Gravity-sensing processes and gravity-dependent gene expression in plants
studied under altered gravity conditions

Dissertation
zur Erlangung des Doktorgrades (Dr. rer. nat.)
der Mathematisch-Naturwissenschaftlichen Fakultät
der Rheinischen Friedrich-Wilhelms-Universität Bonn

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Bonn 2010
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<tr>
<td>ATH1</td>
<td>Arabidopsis thaliana genome array</td>
</tr>
<tr>
<td>BaSO4</td>
<td>barium sulfate</td>
</tr>
<tr>
<td>°C</td>
<td>degree celsius</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>°</td>
<td>degree</td>
</tr>
<tr>
<td>DLR</td>
<td>Deutsches Zentrum für Luft- und Raumfahrt (German Aerospace Center)</td>
</tr>
<tr>
<td>ESA</td>
<td>European Space Agency</td>
</tr>
<tr>
<td>FC</td>
<td>fold change</td>
</tr>
<tr>
<td>g</td>
<td>gravity (1g = 9.81 ms⁻²)</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>IML-2</td>
<td>second International Microgravity Laboratory mission</td>
</tr>
<tr>
<td>K</td>
<td>represents 1000</td>
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<tr>
<td>m</td>
<td>meter</td>
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<tr>
<td>µ</td>
<td>micro</td>
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<td>microgravity</td>
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</tr>
<tr>
<td>#</td>
<td>number</td>
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<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>o/n</td>
<td>over night</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
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</tr>
<tr>
<td>p/n</td>
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<td>RIN</td>
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</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SSC</td>
<td>Swedish Space Cooperation</td>
</tr>
<tr>
<td>TAIR</td>
<td>The Arabidopsis Information Resource</td>
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<tr>
<td>TEXUS</td>
<td>Technological Experiments Under Reduced Gravity</td>
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<td>TIGR</td>
<td>The Institute for Genomic Research</td>
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<td>UniGene</td>
<td>NCBI transcriptome database</td>
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1. INTRODUCTION

1.1 Gravitropism-related processes in plants

Plants need to orientate their organs in the most beneficial way with respect to changing environmental conditions in habitats below and above the surface of the Earth. In order to produce energy-rich metabolites, shoots grow upwards toward the light, while roots grow downwards into the soil to supply the plant with nutrients and water as well as to anchor the plant body. In contrast to fluctuating environmental conditions due to seasonal or photoperiodic changes, gravity is the only constant factor providing plants with reliable information for the spatial orientation of their organs. Therefore, plants have evolved a highly sophisticated gravisensing system whose basic characteristics have persisted throughout plant evolution.

Plants perceive gravity by specialized cells, referred to as statocytes, in which starch-filled amyloplasts (statoliths) are free to sediment in the direction of gravity and, thereby, function as susceptors of the gravistimulus (starch-statoliths theory, Němec, 1900; Haberlandt, 1900). The gravity-induced sedimentation of statoliths leads to graviperception, the transduction of the physical stimulus into a physiological signal. Investigations on starch-less mutants of Arabidopsis thaliana and Nicotiana tabacum showed that these mutants were still gravitropic but their gravisensitivity was strongly reduced, thus, confirming the crucial role of statoliths as primary susceptors of gravistimuli in plants (Kiss et al., 1996; MacCleery and Kiss, 1999; Weise and Kiss, 1999). Furthermore, high-magnetic field studies supported the starch-statoliths theory of gravisensing in plant cells, since gravitropic curvature responses of plant organs were induced solely by the magnetophoretic displacement of statoliths in the statocytes of nominal vertically oriented roots and shoots (Kuznetsov and Hasenstein, 1996, 1997; Kuznetsov et al., 1999; Weise et al., 2000). These experiments provide strong evidence that the displacement of statoliths is the decisive initial step of gravisensing in plant cells.

During the last decades, numerous experimental approaches, based on cellular and molecular assays, were conducted to increase our knowledge about the processes during plant gravisensing and gravitropic response. Beside physiological studies at normal 1g conditions (e.g. various inhibitor-treatment analyses), instruments modifying acceleration conditions on ground by centrifugation or clinorotation became a powerful tool to investigate gravisensing processes in plants. The response of plants to hyper-g during centrifugation (e.g. Sievers and Heyder-Caspers, 1983; Wendt et al., 1987; Braun et al., 2002) and to ‘simulated weightlessness’ by applying a multilateral 1g stimulus during clinorotation (e.g. Sacks, 1887; Sievers and Hejnowicz, 1992; Cai et al., 1997) have been analyzed by physiological and biochemical studies. In recent years, a sensitive analysis method on
genomic level, the microarray technology that had already been successfully established in other gene-expression studies in plants (e.g. Girke et al., 2000; Hanano and Davis, 2007; Kilian et al., 2007; Goda et al., 2008), became an important tool for these ground-based studies on the effect of various acceleration conditions (Moseyko et al., 2002; Martzivanou and Hampp, 2003; Kimbrough et al., 2004; Salmi and Roux, 2008). Oligonucleotide probe microarrays facilitate precise evaluation of gene expression changes for hundreds to thousands of genes in parallel. In contrast to most of the physiological and biochemical studies, which are generally focused on one or a few targets of interest, the array technology provides a research tool to study effects on the whole genome-expression pattern.

1.1.1 The unicellular models Chara rhizoid and Chara protonema for research on gravisensing in plant cells

In contrast to the gravisensing statocytes of higher plants, which are located in compact tissues, the gravitropically growing transparent rhizoids and protonemata of the characean green algae are easily accessible for numerous experimental approaches. In addition, all steps of gravisensing including susception, perception, signal transduction and the gravitropic response are limited to one single cell. These beneficial features of rhizoids and protonemata have allowed for intensive investigations of the cellular and molecular mechanisms underlying the gravisensing mechanisms (for review, see Braun and Wasteneys, 2000; Braun and Limbach, 2005). In particular, experiments that have been performed in microgravity (µg) or on clinostats and centrifuges on ground, have decisively contributed to our current understanding of gravity-suscpetion and -perception processes in characean rhizoids and protonemata (Buchen et al., 1993; Buchen et al., 1997; Cai et al., 1997; Hoson et al., 1997; Braun et al., 2002).

Rhizoids and protonemata, both tube-like cells with a diameter of up to 30 µm, exhibit a very similar polar organization of their cytoplasm. However, they show opposite gravitropic growth orientation, i.e. rhizoids grow downwards (positive gravitropism) in order to anchor the algal thallus in the sediment, whereas protonemata grow upwards (negative gravitropism) by reason of regenerative function. Both cell types originate from nodal cells of the algal thallus. Protonemata are produced in the absence of blue light, e.g. when the thallus was buried by sediment. As soon as protonemata have reached light, tip growth terminates and cell divisions are initiated in order to regenerate the thallus.

