A PARABOLIC FLIGHT PROFILE AS REFLECTED BY FLUCTUATIONS IN CYTOSOLIC CALCIUM AND GENE EXPRESSION OF PLANT CELLS

Maren Neef[(1)], Anne Hennig[(1)], Niklas Hausmann[(1)], Rüdiger Hampp[(1)]

[(1)]University of Tübingen, Physiological Ecology of Plants, Auf der Morgenstelle 1, 72116 Tübingen (Germany). Email: maren.neef@uni-tuebingen.de; ruediger.hampp@uni-tuebingen.de

ABSTRACT

In plants, like in other organisms, calcium is an important secondary messenger which participates in the conversion of environmental signals into molecular responses. There is increasing evidence, that sensing of changes in gravitation is an example for such signaling cascades where calcium is involved. In order to determine g-dependent changes in the cytosolic calcium concentration (Ca_{cyt}) of plant cells, cell cultures of *Arabidopsis thaliana* (A.t.) were exposed to g-forces between µg and 1.8g in the course of parabolic flights. Intracellular calcium was monitored by the calcium sensor YC3.6 ("cameleon"). This modified calmodulin shows calcium-dependent fluorescence resonance energy transfer (FRET). We determined the shift in fluorescence from 480 to 530 nm which rises with increasing Ca_{cyt}. For most of the parabolas a transient elevation of Ca_{cyt} was detected upon onset of the µg phase. In parallel, samples were taken for the analysis of mRNA. Using Affymetrix GeneChip microarrays we could identify both phase-specific as well as general changes in the amounts of transcripts. Examples for Ca-related genes are given.

1. INTRODUCTION

In plant cells, Ca ions play a central role in responses to environmental stimuli such as abiotic and biotic forms of stress. Generally, a sensed signal causes a more or less rapid, but mostly transient increase in the cytosolic Ca^{2+} (Ca_{cyt}) concentration [1]. The Ca^{2+} ions can either originate from intracellular compartments or from outside the protoplast. Gravitation is an abiotic signal which induces a Ca_{cyt} response. This has been intensely investigated with regard to gravity vector dependent root curvature (gravitropism). As the presence of Ca chelators can abolish a gravitropic reaction, it has been concluded that the uptake of extracellular Ca is involved [2, 3]. Using Ca-sensitive probes it could be shown that gravistimulation induced asymmetrical changes in cytosolic Ca concentration in corn coleoptiles (Gehring et al. 1990) and in *Arabidopsis thaliana* (A.t.) seedlings [4]. Clinorotation, an attempt to compensate the gravity vector due to continuous horizontal rotation, resulted in a sustained increase in Ca_{cyt} [4]. Application of hyper g (100 g) by centrifugation of A.t. seedlings also induced an extended increase in Ca_{cyt} [5]. Recent results published by [6] indicate that gravistimulation by clinorotation results in a biphasic Ca^{2+} transient of which only the second peak is due to the gravity vector while the first one can be attributed to rotational movement.

In recent studies we have shown that cell cultures of A.t. exposed to hypergravity, simulated weightlessness or microgravity show distinct changes in gene and protein expression, as well as protein modulation [7, 8, 9]. As a range of the altered transcripts / proteins are Ca^{2+}-dependent, we investigated possible changes in the Ca_{cyt} concentration of these culture cells under different gravitational field strengths. For this purpose we employed cell lines expressing a modified calmodulin (cameleon; YC3.6; [10]) as Ca_{cyt} indicator. In combination with a gene expression analysis we present some idea about the first steps of molecular cellular responses to altered gravity.

2. MATERIALS AND METHODS

2.1 Cell cultures

Wildtype and transgenic (YC3.6 = cameleon) suspension and calli cultures of *Arabidopsis thaliana*

Proc. '20th ESA Symposium on European Rocket and Balloon Programmes and Related Research'
Hyère, France, 22–26 May 2011 (ESA SP-700, October 2011)

(c.v. Columbia) were generated as described previously [7, 8, 9, 11]. Seeds from *Arabidopsis* plants expressing the Ca^{2+} sensor YC3.6 [10, 12] were kindly provided by K. Schumacher (University of Heidelberg).

