromide staining. For the DNA chip analysis of simulated microgravity-exposed and control seedlings, the synthesis of target cRNA probes and hybridization was conducted using Agilent's Low RNA Input Linear Amplification Kit (Agilent Technology, Palo Alto, CA, USA), in accordance with the manufacturer's instructions. In brief, each 1 μg of total RNA and T7 promoter primer were mixed and incubated at 65°C for 10 min. The cDNA master mix (5× first strand buffer, 0.1 M DTT, 10 mM dNTP mix, RNase-Out, and MMLV-RT) was prepared and added to the reaction. The samples were incubated for 2 h at 40°C and then the reverse transcription (RT) and double-strand DNA synthesis was completed for 15 min of incubation at 65°C. The transcription master mix was prepared according to the manufacturer's recommendations (4× transcription buffer, 0.1 M DTT, NTP mix, 50% PEG, RNase-Out, inorganic pyro-phosphatase, T7 RNA polymerase and Cyanine 3/5-CTP). Transcription of dsDNA was carried out by the adding the transcription master mix to the dsDNA reaction samples, followed by 2 h of incubation at 40°C. Amplified and labeled cRNA was purified on the cRNA Clean-up Module (Agilent Technology) according to the manufacturer's recommended protocols. Labeled target cRNA was quantified with an ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA). After checking the labeling efficiency, cRNA fragmentation was conducted via the addition of 10× blocking agent and 25× fragmentation buffer, followed by 30 min of incubation at 60°C. The fragmented cRNA was then resuspended with 2× hybridization buffer and pipetted directly onto the assembled Arabidopsis Gene Expression Microarray (44 K) (Agilent Technology). The arrays were hybridized at 65°C for 17 h using a hybridization oven (Agilent Technology). The hybridized microarrays were then washed as per the manufacturer's washing protocols.

Acquisition and analysis of DNA chip data

The hybridized images were scanned using a DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology). Probe sets with signal values lower than the detectable range were adjusted to 75, and probe sets with the values of 75 for all conditions were removed from subsequent analysis. All data normalization and selection of fold-changed genes were conducted using Gene Spring GX 7.3 (Agilent Technology); scanned DNA arrays were normalized to a baseline array with median overall expression using dCHIP (Li and Wong 2001). The averages of normalized ratios were calculated by dividing the average of the normalized signal channel intensity by the average of the normalized control channel intensity. The standard deviation of the vertical controls (four biological replicates) was employed to identify genes of significant changes relative to the vertical controls (P value < 0.05). Functional annotation of the identified genes was conducted by gene ontology analysis with the EasyGO program (http://www.bioinformatics.cau.edu.cn/easygo) (Zhou and Su 2007).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Highly sensitive reaction qRT-PCR was employed to confirm the expression of the selected genes. At least two independent biological replicates of each sample and three technical replicates of each biological replicate were used for real-time PCR analysis. The genes and their qRT-PCR primers are presented in Table 1. The 18S rRNA (Martzivanou et al. 2006) was amplified as an internal reference for all qRT-PCRs using the following primers: forward (5′-GATGAGCCTGCGTAGTATTAGG-3′)

Table 1 Profiles of the 18 selected genes that were up- and down- regulated in the time frame designated in the present study, including DNA primer sequences for qRT-PCR and locus ID from the Arabidopsis genome database (TAIR) (http://www.arabidopsis.org), as well as the main reference for each gene

Genes	Locus ID	Primers	References
ABF3 (abscisic acid responsive element-binding factor 3)	At4g34000	F TTT GGG CGA GTG AGA AAA AC / R TTT GAG TTG CGC AAT TTC TG	Fujii and Zhu (2009)
AtPUB19 (U-box domain-containing protein)	At1g60190	F ACT CGC GGT GAA GAT ATT GG / R GTG TAA AGC GAC CCC ATG AT	Wiborg et al. (2008)
CML38 (calmodulin-like 38)	At1g76650	F TCA CCA AAG AGG GAA GAT TCA G / R CCC ATC TCC GTC CGT ATC A	Walley et al. (2007)
CYP705A5	At5g47990	F TAG CGG AAG GAG AGC TTG TC / R GAG CCA TGG TCA AGG TCA TT	Ehlting et al. (2008)
ERD6 (early-responsive to dehydration 6)	At1g54730	F TCT CGG CTG GTT AGC AAT CT / R CAC CAC GAA GAC CCT TAG GA	Kiyosue et al. (1998)
Ext (extensin-like protein)	At3g24480	F CCC ATG CAT TTT CAG GAC TT / R TCC TTT AAC GCT CCG TTG TC	Martzivanou and Hampp (2003)
FC-1 (ferrochelatase-1)	At5g26030	F ACA GAT TTC CCA GGT CAT GC / R CAG TCA CAG AAC GAG CTT GC	Nagai et al. (2007)
FLS2 (flagellin-sensitive 2)	At5g46330	F CGG CTC TTC TTC TTG TCC TG / R GCT TGC TCC AAC TCT TTT GG	Chinchilla et al. (2007)
HSP81-2 (heat shock protein 81-2)	At5g56030	F TGA TGA GCC AAA CAC TTT CG / R TCG ACT TCC TCC ATC TTG CT	Wang et al. (2004)
IAA6 (IAA transcriptional factor 6)	At1g52830	F ACT GCC GGT TGT GAA GAG TC / R CGA ACC AAG GTC AAT CTT CC	Overvoorde et al. (2005)
LTP6 (lipid transfer protein 6)	At5g01870	F ATT TGC ATG GGG TTC TGA AG / R GAT GCG CTA GTT GCC TGT TT	DeBono et al. (2009)
ERF sub (ethylene response factor subfamily protein)	At5g51190	F AAAG CAT GAG GAC TTG GGA GA / R GCG TTA ATG GAC AAG CAT CA	Fujimoto et al. (2000)
PDK1 (3-phosphoinositide-dependent protein kinase 1)	At5g04510	F TAG CTC CAG ATC CTG CGT CT / R ATG GAA CCC GAT GAT TCA G	Zegzouti et al. (2006)
PLP2 (patatin-like protein 2)	At2g26560	F TGC CGT TAT CCT TGG TTT TC / R AGG GTC GGC CTT CCT TAT TA	La Camera et al. (2009)
SAM-MT (S-adenosylmethionine-dependent methyltransferase)	At5g37970	F ACC AGT TGT CAG CAT TGT GG / R GAC CAT CAC CAC CGT TCA TT	Finnegan and Kovac (2000)
VSP1 (vegetative storage protein 1)	At5g24780	F ACC TCT TGG AAC TCG GGA TT / R ACT TGC GTC AAC TTC GAT CC	Berger et al. (2002)
WRKY40	At1g80840	F GTG GAG GAT CAG TCC GTG TT / R TTC TTC TGT TTG CTG CAA CG	Xu et al. (2006)

Table 1 continued

Genes	Primers	Locus ID	References
XTR6 (xyloglucanendo-transglycosylase 6)	F GGT TGC TCT GCT TGC TTC TT R TCC TGA GCC TGA AGC TTT GT	At4g25810	Claisse et al. (2007)
18S rRNA	F GAT GAG CCT GCG TAG TAT TAG R AGT CAT TCC GAA GAA CAC TTG C		Martzivanou et al. (2006)