The polar organization of the cytoplasm in rhizoids and protonemata is based upon the highly dynamic arrangement of actin microfilaments. Microtubules are also involved in the organization of the sub-apical and basal region, but they are absent from the apical tip region and are not involved in the primary steps of gravisuception and -perception. A multitude of actin-binding proteins manage the distinct arrangement of the actin cytoskeleton in the different zones of
rhizoids and protonemata. In the basal zone two populations of actin bundles with opposite polarities underlie the cytoplasmic streaming, which surrounds the large vacuole of the cell. The subapical region is characterized by a dense meshwork of mainly axially oriented, fine actin microfilament bundles (Braun et al., 2004). In the apical region, actin microfilaments and actin-associated proteins organize the so-called Spitzenkörper consisting of a dense aggregate of endoplasmic-reticulum (ER) membranes and secretory vesicles. The position of the Spitzenkörper defines the center of growth and, thus, the plasma membrane area with the maximal exocytosis rate of vesicles (Hejnowicz et al., 1977; Sievers et al., 1979; Braun, 1996; Limbach et al., 2008). Inhibitor treatment experiments with actin disrupting drugs and calcium (Ca\(^{2+}\)) ionophores have demonstrated that the structural integrity of the Spitzenkörper and the function of the center of growth are dependent on a specific subset of actin-associated proteins (Braun and Richter, 1999; Braun, 2001). A gradient of cytoplasmic free Ca\(^{2+}\) with the highest concentration at the outmost tip regulates the activity of spectrin-like proteins and the distribution of secretory vesicles (Braun and Richter, 1999; Braun et al., 2004).

The actomyosin system of rhizoids and protonemata precisely controls the positioning and the sedimentation of statoliths, small vacuoles containing barium-sulfate crystals (Schröter et al., 1975). Actomyosin forces prevent statoliths from settling into the cell tip by acting net-basipetally in tip-downward growing rhizoids. In tip-upward growing protonemata, the actomyosin system acts net-acropetally in order to prevent statoliths from sedimenting toward the cell base (Hodick et al., 1998; Braun et al., 2002; Braun, 2002). In downward growing rhizoids and in upward growing protonemata under normal \(1g\) conditions (\(g = 9.81 \text{ m s}^{-2}\)), statoliths are kept in a dynamically stable position of balance in an area a few \(\mu\)m from the cell tip by actomyosin forces compensating the oppositely acting gravity force (Braun et al., 2002). Inhibitor studies with Cytochalasin D have shown that disrupting the actin cytoskeleton in rhizoids and protonemata leads to termination of tip growth and causes the sedimentation of statoliths in the direction of gravity. After removing the drug, statoliths are retransported to the original position and tip growth continues (Braun et al., 2004).

In contrast to the highly regulated statolith positioning in both axial directions of the rhizoid, the control by the actomyosin system is less pronounced in lateral direction. The rather weakly regulated statolith position in lateral direction of the rhizoid is the prerequisite for a fast and sensitive gravisensing mechanism. In rhizoids that are stimulated by \(90^\circ\), the sedimenting statoliths mainly follow the gravity vector and settle onto the physically lower cell flank where graviperception takes place and the gravitropic response is initiated. In rhizoids reoriented by angles different from \(90^\circ\), statoliths no longer simply sediment along the gravity vector, but are actively guided by actomyosin forces against the gravity force onto the beltlike gravisensitive
plasma-membrane area (10-35 µm from the tip) — the only membrane area where gravireceptors are located (Braun, 2002). Pushing statoliths onto other plasma-membrane regions by centrifugation or laser-tweezer micromanipulations does not initiate a curvature response of the rhizoid. However, the displaced statoliths are retransported to the gravisensitive subapical membrane area, where graviperception occurs (Leitz et al., 1995; Braun, 2002). In contrast to rhizoids, the gravisensitive plasma-membrane site in protonemata is located at 5-10 µm basal to the tip (Braun, 2002). During reorientation of protonemata, net-acropetally actomyosin forces guide sedimenting statoliths to the graviperception site initiating gravitropic response (Hodick et al., 1998).

Fluorescence imaging of Ca\textsuperscript{2+} and of dihydropyridine, an L-type Ca\textsuperscript{2+} channel blocker, in rhizoids and protonemata indicated that the gradient of cytoplasmic free Ca\textsuperscript{2+} in the cell tip is altered by gravireceptor activation. During the readjustment of the nominal growth direction in protonemata, a drastic shift of the tip-high calcium gradient toward the upper flank is observed. This asymmetric Ca\textsuperscript{2+} gradient results most likely from the statolith-induced differential activation or local inhibition of Ca\textsuperscript{2+} channels in the protonema tip. The Spitzenkörper and, therewith, the growth center, is most likely displaced by Ca\textsuperscript{2+}-dependent actomyosin-generated movements toward the upper cell flank and the new outgrowth occurs at that site ('bending by bulging'; Braun and Richter, 1999; Braun, 2001). In contrast to protonemata, the growth center remains symmetrically positioned in the center of the tip during the gravitropic response of rhizoids (Braun, 2001). The sedimentation of statoliths onto the gravisensitive site results in the local inhibition of Ca\textsuperscript{2+} influx leading to differential growth of the opposite cell flanks in rhizoids ('bending by bowing'; Braun and Richter, 1999).

Although the molecular nature of the gravireceptors in plants is still unknown, the mechanism of gravireceptor activation has recently been functionally characterized in the rhizoid of the green alga Chara (Limbach et al., 2005). Earlier experiments have shown that statoliths have to be fully sedimented onto the gravisensitive area of the plasma membrane. The sedimentation process of statoliths itself does not induce a curvature response (Braun, 2002). Parabolic flight experiments aboard the Airbus A300 Zero-G were conducted to reveal the activation mechanism of the gravireceptors in rhizoids. It was found that the sedimented statoliths in gravistimulated rhizoids remained settled onto the plasma membrane during the different acceleration levels of the parabolic flight profile (1g, hypergravity and microgravity) and did not lose contact with the gravireceptors (Limbach et al., 2005). Flight samples experienced all 31 periods of microgravity during the 2-h flight. In-flight control samples were centrifuged at 1g during the microgravity phases. Surprisingly, the final curvature angles of flight samples were not different from in-flight controls. This provided clear evidence that graviperception was not interrupted.
1. INTRODUCTION

when statoliths became weightless during microgravity. Weightless statoliths were able to activate the gravireceptors and, thus, pressure exerted by the weight of statoliths is not required for gravireceptor activation and graviperception to occur in characean rhizoids (Limbach et al., 2005). This finding was supported by ground-control experiments, which demonstrated that an increase of pressure of statoliths exerted on gravireceptors by the application of hyper-g accelerations did not result in enhanced gravitropic responses. On the other hand, disruption of the contact between statoliths and gravireceptor molecules by inverting roots in further ground-control experiments led to significantly smaller curvature responses. Even short-term removal of statoliths from the gravisensitive plasma membrane for only 10 s was found to significantly impair gravitropic curvature. Considering the results of the flight and ground-control experiments the gravireceptor in characean rhizoids must be referred to as a receptor, which is activated by direct interactions with components of the statolith surface (Limbach et al., 2005).