2.2 Fluorometric analysis

Cytosolic calcium changes in YC3.6 transgenic plant cell cultures were monitored with a filter-based microplate fluorometer (POLARstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany) equipped with a simultaneous dual emission detection system. Samples were exposed in 96-well microtiter plates (Nunc, Germany) containing 200 µl solid Murashige and Skoog basal medium (SigmaAldrich, St.Louis, USA) per well. About 100 mg YC3.6 calli were transferred into each well, and the plates sealed with foil (Dynatech microtiter system, Denkendorf, Germany)at least one hour before the start of the experiment. Fluorescence ratios (535 nm / 480 nm) were calculated via MARS software (BMG LABTECH GmbH, Offenburg, Germany). Cytosolic Ca^{2+} concentrations were calculated according to the equation $Ca^{2+} = Kd\ (R-R_{min})/(R_{max} - R)^{1/n}$ [13] (R = the YFP/CFP ratio measured during the experiment [10]; n = the Hill coefficient= 1 for YC3.6 and Kd for Ca^{2+} = 250 nM [14]; R_{min} = 0.6 (cold stress); R_{max} = 2.1 (mechanical stimulation)).

2.3 Gene expression study

Calli of A.t. (average diameter about 1 mm) are spread on agar in cultivation flasks 8 days before the experiment. On the day of the experiment, the cultivation flasks were connected to syringes containing the fixative (RNAlater, Qiagen, Hilden, Germany; 9 times sample volume). Upon injection, RNA fixation takes place immediately (≤ 2s; own data). The whole experimental set up was contained in a glove box, the edges consisted of strut-elements (30x30). The base- and site plates are made of aluminum, the lid of transparent plexiglass, with two gloves inserted. The aluminum plates were sealed with silicone. The lid was fixed with bolt-and-nut screws and was water-proof (Fig. 1). Extraction of total RNA was as detailed in [15].

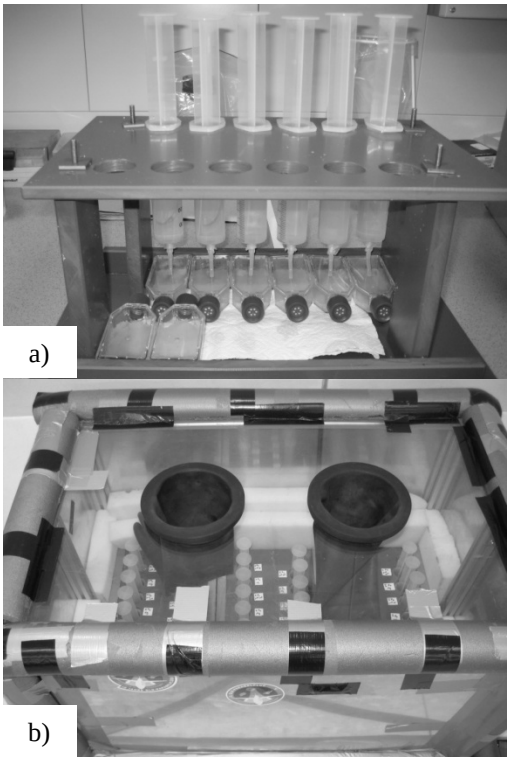

Figure 1. Arrangement of cell culture flasks and syringes, containing RNALater (a); whole experimental setup within glove box (b)

One µg/µl of total RNA in a final volume of 10 µl, was used for double-stranded cDNA synthesis (SuperScript Choice System, Invitrogen, Karlsruhe, Germany). Biotin-labelled cRNA was synthesized using the BioArray High Yield RNA Transcript Labelling Kit (Enzo Life Science Inc., Farmingdale, USA). All cRNA samples were tested for degradation by gel analysis according to the Affymetrix GeneChip expression analysis technical manual [16]. The cRNAs obtained were hybridized with the Affymetrix Arabidopsis Full Genome Array (ATH1; P/N: 510690; Lot No: 4038587). Hybridization, washing, staining and scanning procedures were performed as described in the Affymetrix technical manual [16]. Expression analysis and evaluation was performed by using the GeneSpringGX software program (Agilent Technologies, Böblingen, Germany).

2.4 Parabolic flight campaign (PFC)

Parabolic flight experiments were performed in an A300 airplane (Novespace, France) during the 16th DLR parabolic flight campaigns. A typical parabolic flight maneuver provides alternating acceleration levels of regular gravity (1 *g*), microgravity ($<10^{-4}$ *g* for 22 s per parabola), and hypergravity (up to 1.8 *g* for 20 s both before and after each period of microgravity). One campaign comprises three flight days with 31 flown parabolas each. For the fluorometric analysis, a microplate reader (POLARstar OPTIMA, BMG, Germany) was installed in a custom-made aluminum rack on board the aircraft (Fig. 2).