The 18S rRNA was used as an internal reference for qRT-PCR

and reverse (5′-AGTCATTCCGAAGAACACTTGC-3′). Reverse transcription was conducted to synthesize the complementary DNA by mixing the following components in order: 5 μg total RNA, 5 μl 5× RT buffer, 1.14 μl oligo-dT (18-mer) (final conc. 1.5 pmol/μl), 1.25 μl 10 mM dNTPs (final conc. 0.5 mM), 0.05 μl MMLV reverse transcriptase (200 U/μl) (Promega, Madison, WI, USA) (final conc. 10 U), and distilled water (DNase free) up to a total volume of 25 μl. After the reaction was incubated for 1 h at 37°C, real-time PCR was conducted by adding the following reagents: 3 μl synthesized cDNA, 1 μl primer (final conc. 0.25 mM), and 10 μl 2× SYBR mixture solution (Qiagen, Valencia, CA, USA), and distilled water up to a total volume of 20 μl. The cDNA amplification was conducted in a Rotorgen 3000 Real-time Amplification System (Corbett Research, Mortlake, NSW, Australia) with the following thermal profile: 40 cycles at 94°C (10 s), 59°C (20 s), 72°C (15 s), and, finally, 95°C. The PCR products were analyzed on 1.5% ethidium bromide-stained agarose gels. Amplification of all cDNAs with specific primers yielded PCR products of the predicted sizes (150–200 bp) from the respective gene sequences. The data were normalized using a reference (the ribosomal 18S transcript), and the relative quantity of mRNA was calculated by the comparative Ct method based on the program provided by the manufacturer (Corbett Research) for the determination of melting and standard curves. Oligo-nucleotide primers were designed with optimal Tm (55–60°C) and G/C content (40–60%) using Primer 3 Output Software (http://www.justbio.com/primer/index.php). Data analysis and graphs were plotted on GraphPad Prism v.5 (GraphPad Software, San Diego, CA, USA). The statistical significances of differences in expression levels were determined by one-factor analysis of variance (ANOVA) followed by Tukey's test, and differences were not significant at P value >0.05.

Results

Simulated microgravity treatment

In the initial (1 day) 3-D clinorotation, no distinct phenotypic differences were observed between the intact seedlings (the control) at 1g force and the exposed Arabidopsis seedlings to simulated microgravity condition. However, at the fourth day of clinorotation treatment, multi-directional growth occurred in both the shoots and roots of the treated seedlings, as compared to the intact controls with the normal vertical growth pattern; the observed morphological changes were maximized at 6 days of 3-D clinorotation (Fig. 1a).

DNA chip analysis, gene screening and quantitative real-time polymerase chain reaction (qRT-PCR)

DNA chip experiment were conducted only for *A. thaliana* seedlings collected at the initial and the final time points (1 h and 6 days) of 3-D clinorotation treatment. Time-course based expression patterns for the selected genes were investigated for the exposed seedlings to 3-D clinorotation at all the clinorotation time points (1 h and 1, 4, 6 days) by using qRT-PCR (Fig. 1b).

The functional annotation for the screened genes

The results showed that more than 900 diverse genes were up-regulated (more than 600 genes) or down-regulated (about 300 genes) in the combined DNA chip data (Fig. 2a, b). For the 1 h of 3-D clinorotation, approximately 400 genes exhibited differential expressions, with 302 up-regulated and 110 down-regulated genes (Fig. 2a). These genes were classified into functionally diverse groups, such as those encoding for ROS-related enzymes (oxido-

reductase), transporters (substrate-specific/*trans*-membrane proteins), transcription (transcription regulator), gene regulation/nucleic acid metabolism (nucleic acid binding, nucleotide binding), or general metabolism related proteins involved in response to other environmental stresses (ion binding, isomerase, protein binding, structural molecule binding, ligase, lyase, lipid binding, hydrolase, and transferase). Interestingly, isomerase-related genes were detected only in the seedlings with the initial exposure (1 h) to simulated microgravity. The genes of lipid-binding proteins were suppressed completely, whereas the genes representing diverse functions such as structural molecule, nucleotide binding, lyase, ligase, and isomerase were up-regulated only at the initial (1 h) exposure to simulated microgravity.

On the other hand, 325 up-regulated and 177 down-regulated genes (totaling ∼500 genes) were scored for the long-term (6 days) exposure to simulated microgravity (Fig. 2b). The genes were categorized into the same functional groups as the screened genes for the 1 h of 3-D clinorotation. Genes for antioxidant and enzyme regulators
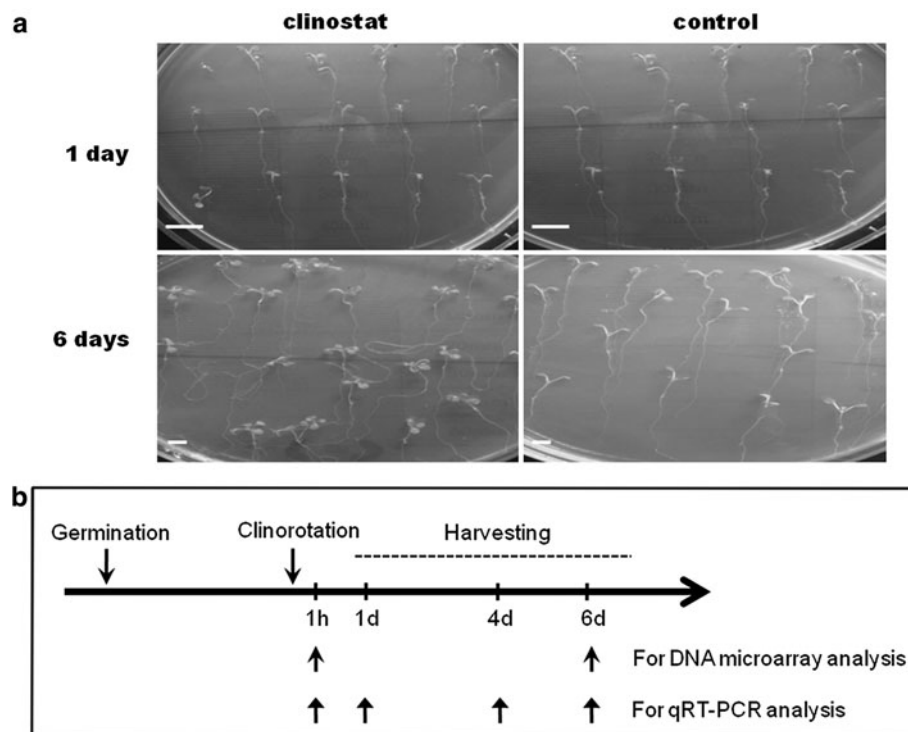