1.1.2 Arabidopsis as a multicellular plant model system for research on gravitropism-related signaling pathways

The pioneering studies on graviresponsive characean rhizoids and protonemata have resulted in a considerable progress in the understanding of the primary mechanisms of gravitropic sensing and signaling in relatively simple lower plant cell types. Gravisensing processes in the statocytes of higher plants are less well understood and results of experiments were often contradictory. Models of graviperception in higher plant statocytes postulated that sedimentation of statoliths generates tension or shearing forces, which are transferred via actin-dependent mechanisms to putative mechanosensitive receptors in the cortical ER membrane or the plasma membrane (Sievers et al., 1991; Yoder et al., 2001; Zheng and Staehelin, 2001; Boonsirichai et al., 2002, Sievers et al., 2002; Blancaflor and Masson, 2003; Perbal and Driss-Ecole, 2003). However, until today the nature of the gravireceptors in higher plants is unknown and studies have so far not given proof of mechanosensitive receptors involved in plant gravitropism.

As already mentioned above, in contrast to the relatively big and transparent single-celled rhizoids and protonemata, the major problem for addressing questions about gravitropic mechanisms in higher plants is the complexity of the gravisensing tissues and the transmission and integration of the gravity-induced signals from a multitude of cells. Therefore, experimental investigations, e.g. microscopic or molecular applications on gravity-susception and –perception mechanisms in higher plants are quite difficult. However, previous findings have identified second messengers that are most likely involved in the early processes of gravisignal transduction including cytoplasmic free Ca\(^{2+}\) (Lu and Feldman, 1997; Plieth and Trewavas, 2002; Belyavskaya, 2004), cytosolic protons (pH) (Scott and Allen, 1999; Fasano et al., 2001), and inositol-1,4,5-triphosphate (IP3) (Perera et al., 2001, 2006). According to the Cholodny-Went theory,
the physiological signal generated by graviperception is transmitted from the sensing statocytes to the responding cells by means of the directional transport of the growth hormone auxin (Cholodny, 1928; Went, 1933). A lateral gradient of auxin in the gravistimulated organ results in the differential growth of the opposite flanks of the organ. The establishment of the auxin gradient during gravitropic reorientation has been intensively investigated. Auxin influx carriers and efflux carriers, PIN-formed (PIN) proteins, were identified as key components of the auxin transport mechanism (Friml, 2002, 2003; Ottenschläger, 2003; Blilou et al., 2005). Results of previous experiments have also implicated a role for the hormones cytokinin (Aloni et al., 2004), ethylene (Lee et al., 1990; Madlung et al., 1999; Buer et al., 2006), brassinosteroids (Kim et al., 2000; Li et al., 2005) and gibberellic acid (Brock and Kaufmann, 1988) in the regulation of growth during gravitropic responses.

In recent years, several studies reported on gravity-related alterations in protein expression involved in stress responses, primary metabolism, general signaling, protein translation and ion homeostasis (Wang et al., 2006; Barjaktarović et al., 2007, 2008, 2009). Treatment-related changes were detected in Arabidopsis cell cultures exposed to hypergravity or 'simulated' microgravity conditions (clinorotation or random-positioning machine). Within 10 min, first changes in expression patterns of phosphorylated proteins were detected (Barjaktarović et al., 2009). In addition to analyses on protein-regulation level, in the last years, the analyses via oligonucleotide-microarray technology has become a sensitive tool for investigating changes in gene-expression patterns in response to altered gravitational conditions in ground and flight experiments. It was unclear, for a long time, whether the regulation of gravity-related genes is also involved in the plant response to altered g conditions. However, recent studies on Arabidopsis demonstrated that altered g forces affect plants, causing changes in metabolic processes, signaling pathways, cellular organization and stress-related responses, which are regulated by gene expression (Moseyko et al., 2002; Martzivanou and Hampp, 2003; Martzivanou et al., 2006; Kimbrough et al., 2004). Findings indicated that both, complex interacting and independent regulatory pathways, are affected by different g conditions (Centis-Aubay et al., 2003). The genomic studies reported on a high sensitivity for gravity-related gene responses to only transient stimuli, e.g. the transient reorientation by rotating the plant (360°) within 10 s (Moseyko et al., 2002). Furthermore, it was shown that the significant up- or rather down-regulation of gravitropism-related genes was detected even 2 min after stimulation (Moseyko et al., 2002).
1.2 Objectives of the study

In this study, experiments were conducted under altered gravitational conditions provided by sounding rocket and parabolic plane flights in order to unravel the fundamental mechanisms, which underlie the sensitivity and the effectivity during gravisusception and -perception in plants.

1.2.1 Threshold acceleration level required for lateral statolith displacement in Chara rhizoids

In downward growing rhizoids, the equilibrium position of the statoliths results from the actomyosin forces acting against the gravity force. Sounding-rocket and clinostat experiments demonstrated that, when the influence of gravity was abolished, the basipetal acting actomyosin forces caused the displacement of statoliths against the former direction of gravity (Buchen et al., 1993; Cai et al., 1997; Braun et al., 2002). Earlier studies conducted on sounding rocket MAXUS-3 and MAXUS-5 missions have already shown that the acceleration level required for statoliths to overcome the cytoskeletal positioning forces and to induce lateral statolith displacement, i.e. sedimentation, is \( \leq 0.14 \text{g} \) in Chara rhizoids (Limbach et al., 2005). In the present study, a microgravity experiment during the MAXUS-8 campaign in spring 2010 aimed at narrowing down this approximation of acceleration value. The results allowed for calculating of the energetic concept underlying the gravity-susception and -perception mechanisms in such a precise way, as it had never been possible before for any plant- or animal-model system.

1.2.2 Statolith-mediated graviperception in Arabidopsis root statocytes

Encouraged by the findings on the gravireceptor-activation mechanism in Chara rhizoids, in this study, higher plants were investigated in a similar way under microgravity conditions provided by parabolic plane flights aboard the Airbus A300 Zero-G. Comparison of the growth curvatures of characean rhizoids, which experienced 31 microgravity phases during 2-h flights and of those, which were centrifuged at 1\( \text{g} \) to avoid the microgravity phases (in-flight controls), revealed no significant differences. Therefore, abolishing the weight of sedimented statoliths during the microgravity phases did not interrupt the activation of gravireceptors in Chara rhizoids (Limbach et al., 2005). In the present study, it was tested for the first time if the statoliths in higher plant root statocytes also stay in contact with the gravireceptors and are not displaced during the \( \mu \text{g} \) phases of parabolic plane flights, as it was shown for characean rhizoids (Limbach et al., 2005). It has already been shown that the final curvature angles of primary Arabidopsis roots, which experienced all conditions of a parabolic plane flight including the \( \mu \text{g} \) phases, were sometimes slightly higher but never smaller than those of the in-flight controls, which did not experience microgravity (Greuel, 2007; Hauslage, 2008). Therefore, showing that the sedimented statoliths in the horizontally positioned Arabidopsis seedlings during flight are not displaced...
from the lower cell flank but stay in contact with the gravireceptors during flight, would support the idea that graviperception in higher plant statocytes relies on graviperception by contact-interactions between sedimented statoliths and receptor molecules. For this purpose, the primary roots of Arabidopsis seedlings were fixed with potassium permanganate (KMnO₄) in the different phases of parabolic plane flights. Semithin sections of the plant samples allowed for analyses of the statoliths' position.