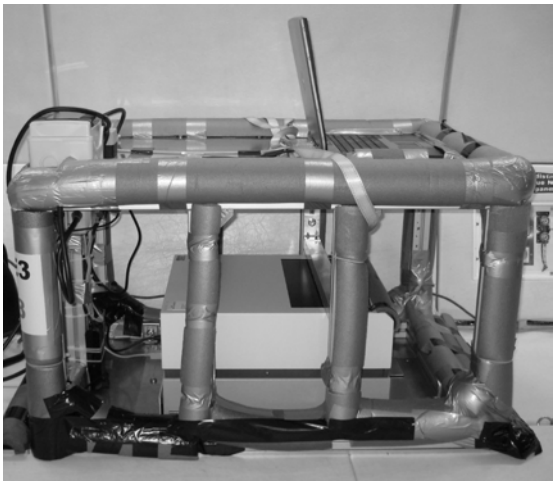

Figure 2. Rack arrangement of microplate-reader

3. RESULTS AND DISCUSSION

3.1. Cytosolic calcium fluctuations in response to a parabolic flight *g*-profile

The purpose of this experiment was to find out whether there is a response of the cytosolic calcium concentration towards altered gravitation. Fig. 3 shows the respective flight profiles. In Fig. 4 a typical example of corresponding changes in Ca_{cyt} is given. While wild type cells showed no specific change (fluorescence background; Fig. 4a), transition from 1 g to 1.8 g results in an immediate increase of Ca_{cyt} in the transformed cells (Fig. 4b). This persists until the end of the 20s-µg-phase. Transitions from 1g to 1.8g and *vice versa* were without effect. The same observation has been made during an earlier parabolic flight campaign and appears to be fully reproducible [17].

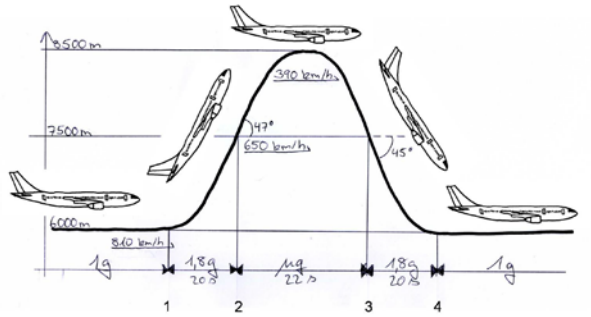

Figure 3. Parabolic flight profile. Numbers 1 to 4 identify points of sampling (injection of RNALater)

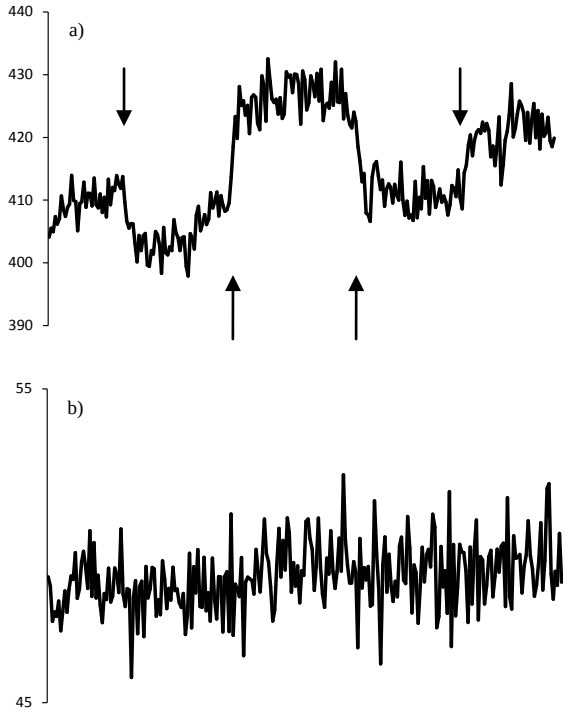

Figure 4. Typical changes in Ca_{cyt} during a parabola. Y-axis: calculated Ca concentrations (nM). Arrows indicate end of 1g, end of 1.8g, end of µg, end of 1.8g (see numbers 1 to 4 in Figure 3. a), YC3 cells (cameleon); b) wild type.