Fig. 1 Growth of Arabidopsis seedlings under 3-D clinorotation and the experimental scheme. **a** Morphological alteration was not detected on 1 day of the 3-D clinorotation for the treated seedlings (*left* clinostat) and the untreated control (*right*). However, the shoots and roots of the treated seedlings showed multi-directional growth patterns at 6 days of the 3-D clinorotation, which can be indicative to the effect of minimized gravitational strength by 3-D clinorotation (*magnification bars* 1 cm for 1 day, 2 cm for 6 days). **b** DNA chip analysis on 6-day-old seedlings sampled from two distinct time

regimes (1 h and 6 days) when plants were exposed to 3-D clinorotation. Subsequently, qRT-PCR was conducted to monitor continual expression patterns for two intermediate time points (1 and 4 days), along with the 1 h and 6 day time points for the selected genes from the DNA chip analysis. The intact seedlings as the control were grown on the shelf during whole of time regimes for the experiment. This approach allowed for the investigation of the continual response of the Arabidopsis transcriptome during short- to long-term exposure to simulated microgravity stress [*h* hour, *d* day(s)]
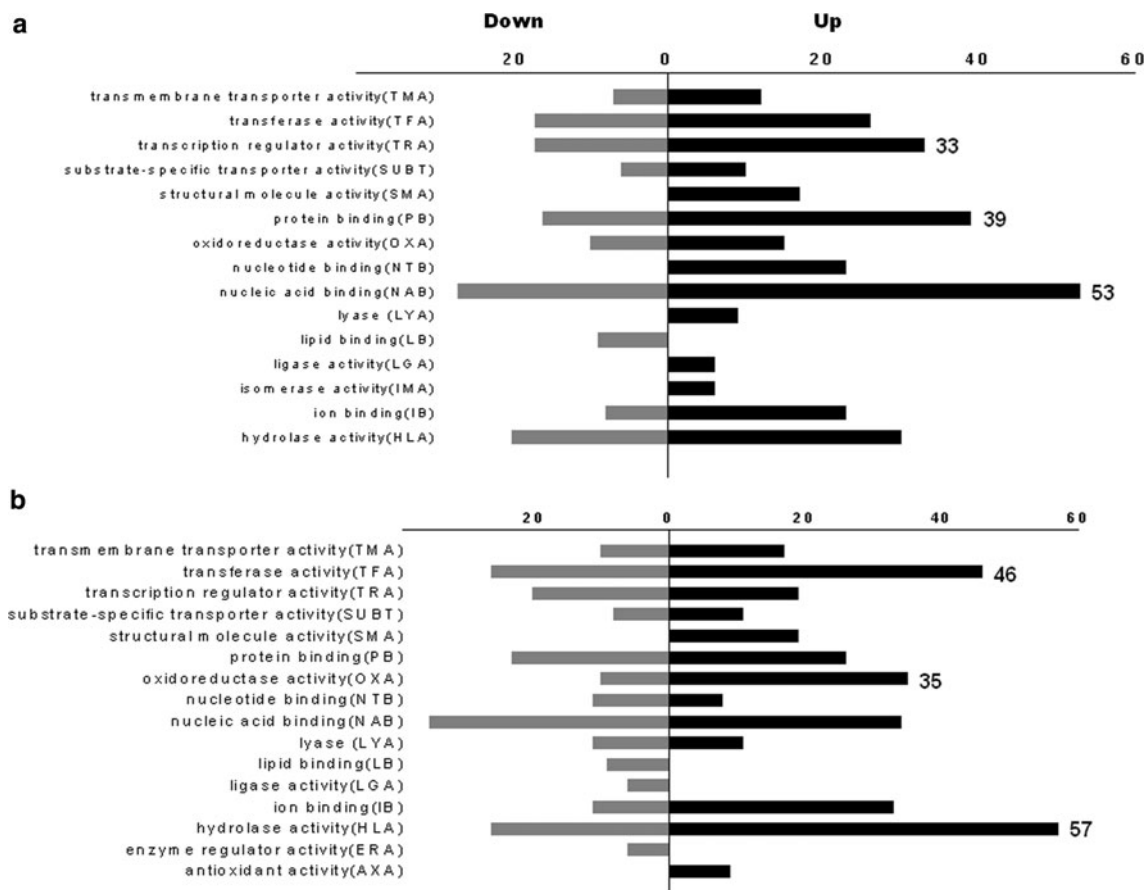
Fig. 2 Functional annotations for the transcriptome of Arabidopsis seedlings exposed to 1 h and 6 days of 3-D clinorotation. Gene ontology analysis yielded up-regulated and down-regulated genes with functional diversities for 1 h (**a**) and 6 days (**b**) of simulated microgravity. The *numbers* show the identified gene numbers for each of the predominant functional categories. Refer to the text for further details

were expressed only in the seedlings exposed to 6 days of 3-D clinorotation. Three functional groups (lipid binding, ligase activity, and enzyme regulator) evidenced only down-regulation trends. The only up-regulated genes were those associated with structural molecules and anti-oxidation.

Response patterns of the genes under simulated microgravity

The gene response patterns under simulated microgravity condition were proposed on the basis of rescreening and primary gene ontology analysis for the initially annotated genes in Fig. 2. The screening work was made on a twofold increase or reduction in gene expression levels on the DNA chip data, and generated up-regulated and down-regulated genes for 1 h only and 6 days only for 3-D clinorotation (Supplementary Tables S1–S4). The up-regulated genes were then categorized into three patterns: 1 h only (with No change at 6 days), 6 days only (with No change at 1 h), and 1 h/6 days (constant Up in 1 h and 6 days both).

Likewise, the down-regulated genes were sub-grouped into three patterns: 1 h only (with No change at 6 days), 6 days only (with No change at 1 h) and 1 h/6 days (constant Down in 1 h and 6 days both) (Fig. 3).

Venn diagram on the gene classification

Alternatively, the classified genes in Fig. 3 were collectively presented into a Venn diagram (Fig. 4). For each of the functionally annotated gene groups, the numbers of genes (with twofold change on gene expression levels) were shown in parentheses. For the up-regulated genes, isomerase activity (IMA) and antioxidant activity (AXA)-associated genes were found to be up-regulated in 1 h only and 6 days only, respectively. The genes associated with tetrapyrrole binding (TPB) belonged to the constant up-regulation category at both 1 h and 6 days (1 h/6 days). The commonly up-regulated genes in the three categories were identified as those relevant to: hydrolyase activity (HLA), ion binding (IB), lyase activity (LYA), nucleic acid binding (NAB), nucleotide binding (NTB), oxido-reductase
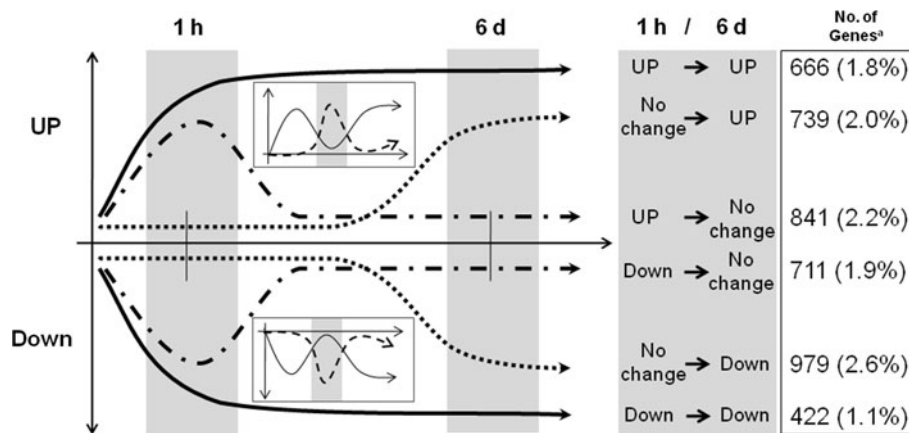
Fig. 3 Gene expression patterns in two time regimes (1 h, 6 days) for 3-D clinorotation. Up-regulated genes were categorized into three patterns: 1 h only (with No change at 6 days), 6 days only (with No change at 1 h) and 1 h/6 days (Up-regulated in both 1 h and 6 days). Likewise, down-regulated genes were also sub-grouped into three patterns: 1 h only (with No change at 6 days), 6 days only (with No change at 1 h) and 1 h/6 days (Down-regulated in both 1 h and 6 days). The *small boxes* contain predicted gene expression patterns between 1 h and 6 days. The screening work was based on twofold ($2\times$) increases or decreases in gene expression levels in the DNA chip data. The genes with expression values of ≥ 2.0 were classified into the up-regulation group via the reference value of 1.0 for the expression rate in the DNA chip data, whereas the down-regulated genes evidenced expression values of ≤ 0.5. On the other hand, the genes with expression values between 1.0 and 2.0 were assigned to 'No change' in the background of the up-regulation. Likewise, the genes between 0.5 and 1.0 were grouped into the 'No change' group in the background of the down-regulation. Superscript *a* indicates the number of genes with twofold up-regulation and/or down-regulation after clinorotation treatment (*numbers in parentheses* are the percentage of the screened genes based on the total genes analyzed in the DNA chips)