1.2.3 Gravity-dependent gene expression in plants

After the successful functional characterization of the gravireceptor activation in characean rhizoids and in higher plant statocytes, the consequent next step comprises the investigation of the molecular basis of gravity sensing in plants. To provide a basis for the identification and detailed molecular characterization of key components involved in graviperception, e.g. the gravireceptors or crucial membrane components on the statoliths' surface, the general effect on the protein- and gene-expression pattern during plant reorientation under different gravitational conditions has to be evaluated. In this respect, the third part of the present study is focused on the regulation of gene-expression during gravity-related responses in Arabidopsis seedlings. As already mentioned above, genome analyses on the effect of altered gravitational conditions in plants have already been conducted (Moseyko et al., 2002; Martzivanou and Hampp, 2003; Kimbrough et al., 2004; Paul et al., 2005). However, none of these studies comprised experiments during parabolic plane flights providing fast and successive changes of different acceleration conditions (1g, hyper- and microgravity). For the first time, this study aimed at investigating the changes in gene expression patterns in response to the different gravitational conditions of parabolic plane flights using the aircraft A300 Zero-G (10th DLR (Deutsches Zentrum für Luft- und Raumfahrt) campaign). In parallel, a comprehensive experiment series on ground was performed, in order to evaluate the effects of plant stimulation by reorientation under 1g and 2g conditions. In contrast to the previous studies using oligonucleotide probe arrays with maximum 8,300 transcripts, in the present study, an Arabidopsis-DNA microarray covering the entire Arabidopsis genome (43,803 transcripts) was applied, ensuring a whole-genome analysis.
2. MATERIALS AND METHODS

2.1 Plant Material

2.1.1 Chara rhizoids
Thallus of Chara globularis Thuill. was collected from a pond in the Botanical Garden of the University of Bonn, Germany. For the production of rhizoids, thalli were cut into segments comprising two nodes and one internodal cell. The side branches of the lower node were cut off to induce rhizoid development. Thallus segments were embedded in a thin layer of agar (1.2% in distilled water) in TEXUS (Technological Experiments Under Reduced Gravity) cuvettes (Fig. 1, constructed by Astrium Space Transportation, Bremen, Germany and first described by Volkmann et al., 1991) in 1.2% agar in distilled water at RT and under constant illumination at 200 µmol m\(^{-2}\) s\(^{-1}\) for 3-4 d.

2.1.2 Arabidopsis
Arabidopsis thaliana ecotype Wassilewskija and ecotype Columbia were cultivated in a cultivation room at alternating illumination with 16 h of light and 8 h of darkness. Mature seeds were harvested and stored at 4°C.

2.1.2.1 Arabidopsis root seedlings for fixation by KMnO\(_4\)
Seeds of Arabidopsis thaliana ecotype Wassilewskija were sterilized with 75% EtOH for 3 min and 1% NaOCl for 10 min. Sterilized seeds were washed 4 times with distilled water. Arabidopsis seedlings were cultivated on filter paper in TEXUS cuvettes (Fig. 2). The cuvettes comprised a core frame (V2A steel), two Makrolon\(^®\) window and two aluminium frames on either side. The core frame and the two Makrolon\(^®\) windows were sealed with o-rings. Between the core frame and one Makrolon\(^®\) window the filter paper with the Arabidopsis seeds (15-20 seeds per filter paper) was embedded (Fig. 2). Inlet and outlet ports on each side of the cuvette facilitated the injection of liquids and the ventilation. Distilled water (0.5 mL) was injected into the...

Fig. 1. Components of a TEXUS cuvette. The core frame (D) and a Makrolon\(^®\) window (B, F) on either side are sealed with o-rings (C, E). Two aluminium frames (A, G) on either side protect the cuvette against pressure disturbances. The components are screwed together on either side. (The in- and outlet ports (H, I) are attached to the core frame only for the experiments with Arabidopsis. These ports are sealed for the MAXUS experiment.)

Fig. 2. Arabidopsis seeds on wet filter paper in a TEXUS cuvette.
cuvettes. After a cold stratification at 4°C in darkness for 2 d, germination was induced and seedlings were cultivated at 24°C under constant illumination (Radium Bonalux super, NL24W/11-860 Spectralux daylight). The cuvettes were daily vented with 10 mL O₂. Three-days old seedlings were used for the experiments.

2.1.2.2 Arabidopsis seedlings for dry-ice fixation
Seeds of Arabidopsis thaliana ecotype Columbia were sterilized with 75% EtOH for 3 min and 5% NaOCl for 15 min. Sterilized seeds were washed 4 times with distilled water. Arabidopsis seedlings were cultivated on filter paper in 60-mm Petri dishes. The filter paper was saturated with 1% bacto-agar in medium containing micronutrients (modified after Legué, 1997). Petri dishes were sealed with Parafilm® to avoid contamination. After a cold stratification at 4°C in darkness for 2 d germination was induced in an incubator at 24°C under constant illumination (Radium Bonalux super, NL24W/11-860 Spectralux daylight). In the sterile environment of the incubator a small hole in the Petri dishes ensured air exchange during cultivation. Four days old seedlings were used for the experiments (Fig. 3). Approximately 12 h before start of the experiments, seedlings were habituated to darkness, since all flight and ground experiments were performed in darkness.

2.2 MAXUS-8 sounding rocket flight experiment
MAXUS is an enlarged version of TEXUS. Both, MAXUS and TEXUS missions, are part of the European sounding rocket program that provides a platform for scientific experiments under microgravity conditions. The MAXUS program is a joint venture between SSC (Swedish Space Cooperation) and Astrium Space Transportation funded by ESA (European Space Agency). The parabolic flight of MAXUS rockets provides scientists a reliable access to high quality microgravity (µg) conditions of up to 10⁻⁴g for 12-14 min. The experiment modules can be accessed until about one hour before lift-off. In-flight data recording via telemetry is possible. Telecommand and video transmission provides controlling and monitoring of the experiment during the 12 to 14 min of µg. Biological samples are recovered directly after landing and brought back to the laboratories at Esrange (near Kiruna, Sweden) usually within 1-2 h after launch.
Previous experiments have demonstrated that parabolic flights of sounding rockets are consummately suited to study the actomyosin mediated statolith sedimentation in Chara rhizoids (Volkmann et al., 1991; Buchen et al., 1997; Braun et al., 2002). The MAXUS-8 experiment was conducted in order to determine the cytoskeletal forces underlying the gravisensing mechanisms of characean rhizoids.