3.2. Gene expression analysis

In order to further this investigation, we performed a gene expression analysis in parallel. The microarray

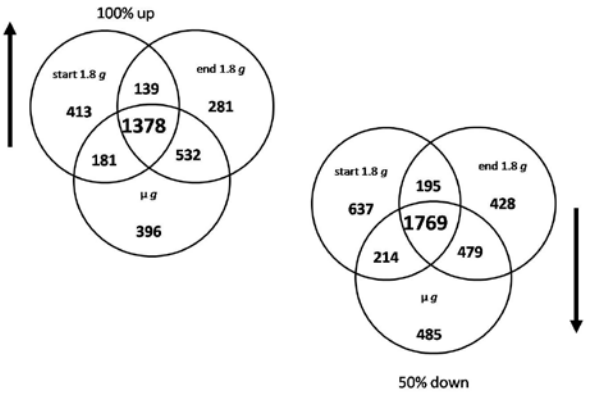

Figure 5. Venn-diagram of the microarray data showing the gene expression overlap for phases 1 to 3 (Fig. 3). The data are compared to the in-flight 1g control. Left side = transcripts increased by at least 100 %; right side = transcripts decreased by at least 50 %

data summarized in Fig. 5 show a surprisingly high number of alterations in the amount of transcripts, even after such short periods of exposure. A large amount of transcripts obviously exhibits a general stress response as they appear up- or down-regulated under all conditions different from 1g (number in the central overlap; 1378 and 1769 respectively). There are, however, also phase-specific responses. At the end of the 20s-µg-period, for example, 396 unique transcripts were increased by >100%, while 485 were decreased to 50% or less.

In Table 1 we have compiled some of the genes, the products of which have a relationship to Ca^{2+}.

The increased expression of catalase, superoxide dismutase/related transcription factor, and glutathione peroxidase indicate a role of reactive oxygen species (ROS) in the response upon transition to microgravity. We have recently shown that an important member of ROS, namely hydrogen peroxide, changes its concentration in parallel to that of Ca^{2+} [17], and that short-time changes in protein phosphorylation comprise enzymes involved in the detoxification of ROS [9]. ROS are parts of stress signaling, and fine tuning of their concentrations is an important regulatory step ([18, 19]. Obviously, cytosolic Ca^{2+} pools and ROS closely interact, and it is not clear what is first, an increase in hydrogen peroxide, followed by an increase of cytosolic Ca^{2+} or vice versa ([18, 20]. The selected proteins presented in Table 1 support the role of Ca^{2+} (calcium-dependent lipid binding family protein; Ca^{2+}-binding protein; Ca^{2+}-dependent protein kinase; proteins taking part in Ca^{2+} homeostasis), as well as the participation of lipid signaling (inositol polyphosphate 5-phosphatase.

Tabel 1: µg specific gene expression during a parabolic flight. Examples for Calcium and ROS (reactive oxygen species) dependent genes

Gene identifier	Gene description	Protein function
AT4G34150	Calcium-dependent lipid-binding (CaLB domain) family protein	Calcium-dependent membrane targeting
AT1G02270	Calcium-binding family	Calcium ion binding
AT1G05630	Inositol polyphosphate 5-phosphatase	Ins(1,4,5)P3 dependent calcium release
AT1G35670	Calcium-dependent protein kinase 2 (CDPK2)	Calcium sensor; MAPK-crosstalk
AT4G35290	Glutamate receptor 2 (GLUR2)	Cellular calcium ion homeostasis
AT5G11210	Ligand-gated ion channel	Cellular calcium ion homeostasis
AT1G20630	Catalase 1 (CAT1)	Detoxification of hydrogenperoxide
AT2G28190	Copper/zinc superoxide dismutase 2 (CSD2)	Detoxification of superoxide radicals
AT1G14920	Transcription factor	Reduces ROS accumulation by up-regulating the transcription of superoxide dismutases.
AT2G43350	Glutathione peroxidase 3 (GPX3)	Functions as both a redox transducer and a scavenger in stress responses

The data presented are in good support of the notion that Ca^{2+} and ROS are part of signaling in response to gravitational stress, and, although changes in gene expression are obviously very fast, immediate effects should be more due to protein modification. Making use of advanced methods in protein analysis [21] we will now focus on phosphoproteom studies.

4. REFERENCES

1. Dodd, A.N., Kudla, J., Sanders, D. (2010). The language of calcium signaling. *Annual Review of Plant Biology* **61**, 593-620.

2. Lee, J.S., Mulkley, T.J., Evans, M.L. (1983). Reversible loss of gravitropic sensitivity in maize roots after tip application of calcium chelators. *Science* **220**, 1375–1376.

3. Daye, S., Biro, R.L., Roux, S.J. (1984). Inhibition of gravitropism in oat coleoptiles by the calcium chelator, ethyleneglycol-bis-(beta-aminoethyl ether)-N,N'-tetraacetic acid. *Physiol. Plant.* **61**(3):449-54.

4. Plieth, C. & Trewavas, A.J. (2002). Reorientation of seedlings in the earth's gravitational field induces cytosolic calcium transients. *Plant Physiol.* **129**(2):786-96.