activity (OXA), protein binding (PB), structural molecule activity (SMA), substrate-specific transporter activity (SUBT), transferase activity (TFA), *trans*-membrane transporter activity (TMA), and transcription regulator activity (TRA). The genes relevant to ligase activity (LGA) were categorized into the up-regulation group of 1 h only and 1 h/6 days of exposure to simulated microgravity.

On the other hand, the genes for enzyme regulator activity (ERA), nucleotide binding (NTB), ligase activity (LGA), and lyase activity (LYA) were grouped into the down-regulation category for 6 days only. Lipid-binding protein (LB) encoding genes belonged commonly to the down-regulation category in the 1 h only and 6 days only groups. The commonly down-regulated genes in the three categories were relevant to the following activities: hydrolase activity (HLA), ion binding (IB), nucleic acid binding (NAB), oxido-reductase (OXA), protein binding (PB), substrate-specific transporter activity (SUBT), transcription regulator activity (TRA), transferase activity (TFA), and *trans*-membrane transporter activity (TMA).

Time-course expression patterns for the responsive genes to simulated microgravity

All of the genes for time-course expression in this study were basically selected with the expression level of >twofold, significant GO analysis output and preliminary RT-PCR validation. Initially, from the regrouped genes in Fig. 3, we selected 12 representative genes (six genes for

each of up-regulation and down-regulation) and, subsequently, conducted quantitative real-time polymerase chain reactions to characterize their gene expression patterns on 1 h–1 day–4 days–6 days under simulated microgravity condition. For this, the intact seedlings which were not exposed to 3-D clinorotation were included as the control.

The six up-regulated genes belong to each of three categories: (I_{up}) four genes for 1 h only (Up in 1 h and No change in 6 days)—abscisic acid responsive element-binding factor 3 (ABF3) (At4g34000), extensin-like protein (Ext) (At3g24480), calmodulin-like 38 (CML38) (At1g76650), heat shock protein 81-2 (HSP81-2) (At5g56030); (II_{up}) one gene for 6 days only (No change in 1 h and Up in 6 days)— XTR6 (At4g25810); (III_{up}) one gene for 1 h/6 days (constant Up in 1 h and 6 days both)—ferrochelatase 1 (FC-I) (At5g26030).

Figure 5 shows the expression patterns of the selected up-regulated genes on time-course (1 h–1 day–4 days– 6 days) based exposures to simulated microgravity conditions. The overall gene expression levels were substantially higher under simulated microgravity conditions than under normal 1g conditions. In general, initial high inductions of transcript abundance at 1 h of clinorotation (except XTR6) were followed by a dramatic reduction on the expression levels through 4-day clinorotation, although HSP81-2 (At5g56030) evidenced a slight up-regulation during mid-term clinorotation (1–4 days). For the four selected genes in category (I_{up}), the expression levels finally approximated those of the control at 6 days of exposure in Ext, CML38,
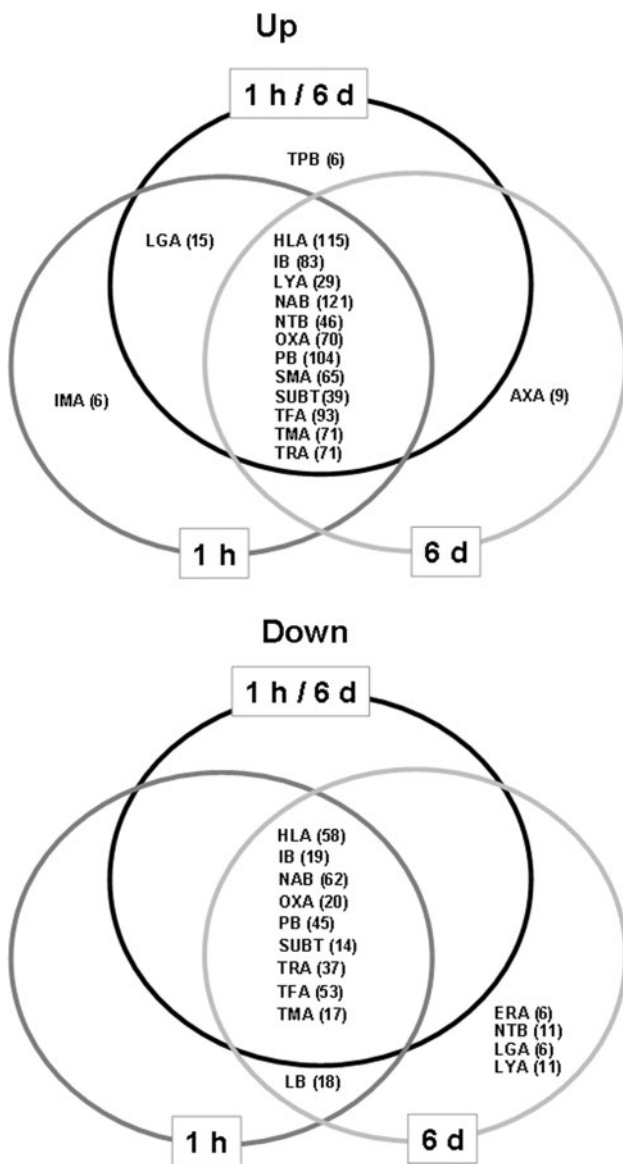
Fig. 4 A Venn diagram on the functional classification of up-regulated and down-regulated genes for three time regimes (1 h, 6 days, and 1 h/6 days) of 3-D clinorotation. The gene categories in Fig. 3 were presented in a Venn diagram for three types of up- and down-regulated gene expression patterns. The *numbers in parentheses* are the numbers of genes (twofold up-regulation/and down-regulation) for each of the functional annotation groups screened out on the basis of further gene ontology analysis of the responsive genes shown in Fig. 3. For instance, LGA (15) indicates that some genes are up-regulated at 1 h only and others are up-regulated constantly at 1 h and 6 days both during 3-D clinorotation for the selected 16 genes for LGA. Refer to the text for details