Fig. 3. Arabidopsis seedlings for dry-ice fixation. Plants are cultivated on filter paper in 60-mm Petri dishes.
2. MATERIALS AND METHODS

2.2.1 Sample preparation
Internodal segments of Chara thalli were prepared in the laboratory at Esrange and Chara rhizoids were cultivated in TEXUS cuvettes (see 2.1.1). The cuvettes comprised a core frame (V2A steel), which was covered by two Makrolon® windows on either side. The core frame and the Makrolon® windows were sealed with o-rings. Two aluminium frames on either side protected the cuvette against the vacuum in the orbit. Three hours before lift-off, 3 TEXUS cuvettes with rhizoids showing good growth rates (~100-200 µm h\(^{-1}\)) and well positioned statoliths were selected and mounted on late-access units (see Fig. 4, C), which were mounted in the payload module about 2 h before lift-off.

Fig. 4. Chara module TEM 06-RO1M of MAXUS-8. The module (side view on the left and top view on the right) consists of a motor and telemetry unit (B) and a rotatable platform (A), on which 3 microscopic units are attached. The flight cuvettes with rhizoids are mounted on late-access units (C), which are mounted in the module approximately 2 h before lift-off.

2.2.2 Chara module TEM 06-5RO1M
A precursor version of the payload module TEM 06-RO1M (Fig. 4), constructed by Astrium Space Transportation (Bremen, Germany), had already been successfully flown on MAXUS-3 and MAXUS-5. The precursor module was slightly modified for the MAXUS-8 experiment. The Chara module consisted of a stationary lower motor and telemetry unit and an upper rotatable platform, on which 3 horizontal microscopic units were installed (Zeiss, Oberkochem, Germany). Each microscopic unit comprised a 20x objective lens and a 2.5x optovar system resulting in a total magnification of 50x. Observing and recording was performed with the black/white CCD camera XC-ST50 (Sony, Cologne, Germany). Three flight cuvettes with rhizoids were mounted in late-access units (Fig. 4), which were inserted into the module approximately 120 min before lift-off. The three cuvettes were located on three different radii of the rotatable platform. The position of the cuvettes with regard and the focus was adjusted by telecommand. The rhizoid samples in the cuvettes were observed via in-vivo video microscopy during the sounding rocket flight. Temperature was kept at 22°C ± 1 °C.
2.2.3 Experiment procedure
The ESA MAXUS-8 sounding rocket was launched from the SSC’s launch facility Esrange Space Center outside Kiruna in Northern Sweden in March 26th, 2010. MAXUS-8 reached an apogee of 700 km and provided μg conditions of 10^-4g for 836 s. Rotation of the platform of the experiment module TEM 06-5RO1M was started 75 s after lift-off, at the beginning of microgravity, and continued until re-entry of the payload (836 s after lift-off). Dependent on the different distances of the three cuvettes from the center of the platform, the rhizoids experienced lateral accelerations of 0.060g, 0.080g or 0.100g (Fig. 4). Via telecommand access on the rotation of the platform, at 400 s after lift-off, lateral accelerations were decreased to 0.055g, 0.073g and 0.092g. At 550 s after lift-off, lateral accelerations were further decreased to 0.050g, 0.067g and 0.083g and at 720 s after lift-off they were upregulated again (0.055g, 0.073g and 0.092g) until re-entry. During flight, the rhizoids were observed and checked for lateral displacement of the statolith complex.

2.2.4 Data analysis
The video-microscopic images were analyzed to identify and track the movements and, in particular, any lateral displacements of statoliths during the lateral centrifugation under the microgravity conditions of the MAXUS-8 sounding rocket flight. The original video format VOB of the video microscopy recording was converted to WMV1 codec (Aura Video Converter 1.21, http://download.cnet.com/Aura-Video-Converter/3000-2194_4-10966793.html). Single video images were extracted with Ulead VideoStudio 6 (Bitmap format) and processed with Adobe® Photoshop® (JPEG format). Tracking of the statoliths and the statolith complex was performed with ImageJ (http://rsbweb.nih.gov/ij/download.html). Because the images during the first 110 s after lift-off were out of focus (re-focusing was performed), tracking analysis of the statoliths was done from +110 s until +800 s after lift-off.

2.3 Parabolic plane-flight experiments
Parabolic plane-flight experiments were performed during the 10th DLR parabolic plane-flight campaign at Cologne airport, Germany, in September 2007 (2.3.2), and during the 13th DLR parabolic plane flight campaign at Bordeaux Airport, France, in February 2009 (2.3.1).

Parabolic plane flights with the Novespace Airbus A300 Zero-G provide alternating levels of 1g, microgravity and hypergravity conditions. Therefore, parabolic plane-flight experiments are well suited to study the effect of different acceleration levels on gravitropism-related processes, in particular, on gravity sensing mechanisms in biological organisms. At the beginning of each parabola, the aircraft is pulled up with full engine power from the horizontal flight to an ascending angle of 47°. The aircraft is subjected to approximately
2. MATERIALS AND METHODS

Fig. 5. Scheme showing the parabolic flight profile of the aircraft A300 Zero-G on one flight day with the alternating acceleration levels of 1g, hypergravity and microgravity. Within approximate 109 min 31 parabolas are flown at intervals of about 3 min. After a first test parabola, 6 sets of 5 parabolas are interrupted by breaks of four, five and eight minutes with 1g conditions. The real acceleration values in the direction of the z and x axis of the aircraft are shown for the parabolas number 22 to 26.

twice of normal Earth gravity (1g = 9.81 ms⁻²). After 20 s of hypergravity, the aircraft engines are throttled down to a minimum thrust (‘injection’). During this phase of the parabolic flight curve, all forces acting on the aircraft compensate each other and the aircraft freely falls in the gravitational field of the Earth. The aircraft is exposed to microgravity (< 10⁻²g) for 22 s per parabola until at a descending angle of 42° the engines are reset to full power again. The aircraft is pulled out of the parabola in a second 2g phase of 20 s followed by normal horizontal flight phase (Fig. 5). The flight profile consists of 31 parabolas, which were flown within approximately 2 h on each of the three flight days per campaign (Fig. 5), thus, total microgravity time per flight is approximately 12 min or 10 % of the entire flight time. The 10th DLR parabolic flight campaign provided another two flight days with 11 parabolas, which were flown within approximately 50 min (8% total µg).