5. Toyota, M., Furuichi, T., Tatsumi, H., Sokabe, M. (2007). Hypergravity stimulation induces changes in intracellular calcium concentration in Arabidopsis seedlings. *Adv Space Res* **39**, 1190–1197.

6. Toyota, M., Furuichi, T., Tatsumi, H., Sokabe, M. (2008). Cytoplasmic calcium increases in response to changes in the gravity vector in hypocotyls and petioles of Arabidopsis seedlings. *Plant Physiol.* **146**(2), 505-514.

7. Martzivanou, M., Babbick, M., Cogoli-Greuter, M., Hampp, R. (2006). Microgravity-related changes in gene expression after short-term exposure of *Arabidopsis thaliana* cell cultures. *Protoplasma* **229**, 155-162.

8. Barjaktarovic, Z., Nordheim, A., Lamkemeyer, T., Fladerer, C., Madlung, J., Hampp, R. (2007). Time course of changes in protein amounts of specific proteins upon exposure to hyper-*g*, 2-D clinorotation and 3-D random positioning of *Arabidopsis* cell cultures. *Journal of Experimental Botany* **58**, 4357-4363.

9. Barjaktarovic, Z., Schütz, W., Madlung, J., Fladerer, C., Nordheim, A., Hampp, R. (2009). Changes in the effective gravitational field strength affect the state of phosphorylation of stress-related proteins in callus cultures of *Arabidopsis thaliana*. *Journal of Experimental Botany* **60**, 779-789.

10. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikurak, M., Tsien, R.Y. (1997). Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. *Nature* **388,** 882-887.

11. Martzivanou, M. & Hampp, R. (2003). Hyper-gravity effects on the *Arabidopsis* transcriptome. *Physiol. Plant.* **118,** 221-231.

12. Yang, Y., Costa, A., Leonhardt, N., Siegel, R.S., Schroeder, J.I. (2008). Isolation of a strong *Arabidopsis* guard cell promoter and its potential as a research tool. *Plant Methods* doi: 10.1186/1746-4811-4-6.

13. Monshausen, G.B., Messerli, M.A., Gilroy, S. (2008). Imaging of the Yellow Cameleon 3.6 indicator reveals that elevations in cytosolic Ca^{2+} follow oscillating increases in growth in root hairs of *Arabidopsis*. *Plant Physiology* **147**, 1690-1698.

14. Nagai, T., Yamada, S., Tominaga, T., Ichikawa, M., Miyawaki, A. (2004). Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *PNAS* **101**, 10554-10559.

15. Babbick, M., Dijkstra, C., Larkin, O.J., Anthony, P., Davey, M.R., Power, J.B., Lowe, K.C., Cogoli-

Greuter, M., Hampp, R. (2007). Expression of transcription factors after short-term exposure of *Arabidopsis thaliana* cell cultures to hypergravity and simulated microgravity (2-D/3-D clinorotation, magnetic levitation). *Advances in Space Research* **39,** 1182-1189.

16. http://www.affymetrix. com/support/index.affx

17. Neef, M., Costa, A., Schumacher, K., Krebs, M., Hampp, R. (2011). Parabolic flight microgravity affects contents of hydrogen peroxide and cytosolic calcium in Arabidopsis cell cultures. *Journal of Gravitational Physiology* in press.

18. Costa, A., Drago, I., Behera, S., Zottini, M., Pizzo, P., Schroeder, J.I., Pozzan, T., Schiavo, F.L. (2010). H_2O_2 in plant peroxisomes: an *in vivo* analysis uncovers a Ca^{2+}-dependent scavenging system. *The Plant Journal* **62,** 760-772.

19. Swanson, S. & Gilroy, S. (2010). ROS in plant development. *Physiologia Plantarum* **138**, 384-392.

20. Mazars, C., Thuleau, P., Lamotte, O., Bourque, S. (2010). Cross-talk between ROS and Cclcium in regulation of nuclear activities. *Molecular Plant* **3**, 706-718.

21. Schütz, W., Hausmann, N., Krug, K., Hampp, R,. Macek, B. (2011). Extending SILAC to proteomics of plant cell lines. *Plant Cell* doi: 10.1105/tpc.110.082016.

5. ACKNOWLEDGEMENTS

We gratefully acknowledge excellent technical assistance by Margret Ecke, help in sample acquisition during parabolic flights by Fabian Bergwitz, and the supply of seeds of YC3.6 plants by Karin Schumacher, University of Heidelberg (Germany). This work was made possible by a grant from the DLR (Deutsches Zentrum für Luft- und Raumfahrt).