Fig. 5 Time-course monitored continual expression patterns for the up-regulated genes of interest. Six genes were selected for three categories (I_{up}, II_{up} and III_{up}) in simulated microgravity stimulation among the up-regulated genes in Fig. 3: (I_{up}) 1 h only (Up in 1 h and No change in 6 days) (4 genes)—abscisic acid responsive element-binding factor 3 (ABF3) (At4g34000), extensin-like protein (Ext) (At3g24480), calmodulin-like 38 (CML38) (At1g76650), heat shock protein 81-2 (HSP81-2) (At5g56030); (II_{up}) 6 days only (No change in 1 h and Up in 6 days) (1 gene)—xyloglucanendo-*trans*glycosylase 6 (XTR6); (III_{up}) 1 h/6 days (constant Up in 1 h and 6 days both) (1 gene)—ferrochelatase-I (FC-I) (At5g26030). To monitor the expression patterns of the selected genes, qRT-PCR was conducted for the selected genes (The 18S rDNA was used as an internal control for qRT-PCR.). The intact seedlings (which were grown on shelf) were included as the system control. The expression levels relative to the value of the internal control (1.0) were calculated using GraphPad Prism v.5 (*X axis* clinorotation treatment time, *Y axis* transcript abundance in relative expression of mRNA)

and HSP81-2. However, the gene expressions were increased from 4 to 6 days and reached higher levels than the control in ABF3.

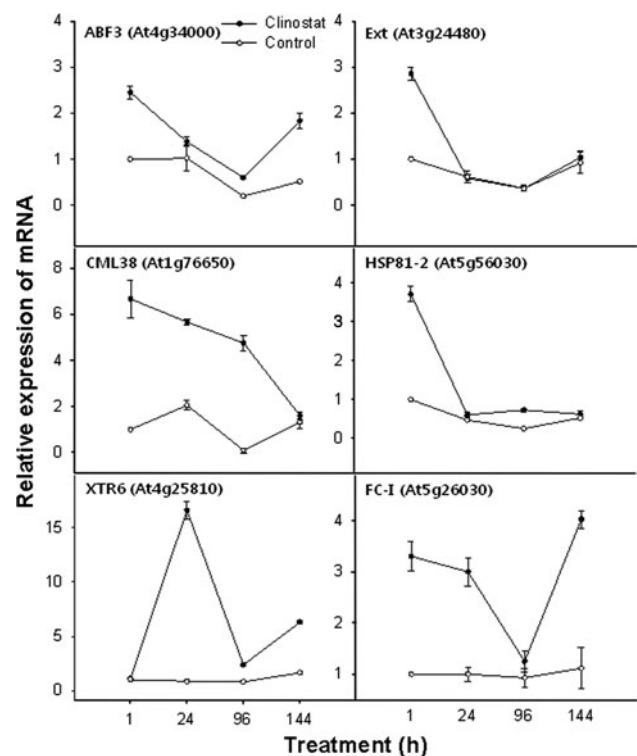On the other hand, the six down-regulated genes were also selected from the same categories as above but, at this time, down-regulated: (I_{down}) one gene for 1 h only (Down in 1 h and No change in 6 days)—IAA transcription factor 6 (IAA6) (At1g52830); (II_{down}) three genes for 6 days only (No change in 1 h and Down in 6 days)—lipid transfer protein 6 (LTP6) (At5g01870), 3-phosphoinositide-dependent protein kinase 1 (PDK1) (At5g04510), patatin-like protein 2 (PLP2) (At2g26560); (III_{down}) two genes for 1 h/6 days (Down in 1 h and 6 days both)—early-responsive to dehydration 6 (ERD6) (At1g54730), flagellin-sensitive 2 (FLS2) (At5g46330). The expression patterns of the down-regulated genes are presented in Fig. 6. IAA6 from category (I_{down}) was generally down-regulated, with negligible differences between the simulated microgravity-treated seedlings and the control. For the genes from category (II_{down}), the gene expression of PDK1 (At5g04510)
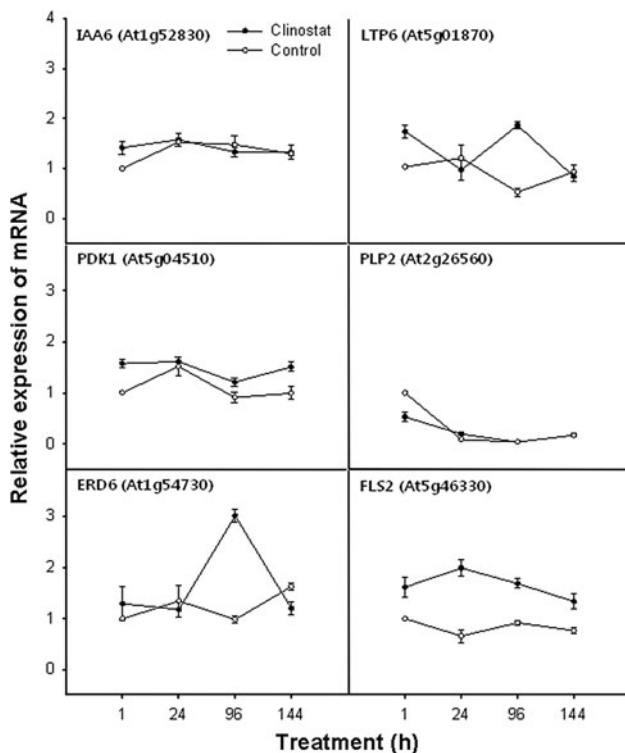
Fig. 6 Time-course monitored continual expression patterns for down-regulated genes of interest. As is shown in Fig. 5, six genes were also selected for three categories (I_{down}, II_{down} and III_{down}) in simulated microgravity stimulation among down-regulated genes in Fig. 3: (I_{down}) 1 h only (Down in 1 h and No change in 6 days) (1 gene)—IAA transcription factor 6 (IAA6) (At1g52830); (II_{down}) 6 days only (No change in 1 h and Down in 6 days) (3 genes)—lipid transfer protein 6 (LTP6) (At5g01870), 3-phosphoinositide-dependent protein kinase 1 (PDK1) (At5g04510), patatin-like protein 2 (PLP2) (At2g26560); (III_{down}) 1 h/6 days (constant Down in 1 h and 6 days both) (2 genes)—early-responsive to dehydration 6 (ERD6) (At1g54730), Flagellin-sensitive 2 (FLS2) (At5g46330). The intact seedlings (which were grown on the shelf) were included as the system control and the expression patterns of the selected genes were also monitored by qRT-PCRs with 18S rRNA used as an internal control. The expression levels relative to the value of the internal control (1.0) were calculated using GraphPad Prism v.5 (*X axis* clinorotation treatment time, *Y axis* transcript abundance in relative expression of mRNA)

evidenced a small change in expression level with continual down-regulation from 1 to 4 days of exposure and finally reached a slight up-regulation at 6 days of stimulated microgravity. The other two genes—LTP6 (At5g01870) and PLP2 (At2g26560)—showed broad stochastic patterns in their expression levels within a low expression rate range from 1 h to 6 days of exposure to simulated microgravity. In the case of ERD6 (At1g54730) and FLS2 (At5g46330) in the category (III_{down}), the expression level ERD6 was increased gradually and reached its highest levels at 4 days of exposure to 3-D clinorotation. Actually, it was down-regulated at both the initial (1 h) and final (6 days) of simulated microgravity. PLP2 gene showed

continual up-regulation patterns from the initial (1 h) time point and reached the highest expression level at 4 days which was followed by decreasing pattern at 6 days. Interestingly, the expression levels were higher than the control at 1 h and 4 days of 3-D clinorotation treatment.