Definition of orientation for the Arabidopsis seedlings in parabolic flight and ground experiments

In the recent study during all in-flight and on-ground experiments (2.3.1 and 2.3.2) the position of the Arabidopsis samples was defined relative to the vector of the applied acceleration, i.e. 1g Earth gravity, hyper- and microgravity, respectively. Therefore, a vertically (0°) positioned plant grew parallel to the direction of the applied gravity vector while a horizontally (90°) positioned seedling was orientated perpendicular to the direction of the gravity vector.
2.3.1 Analysis of the position of sedimented statoliths during parabolic plane flight

In previous parabolic plane-flight experiments, it was demonstrated that in Chara rhizoids and in higher plant root statocytes, weightless statoliths, which do not exert pressure on membrane-bound gravireceptor molecules are still able to activate the gravireceptors (Limbach et al., 2005; Greuel, 2007). This notion has been made under the assumption that, during the microgravity phases of the parabolic plane flight, statoliths remain in contact with the gravireceptors and are not removed from the gravisensitive membrane. In order to investigate if this is also true for higher plant statocytes, the current experiment focused on the microscopic analysis of the position of sedimented statoliths in the root statocytes of Arabidopsis seedlings during the parabolic plane flights of the 13th DLR campaign.

2.3.1.1 Sample preparation
Approximately two to three hours before flight 18 cuvettes with Arabidopsis seedlings showing good growth rates (~120-180 µm h⁻¹) and a good alignment with respect to the gravity vector were mounted in the fixation units. A small PVC (polyvinyl chloride) bar was pinched between the filter paper and the Makrolon® window of the cuvettes to prevent a direct flow-through of the fixation solution and to facilitate a good distribution of the solution on the samples.

2.3.1.2 Flight hardware Charabolix-8
Eight PVC panels, each equipped with two flight cuvettes, were flown on every flight day. The cuvettes were fixed on the panels with cable straps. The inlet port of the flight cuvettes was connected via a LS-2 connecting tube with two 5 mL Omnifix® Luer Lock syringes (B. Braun Melsungen AG, Melsungen, Germany), one filled with 5 mL KMnO₄ fixation solution (3% in tap water) and one filled with 5 mL...
2. MATERIALS AND METHODS

Fig. 8. Arabidopsis root scheme showing the location of S1, S2 and S3 statocytes.

tap water. Connecting tubes were locked with clamps until fixation and elution, respectively. The exit port of the flight cuvettes was connected via Original Perfusor® connecting tube (type MR) with one empty 10 mL Omniflex® Luer Lock syringe (B. Braun Melsungen AG, Melsungen, Germany). The syringes were also fixed with cable straps on the panel (Fig. 6). The whole panel including two fixation units was sealed in a transparent Ziploc® bag (Toppits®) providing a second containment. The panels were arranged in an aluminium box mounted on the experiment rack Charabolix-8 (hardware of Charabolix described in detail by Hauslage, 2008). The temperature in the experiment box was kept at 23-25°C.

2.3.1.3 In-flight procedure

To determine the effect of microgravity on the positioning of sedimented statoliths in the root statocytes of horizontally positioned Arabidopsis roots, the seedlings were fixed with KMnO₄ shortly before the beginning and shortly before the end of the µg-phase (Fig. 7). Fixation was performed during the first and the 16th parabola on all three flight days. The seedlings were horizontally stimulated 20 min prior to the fixation. Thereby, sedimentation of the statoliths in the root statocytes was assured. For fixation, 5 mL KMnO₄ (3% in tap water) were injected into the cuvettes. The KMnO₄ solution was extracted of the cuvettes after 30 s. Immediately, 5 mL tap water were injected to wash out residual KMnO₄. Video recording of the fixation procedure was done for one fixation unit on each flight day to control the optimal distribution of the fixation solution on the plant seedlings. On each flight day, the fixation of control samples was done 5 min before the first parabola. These control samples were also horizontally pre-stimulated 20 min before fixation, and were directly compared with fixed seedlings at the beginning and at the end of the µg phases.

2.3.1.4 Post-flight procedure

Immediately after flight, the root tips of the fixed Arabidopsis seedlings were cut off in a 45° angle with a razor blade. The 45° cut indicated the orientation of the root relative to the gravity vector during parabolic flight (Fig. 9). The root tips were transferred to tap water and stored at 4°C for 4 to 6 d until they were further processed and
prepared for performing semithin sections via a microtome (Ultra-mikrotom OM U3, Reichert Jung, Vienna, Austria). Details of the dehydration, infiltration, embedding and polymerization procedure are summarized in Table I (p 17). During the embedding procedure the root tips were arranged with a needle in the ERL block (Spurr Low Viscosity Embedding Media, Polysciences Europe GmbH, Eppelheim, Germany), such that the longitudinal root axis was positioned parallel to the subsequent microtome section. The embedded samples were trimmed with a razer blade and a mill (TM 60, Reichert Jung, Vienna, Austria). Median semithin sections (0.5 µm) were stained with Methylene Blue/Azure II for 10 s on a heater.

2.3.1.5 Data analysis
Stained semithin sections were examined with the inverse microscope Axiovert 135 (Carl Zeiss Jena GmbH, Jena, Germany). Pictures of the sections were taken using the AxioCam HRC (Carl Zeiss AxioVision software). With the software Segment (developed for MATLAB, The MathWorksTM) the position of the statoliths was determined. The analysis exclusively considered fully sedimented statol-
liths on the physical lower cell flank, i.e. statoliths whose sedimentation was not inhibited by other statoliths. The distances between the outer membrane of the statoliths and the lateral and axial cell wall flanks of the statocytes (the plasma membrane was not visible in the semithin sections) were analyzed. Furthermore, the position of each statolith was described by coordinates relative to a reference point, which was set on the physical lower cell wall flank in the outmost apical corner of the cell (Fig. 10). The pixel values were converted to µm and statistical analyses of the data were performed with Microsoft Excel®. The position of the statoliths in the S2 and S3 statocytes were analyzed (Fig. 8).

2.3.1.6 Ground experiments
In control experiments on ground (1g) Arabidopsis roots were horizontally stimulated for 20 min, subsequently inverted from 90° to 270° for 10 s, 60 s and 120 s and then the seedlings were immediately fixed with KMnO₄ (2% in tap water). In order to determine the sedimentation velocity of the statoliths within a certain period of inversion time, the mean distances between the statoliths and the lateral cell flank of the statocytes were measured in the inverted roots and in the non-inverted controls. The analysis exclusively considered statoliths whose sedimentation was not inhibited by other statoliths (see also 2.3.1.5).