From the primary functional annotation (Fig. 2), single genes were selected from each of predominant functional groups only in respective up-regulated groups of the very early (1 h) and the final (6 days) time points of clinorotation treatment. So, the exemplary three up-regulated genes were selected from predominant functional annotation groups in 1 h of simulated microgravity treatment; WRKY40 (At1g80840), AtPUB19 (At1g60190) and ERF sub (At5g51190) were selected from NAB, PB and TRA group in Fig. 2a, respectively. Likewise, another three of up-regulated genes [VSP1 (At5g24780), SAM-MT (*S*-adenosylmethionine-dependent methyltransferase) (At5g37970) and CYP705A5 (At5g47990)] were selected from three main functional annotation groups (HLA, TFA and OXA group in Fig. 2b), respectively, in the 6-day exposure to simulated microgravity treatment. The results of qRT-PCR showed that WRKY40, AtPUB19, SAM-MT and CYP705A5 were regulated in a stochastic fashion during the time-course regimes (1 h–1 day–4 days–6 days). The expression level of the ERF sub-initially gradually decreased with slight differences in expression rates, and subsequently underwent a dramatic increase during the late exposure duration after 4 days of exposure to simulated microgravity. For VSP1 (At5g24780), the pattern of up-regulation was maintained at almost equal levels after the drastic up-regulation pattern at the early exposure time regime (1 h–1 day).

Discussion

Simulated microgravity treatment

In contrast to most of the previous studies on the relatively early plant gene responses (within 1–24 h) to (simulated) microgravity conditions (Kittang et al. 2004; Martzivanou et al. 2006; Babbick et al. 2007), Arabidopsis seedlings were exposed to 3-D clinorotation up to 6 days in the present study. It was generally assumed that 3-D clinorotation for 6 days generated a constant, minimized gravity signal that acted similarly and evenly upon all parts of a plant/tissue/cell, thus presumably prohibiting specific responses and maximizing weightless conditions.

Two main time points (1 h and 6 days) were selected based on the results of previous studies with significant changes in gene expression from 1 h of gravitational treatment (Kimbrough et al. 2004; Martzivanou et al. 2006) and distinct morphological changes—multi-directional growth of both shoots and roots—from 6 day-clinorotation

(Centis-Aubay et al. 2003) in Arabidopsis. During continual 3-D clinorotation from 1 h to 6 days, morphological changes of the seedlings was observed on 4 days of simulated microgravity treatment and maximized at the 6 days of the simulated microgravity treatment. And, this allowed us to select the 4 days as one of the main checking time points for gene expression under 3-D clinorotation, along with an arbitrarily selected sub-mid-time point (the 1 day) as the main intermediate time points between 1 h and 4 days of clinorotation (Fig. 1a, b).

Functional annotation and response patterns under simulated microgravity

The screening work and ontology analysis for the genes from DNA chip data allowed modeling of gene response patterns in the 3-D clinorotation exposure time regimes designed in this study. The gene response patterns evidenced many similarities between the up-regulated and down-regulated genes during the time course of exposure to simulated microgravity conditions (Fig. 3). The responsive genes were induced early and maintained their own patterns through exposure time regimes to 3-D clinorotation (Up → Up, Down → Down). Alternatively, they were expressed late in a time frame of short- (1 h) to long-term (6 days) exposure to 3-D clinorotation (No change → Up or Down). The early- and late-induced genes can be regarded as genes involved in the process of genome adaptation to simulated microgravity stress conditions. Moreover, it appears highly likely that other genes tended to go back to the original status (Up or Down → No change). These genes could be involved in the recovery of homeostasis within the genome during minimized gravity stress. The classified gene categories in Fig. 3 were employed as a platform for the selection of candidate genes to monitor gene expression trajectories in response to simulated microgravity as shown in Figs. 5 and 6.

The Venn diagram (Fig. 4) presents functional grouping for the classified genes in Fig. 3 and several interesting findings could be derived from this gene grouping. Actually, the up-regulations of antioxidant genes (IMA, AXA, TPB) strongly indicated that plants react actively to simulated microgravity stress conditions. It is generally known that reactive oxygen species (ROS) are notorious for their oxidation capacity, and their cytotoxic properties. They are generated primarily under stress conditions. Thus, it appears most likely that cells had to develop a molecular strategy to overcome ROS-induced cell damage by synthesizing multiple antioxidant proteins. It is interesting to note that genes encoding for TPB evidenced a consistent pattern of up-regulation in short-term (1 h) to long-term (6 days) exposures to simulated microgravity condition. Considering that TPB is a putative protein with antioxidant activities, it

can be speculated that these genes might be involved in homeostasis just from the early response up to the late response to simulated microgravity stress. Therefore, it would be a natural cellular strategy to reduce or at least maintain ROS concentration down to tolerable ranges, to ensure normal cell survival during simulated microgravity stress. On the other hand, the majority of other gene groups (HLA, IB, NAB, OXA, PB, TRA, TMA, SUBT, TFA) were found collectively to be up-regulated and down-regulated under simulated microgravity stress. This connotes that they are dynamically active genes, increasing or decreasing their activities for tuning up genome conditions during adaptation to simulated microgravity conditions.

Time-course based expression patterns in genes responsive to simulated microgravity

Gene expressions were monitored on four time points (1 h–1 day–4 days–6 days) of 3-D clinorotation in order to investigate gene expression trends under simulated microgravity condition. Overall, the monitored expression patterns strongly indicates that some genes in the plant genome generally react dynamically immediately after exposure to simulated microgravity condition and revert to a default state (homeostasis at the nucleic acid level) in a novel genomic environment during prolonged exposure to stress.

In this context, we thus address possible interpretations of the expression patterns for the investigated genes in relation to their functions which are assumed to be involved in response to simulated microgravity in the following section.

ABF3

ABF is a *trans*-acting factor to bind to ABA-responsive genes and regulate them at the transcription level upon multiple environmental stresses (Finkelstein et al. 2005; Tuteja 2007; Fujii and Zhu 2009; Han et al. 2009). ABF3 (At4g34000) is one of its homologues in the Arabidopsis genome, and is assumed to have functions similar to ABF (Fujii and Zhu 2009). Since abscisic acid (ABA) as a 'stress hormone' of plants has been shown to respond to multiple biotic and abiotic stresses, it is quite possible that simulated microgravity, as an abiotic stress, may also trigger ABF3 activity. Presumably, its up- and down-regulation may be correlated with genome homeostasis under minimized gravity conditions.

Ext

Ext (At3g24480) is related to plant tip growth through cell wall changes (Bucher et al. 2002). In fact, plant growth could be distorted under disturbed gravity conditions

(Martzivanou and Hampp 2003) and basically, this can be generated from dynamic changes in the cell wall occurring as the result of the abnormal regulation of cell wall bio-synthesis-related genes such as Ext. It is most likely that high Ext expression at the initial exposure (1 h) to simulated microgravity might be because Arabidopsis seedlings under gravity stress require substantial cell wall-related proteins for the redistribution/reform of composition and cell wall structure.

CML38

CML38 (At1g76650) is one of the calcium sensors for multiple cellular signals in response to different external stimuli or environmental stresses. This protein also directs downstream signaling pathways via the binding and activation of diverse targets for various aspects of plant development and stress response (Walley et al. 2007). Thus, it can be an obvious corollary that CML38 might be abundantly expressed when the plant genome is challenged by simulated microgravity stress conditions. In this study, the up-regulation of CML38 at 1 h of clinorotation can be explained by a sudden surge of signaling-related gene products in the initial instant responses to stress.

HSP81-2

Abiotic stress evokes multiple responses that involve a series of physiological, biochemical, and molecular events. During adaptation to stress, many unnecessary/dysfunctional proteins must be degraded, and new proteins must be synthesized and folded into correct structures. In general, heat shock proteins are responsible for protein folding, assembly, translocation, and degradation in many normal cellular processes, and they stabilize proteins and membranes under stress conditions. Heat shock proteins may perform a crucial function in protecting plants against stress by re-establishing normal protein conformation, and thereby cellular homeostasis (Wang et al. 2004). In this regard, it is possible that the heat shock protein HSP81-2 (At5g56030) might perform some functions in the adaptation process of the plant to simulated microgravity condition.

XTR6

XTR6 correlates with plant cell expansion/elongation by decorating cellulose micro-fibrils and forming cross-links between them (Claisse et al. 2007). Routine cell divisions also occur to form daughter cells during simulated microgravity stress conditions, but new cells need to adjust their physical volumes in order to minimize possible damage on cell morphology under constant/long simulated

microgravity conditions. At this point, especially, the XTR 6 cell expansion gene could be actively expressed. XTR6 expression was increased up to a dramatically high level after 1 day of clinorotation and maximized at 4 days of clinorotation. This indicates that cell volume adjustment reaches a maximum at approximately 4 days of exposure to simulated microgravity condition.

FC-I

FC-I (At5g26030) is known to be involved in ROS generation under oxidative stress conditions (Nagai et al. 2007). The gene was up-regulated at both 1 h and 6 days, during which continual down-regulation was scored at its lowest levels at 4 days and a dramatic increase in gene expression after 4 days up to 6 days of exposure to simulated microgravity. In fact, the FC-I expression patterns observed in the present study suggest an interesting model for the response of the plant to simulated microgravity conditions. The gene classification in Fig. 4 shows that the antioxidant genes (IMA, AXA, TPB) were up-regulated constantly at all categories, such as 1 h, 6 days, and 1 h/ 6 days. It is likely that the plant develops a cellular strategy for the maintenance of physiological integrity under minimized gravity stress via gene interaction between antioxidant genes and ROS-generating genes. In other words, the sudden surge of FC-I expression at initial exposure (1 h) is followed by a dramatic down-regulation occurring at the fourth day via 1-day exposure to simulated microgravity conditions by constant antagonistic action of antioxidant genes against FC-I gene activity. Additionally, as the exposure duration lasts longer from 4 to 6 days, antioxidant gene activities reaches a limit for FC-I suppression and allows for the up-regulation of FC-I activity.

IAA6

IAA6 belongs to the Auxin/IAA gene family and its transcription is regulated positively by auxin transport into the plant cell (Ulmasov et al. 1997; Tiwari et al. 2003; Overvoorde et al. 2005). Interestingly, it was noted that auxin transport was inhibited under simulated microgravity conditions (Ishii et al. 1996). Therefore, the down-regulated pattern of IAA6 could be explained by impaired auxin transport under minimized gravity conditions.

PDK1

As a central/master regulator, PDK1 controls a number of signaling pathways via the activation of downstream protein kinase (Anthony et al. 2004; Zegzouti et al. 2006). The observed expression patterns of PDK1 propose that the signaling pathways (cascades) can be active in plants

initially challenged with simulated microgravity due to sensitive fluctuations of metabolism in an early acute alert state. During adaptation to simulated microgravity condition, it is possible that the signal transduction systems can be gradually stabilized with a relative reduction in the recruitment of critical signaling protein factors in simulated microgravity-treated seedlings. This possibility can be derived from the fact in which a gradual reduction in expression was monitored at the intermediate time points for PDK1, with expression finally going back to the initial expression pattern upon 6 days of exposure to 3-D clinorotation.

LTP6 and PLP2

LTP6, as one of the lipid transfer proteins (LTPs) mediates plant cell wall extension by increasing plant cell wall plasticity when stressed plant cells are stimulated to increase their volumes to overcome stressful conditions (Debono et al. 2009). So, the plant under minimized gravity conditions is likely to generate random/multi-directional growth patterns that could be generated by structural changes in the cell wall. The observed stochastic gene expression pattern for LTP6 may reflect dynamic transcription-level alterations in gene activity. On the other hand, in the case of PLP2, this protein is involved in pathogen-induced cell death (La Camera et al. 2009). Considering cross-talk among stress-related proteins, PLP2 expression may be one of the molecular manifestations of interconnected responsive proteins in response to simulated microgravity condition.

ERD6 and FLS2

ERD6 (At1g54730) function remains elusive in plants. However, the gene is known to be induced by both water (dehydration) and cold stress; thus, ERD6 is believed to possibly function as a putative sugar transporter for the redistribution of sugars used as an energy source for protecting cells against stresses (Kiyosue et al. 1998). The global physiological state is assumed to be drastically compromised at the initial tolerance/adaptation phase in simulated microgravity-exposed plants. Consequently, the transport and/or redistribution of sugars (as metabolic energy sources) do not actively occur. This possible explanation is supported by low expression of the ERD6 gene at initial (1 h) exposure to simulated microgravity. Continual increases in ERD6 expression (1–4 days) are indicative of dynamic metabolic activity for the adaptation of the plant genome to simulated microgravity stress; finally, the gene activity goes back to a level approximately equal to the original status. FLS2 (At5g46330) is a *trans*-membrane receptor-like kinase and enhances plant

resistance against bacteria (Chinchilla et al. 2007). The expression for this gene, which is seemingly unrelated to the simulated microgravity stress, could be explained by a similar logic to that in the case of PLP2 with the cross-talk hypothesis among stress-related proteins.

AtPUB19 and CYP705A5

AtPUB19 (U-box domain-containing protein) (At1g60190) is likely to be involved in the degradation and refolding of abnormal or mis-folded regulatory proteins as a factor of molecular chaperones in response to multiple external stimuli or environmental stresses (Wiborg et al. 2008). On the other hand, CYP705A5 (At5g47990), a member of the plant cytochrome P450 super-family (officially abbreviated as CYP) catalyzes the oxidation of metabolic intermediates such as various fatty acid conjugates and plant hormones (Ehlting et al. 2008). Therefore, their stochastic expression patterns demonstrate that the synthesis and degradation of regulatory proteins such as transcription factors and metabolites occur in a random manner during continuous exposure to simulated microgravity condition. As such, the plant genome may be adjusting to stressful simulated microgravity conditions by maintaining its optimal physiological status.