Table I. Processing of the Arabidopsis root seedlings after fixation with KMnO₄

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<th>incubation time</th>
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2.3.2 Gravity-dependent gene expression in plants

2.3.2.1 Sample preparation
Two to three hours before flight, 35-40 Petri dishes with Arabidopsis seedlings showing good growth rates (~120-180 µm h⁻¹) and a good alignment with respect to the gravity vector were selected. On each of the first three flight days (each with 31 parabolas) 20 Petri dishes with samples were mounted in a fixation chamber (flight samples) and 15 Petri dishes with samples were mounted in the on-board 1g-reference centrifuge (in-flight control samples). Flight and in-flight control samples showed a vertical orientation and were not stimulated by 90°. On the fourth and fifth flight day (each with 12 parabolas) in one fixation chamber, plant seedlings were mounted in a vertical orientation (20 Petri dishes) and in, one fixation chamber, plants were mounted in a horizontal orientation (20 Petri dishes).

2.3.2.2 Flight hardware Carbocryonix
The experiment hardware Carbocryonix, described in detail by Horn (2007) and Hauslage (2008), was mounted on a custom-made aluminium rack and included two steel cylinders as fixation chambers, each with a volume of 3.6 liter and each connected with a 10kg CO₂ (carbon dioxide) gas bottle (Fig. 11). The hardware allows the shock-freezing of biological samples on ground as well as in parabolic plane-flight experiments. A custom-made sample holder for 60 mm Petri dishes allows mounting of Arabidopsis seedlings in vertical as well as in horizontal orientation in the fixation chambers (Fig. 12). For fixation, gaseous CO₂ is conducted into the fixation chambers. The CO₂ gas abruptly expands in the chambers. Due to the released pressure and the spontaneous cooling, CO₂ dry ice with a temperature of -79.5 °C is produced. Within seconds the samples and all biological processes, respectively, are fixed for subsequent processing and genome or proteome analyses. Temperatures inside the fixation chambers are monitored via a temperature measurement system. Temperatures of approximate -60 °C are consistently hold for more than one hour after fixation (Fig. 13).

2.3.2.3 In-flight procedure
In the present experiment it was tested whether acceleration levels differing from normal Earth gravity (1g) have an effect on gene expression level in Arabidopsis seedlings. Therefore, plants were exposed to the hyper- and microgravity conditions of a parabolic plane flight with the Airbus A300 Zero-G. On three flight days vertically oriented flight samples, which experienced all the different acceleration levels of 31 parabolas, were shock-frozen by producing dry-ice after the last parabola. Vertically oriented in-flight control samples, which experienced the same conditions as the flight samples including the hypergravity phases except that they were accelerated with 1g in an on-board centrifuge when the flight samples experienced the µg phases, were fixed by dry-ice production after the last parabola as well. By using swing-out chambers in the on-board centrifuge it was
ensured that all accelerations acted in the same direction. On two additional flight days comprising 12 parabolas, vertically oriented as well as flight samples stimulated by 90° were simultaneously shock-frozen after the last parabola. Fixed samples were immediately recovered after landing and transferred to the lab for further processing.

2.3.2.4 Post-flight procedure
Total RNA of the shock-frozen seedlings (approx. 300 seedlings per experiment) was isolated via guanidinium thiocyanate-phenol-chloroform extraction (modified after Chomczynski and Sacchi, 1987):

RNA extraction via TRIzol® reagent
- homogenize 100 mg of the sample in 1 mL TRIzol®
- centrifuge for 10 min at 12,000 g at 4-8 °C
- transfer supernatant into 200 µL chloroform
- incubate for 2-3 min at RT
- centrifuge for 15 min at 12,000 g at 4-8 °C
- transfer upper aqueous phase into 0.5 mL icecold isopropanol
- incubate for 10 min at RT
- centrifuge for 10 min at 12,000 g at 4-8 °C
- discard the supernatant
- wash the RNA pellet with 75 % ethanol
- mix by vortexing
- centrifuge for 5 min at 7,500 g at 4-8 °C
- air-dry the pellet on ice
- resuspend the pellet in 100 µL H2O
2.3.2 Gravity-dependent gene expression in plants

The extracted RNA was subsequently treated with DNAse and purified according to the protocol of the RNeasy® Mini Kit (p/n 79254, p/n 74104, Qiagen, Hilden, Germany):

DNAse treatment and RNA purification
- mix the following in a microcentrifuge tube:
  - ≤ 87.5 µL RNA solution
  - 10 µL buffer RDD
  - 2.5 µL DNase I stock solution
  - make the volume up to 100 µL with H2O
- incubate for 10 min at 20-25°C
- clean up RNA according to the RNA Cleanup protocol of the RNeasy® Mini Kit
- solve the purified RNA in 30 µL H2O

Quantity and quality of the purified RNA was determined via photometric analyses (UltrospecTM 3100 pro, Amersham Biosciences Europe GmbH, Germany) and gel electrophoresis. Information about the RNA sample integrity was extracted from a bioanalyzer electrophoretic trace (Agilent 2100 Bioanalyzer, p/n G2938A, Agilent Technologies, Germany). Table II (p 21) summarizes the criteria, which had to be satisfied by the RNA samples for further processing.

2.3.2.5 Agilent one-color microarray technology
To detect transcriptional changes in flight and ground samples, which were exposed to different gravity conditions, Agilent’s Arabidopsis thaliana 60-mer oligonucleotide microarrays were processed (Agilent Technologies, Germany). The 4x 44K slide format, covering the entire Arabidopsis genome, was selected (www.agilent.com/chem/dna). Each slide provided four identical microarrays with 43,803 Arabidopsis thaliana probes represented (content sourced from RefSeq, UniGene, TAIR, TIGR and ATH1).
Each sample was hybridized on a separate microarray. In contrast to the two-color ratio-based approach, where all gene expression ratios are generated from two samples compared on the same microarray, the one-color intensity-based microarray solution provided the ability to compare the measured gene expression output of one sample directly across the other samples.

Sample preparation and microarray processing
Sample labeling and microarray processing was performed as detailed in the Agilent “One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling)” protocol (p/n G4140-90040, version 5.7, March 2008). The labeling reactions were performed using the Agilent Quick Amp Labeling Kit One-Color (p/n 5190-0442) in the presence of cyanine 3-CTP dye. For microarray hybridization the cyanine 3-labeled cRNA samples were hybridized on the Agilent *Arabidopsis thaliana* 4x 44K microarrays using the Agilent Gene Expression Hybridization Kit (p/n 5188-5242). Together with the samples the in vitro synthesized spike-in transcripts of the Agilent One-Color RNA Spike-In Kit (p/n 5188-5282) were hybridized onto the microarrays for the purpose of quality checks. The spike-in transcripts show a minimal self- or cross-hybridization and hybridize only onto the complementary control probes on the microarrays. The expected versus the observed log ratios enabled the monitoring of the microarray workflow for sensitivity and accuracy.