WRKY40

A stress-induced response is generally produced from combinational actions of randomly instant or long-lasting molecular signals (or gene expressions) from a complicated network of a myriad of regulatory factors. Thus, WRKY40 (At1g80840) as one of the pathogen-induced transcription factors (Xu et al. 2006) is likely to evidence multi-functionality for loosely defined stress responses in this regard, and this could explain the alternating expression patterns with up- and down-regulation in WRKY40 under simulated microgravity condition.

SAM-MT

Many endogenous gene activities are controlled by DNA methylation, in which DNA sequences are modified by methyl groups (-CH3) from *S*-adenosylmethionine (SAM) and this process is completed through methyltransferase. So, methyltransferase is a critical factor for DNA methylation to the target DNA sequence (Finnegan and Kovac 2000). Furthermore, it has been reported that fluctuating DNA methylation occurred in plant genomes challenged by multiple stress conditions as a molecular strategy to reset gene regulation patterns during tolerance and adaptation to stressful conditions (Kaeppler et al. 2000). So, the gene expression pattern for SAM-MT (*S*-adenosylmethionine
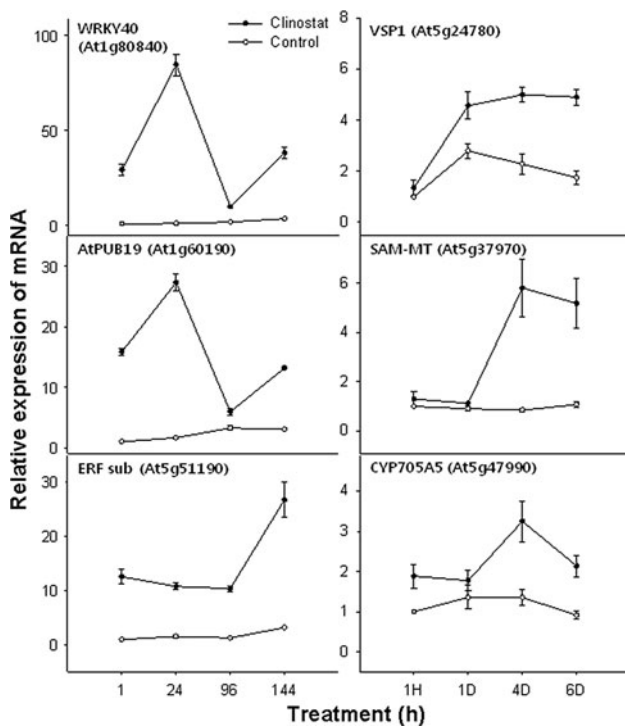
Fig. 7 Time-course monitored continual expression patterns for the genes with predominant functionalities in the gene annotation analysis (Fig. 2) for 1 h only and 6 days only of simulated microgravity. Three genes from predominant functional annotation groups (NAB, PB, TRA) were selected among the up-regulated genes after 1 h of exposure to simulated microgravity; WRKY40 (At1g80840), AtPUB19 (At1g60190), ERF sub (At5g51190). Likewise, three up-regulated genes were selected from the predominant functional annotation groups (HLA, TFA and OXA) upon 6 days of exposure; VSP1 (At5g24780), SAM-MT (S-adenosylmethionine-dependent methyltransferase) (At5g37970), CYP705A5 (At5g47990). The intact seedlings (which were grown on the shelf) were included as the system control. The expression patterns of the selected genes were also monitored via qRT-PCR with an internal control of 18S rRNA and the expression levels relative to the value of the internal control (1.0) were calculated via GraphPad Prism v.5 (*X axis* clinorotation treatment time, *Y axis* transcript abundance in relative expression of mRNA)

methyltransferase) (At5g37970) in Fig. 7 may reflect the dynamic DNA methylation status in the Arabidopsis genome under simulated microgravity stress condition.

ERF sub and VSP1

ERF sub-proteins are a subfamily of the APETALA2 ethylene responsive element-binding protein (EREBP) transcription factor family which are linked to a broad range of (a)biotic stresses, including cold, drought, pathogen infection, and wounding (Fujimoto et al. 2000; Singh et al. 2002). It was reported that ERF sub of cultured Arabidopsis cells was up-regulated at most of exposing time regimes to clinorotation and other gravity stress sources (Babbick et al. 2007). In our case, the expression pattern of

ERF sub was generally compatible to the previous report even though it was initially down-regulated (Fig. 7). The up-regulation pattern was observed as 3-D clinorotation further proceeded up to 6 days. This trend indicates gradual adaptation of Arabidopsis genome to simulated microgravity condition by activating ERF-related *trans*-acting factors. For VSP1, since vegetative storage proteins (VSPs) are believed to promote the sequestration of plant nutrients in stress challenge and support resumed plant growth and reproduction during stress adaptation (Staswick 1994; Berger et al. 2002), the observed consistent high expression levels of VSP1 after high induction can be also interpreted as an essential adaptation response in plants to disrupted (minimized) gravity condition.

For our time-course study about gene expressions, there might be many of in-between gene expression changes for the time points (1 h–1 day–4 days–6 days). However, those changes were not investigated in this study. Instead, the possible patterns were predicted (Fig. 3). Moreover, the whole data assumes that the 6-day state indicates a new steady state in our experimental design. However, a clear finding is that genome change was actively triggered in Arabidopsis, at least at the transcriptional level owing to simulated microgravity condition. Dynamic expression patterns for the genes are another indication that simulated microgravity condition reprograms Arabidopsis genome by re-setting of gene activities (up- and down-regulation). Some of the selected genes had expression levels equal or approximately equal to (slightly higher or lower) those in the untreated intact seedlings during prolonged exposure to simulated microgravity condition, regardless of whether the initial expression levels were high or low. This implies that Arabidopsis genome has an intrinsic nature to recover its original default state for survival, which is a strategy for adaptation for plants encountering a stressful environment (Bohnert et al. 2006).

The selected genes (except ERF sub and Ext) were not specific ones to gravi-sensing-related signal transduction, stress response and metabolic pathways. The raw DNA chip data included gravity-related genes, but they were not included in the gene selection for time-course expression patterns due to their expression levels of <twofold and insignificant GO analysis output in the current study. Instead, they are briefly described in Supplementary Table S5. Therefore, it was difficult to propose a model for a response cascade or gene network with biological significance in sensing minimized gravitational strength. However, functional diversity of the selected genes reflects genome-wide response of Arabidopsis to react to simulated microgravity condition via orchestration of a broad range of genes, owing possibly to similar mechanism to stress cross-talk and tolerance/adaptation in plants (Lichtenthaler 1996; Bowler and Fluhr 2000; Knight and Knight 2001).

Furthermore, in the present study, the gene expression patterns were investigated in long-term based time scale (up to 6 days) on 3-D clinorotation in order to mimic genuine space (flight) environment as closely as possible and the results were significant. The obtained information would provide valuable clues to predict molecular response of living organisms to real space environment. Recently, a follow-up study was launched about analysis on promoter regions and some of the *cis*-regulatory elements for the identified genes in response to simulated microgravity condition and the findings will be informative in space biology.

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