The hybridized microarrays were washed in Agilent Gene Expression Wash Buffer 1 (p/n 5188-5325) for one minute at RT and subsequently in Agilent Gene Expression Wash Buffer 2 (p/n 5188-5326) for one minute at 37 °C. The processed microarrays were scanned with the Agilent DNA microarray scanner (p/n G2565BA), and extracted with Agilent Feature Extraction software (version 10.5). The spot intensity data was loaded into the Agilent GeneSpring® GX software (version 10.0) for data analysis.

2.3.2.6 Data analysis
The technical replicates comprised two to five microarrays. Each of the 11 biological experiments consisted of two to six independent biological replicates to achieve good reproducibility of microarray analyses. The Arabidopsis seedlings for all of the 11 biological experiments were cultivated from three different harvests of seeds (see overview

<table>
<thead>
<tr>
<th>RNA concentration [ng/µL]</th>
<th>OD260/OD280 (indication of protein contamination)</th>
<th>OD260/OD230 (indication of phenol, isothiocyanate, polysaccharides)</th>
<th>rRNA ratio (28s/18s)</th>
<th>RIN (RNA integrity number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 200</td>
<td>&gt; 2.0</td>
<td>&gt; 1.8</td>
<td>&gt; 1.2</td>
<td>&gt; 7.0</td>
</tr>
</tbody>
</table>

Table II: Optimal values for RNA concentration, OD ratio, rRNA ratio and RNA integrity number, which had to be satisfied by the RNA samples for further processing.
about all experiments in Table III in the appendix). The median intensity value of all samples was taken for further calculation of intensity dependent normalization of spots for each microarray slide (standard normalization: threshold raw signals to 1.0, normalization algorithm percentile shift of 75). The entities were filtered based on their flag values. The acceptable flags for at least one out of the samples were present or marginal. Up-/down-regulated genes were determined by a higher/lower than 2-fold change in their expression ratio (experiment versus control “0° 2h 1g”) in all microarrays. The normalized values of up-/down-regulated genes were tested for statistical confidence. Student’s t-test analyses showed all changes in gene expression to be statistically significant at a $P$-value of 0.05 or less. Self-organizing cluster analyses were performed on the log(2) fold changes of normalized expression values. Classification of genes into functional categories for biological processes, molecular functions or cellular components by gene ontology annotation was performed at The Arabidopsis Information Resource (TAIR, www.arabidopsis.org/tools/bulk/go/index.jsp, status April 2010).

**2.3.2.7 Ground experiments**

The parabolic plane-flight experiments offer the possibility to study changes in plant gene regulation as a response to the successively changing acceleration stimuli of $\mu g$, $1g$ and $2g$ as well as to the other flight conditions, e.g. vibrations. However, to evaluate the effects of different $g$-conditions on gene expression, various ground experiments were performed.

**Vertically oriented control seedlings at 1g**

Vertically oriented plants under normal Earth $1g$ conditions were the corresponding controls for all ground and flight experiments.

**Horizontal stimulation at 1g**

To assess the effect on gene expression during stimulation by 90°, seedlings were horizontally positioned for 2 h (typical gravitropic response).

**Inversion experiment at 1g**

Horizontally stimulated Arabidopsis seedlings were inverted from 90° to 270° 31 times for 10 s within a total gravistimulation time of 2 h, to determine the effect of repeated short-term inversions on gene expression.

**Hypergravity experiments at 2g**

In order to analyze whether hyper-$g$ conditions have an effect on gene expression, seedlings were centrifuged parallel to the direction of the resulting acceleration vector for 2 h at $2g$. Furthermore, for simulating the short-term hyper-$g$ phases of the parabolic flight profile, plants were intermittently centrifuged 62 times at $2g$ for 20s within a total experiment time of 2 h. The hyper-$g$ experiments were performed with vertically and horizontally oriented Arabidopsis seedlings.
Vibration experiments at 1g

During parabolic flights vibrations are generated particularly by the operation of the aircraft engines. Schmidt (2004) demonstrated that the vibrations during a parabolic flight were transmitted to the experiment hardware and to the experiment samples, respectively. In order to evaluate the effect of the vibrations, specific for parabolic plane flights, on the gene expression pattern of Arabidopsis, ground controls were performed. The analysis during parabolic flights in the aircraft A300 Zero-G, conducted by Schmidt (2004), was the basis for the vibration simulation on Vibraplex, a newly developed hardware (developed and constructed by Dr. Jens Hauslage, DLR, Germany). The pattern of various vibrations of different frequencies (0.2Hz - 14kHz) and intensities (maximum 56 Wm⁻²), as specific for a parabolic plane flight, was programmed in terms of an audio sound file (WAV format). The digital file signal was amplified with an audio amplifier and directly transmitted to the vibration platform of the Vibraplex unit, on which the vertically oriented Arabidopsis seedlings were attached. The applied signal strength in terms of vibrations was controlled via a piezoelectric crystal, which was installed directly on the vibration platform and, therefore, allowed for oscilloscope monitoring of the signal. During the 2-h ground experiment, the vibration spectrum of a 2-h parabolic flight with 31 parabolas including the parabola-phase specific changes in vibration intensities (Fig. 14) was simulated.

Fig. 14. During vibration experiments on ground, the vibration spectrum of a parabolic flight with the aircraft A300 Zero-G is applied to Arabidopsis seedlings. The vibration pattern and its parabola-phase specific changes for one parabola is illustrated (amplitude in logarithmic scale, frequency in linear scale).
2.4 Chemicals and Reagents

A. dest : aqua destillatum

acetone : Carl Roth GmbH & Co. KG

agar : extra pure, fine powder, Merck KGaA, Darmstadt, Germany

Azure II : Merck KGaA, Darmstadt, Germany

bacto-agar : Sigma Aldrich Chemie GmbH, Steinheim, Germany

EtOH : ethanol, Carl Roth GmbH & Co. KG

Gene Expression Hybridization Kit : p/n 5188-5242, Agilent Technologies, Germany

Gene Expression Wash Buffer 1 : p/n 5188-5325, Agilent Technologies, Germany

Gene Expression Wash Buffer 2 : p/n 5188-5326, Agilent Technologies, Germany

H₂O : water

KMnO₄ : potassium permanganate, crystalline, Sigma Aldrich Chemie GmbH, Steinheim, Germany

Methylene Blue : Merck KGaA, Darmstadt, Germany

NaOCl : sodium hypochlorite solution

Quick Amp Labeling Kit, One Color : p/n 5190-0442, Agilent Technologies, Germany

RNA Spike-In Kit, One Color : p/n 5188-5282, Agilent Technologies, Germany

RNeasy® Mini Kit : Qiagen, Hilden, Germany

sodium tetraborate (Borax) : Merck KGaA, Darmstadt, Germany

TRIZol® : Invitrogen GmbH, Karlsruhe, Germany

TWEEN® : TWEEN® 20 pure (polyoxyethylene sorbitan monooleate), Serva Electrophoresis GmbH, Heidelberg Germany
2. MATERIALS AND METHODS

2.5 Solutions and Media

Medium for Arabidopsis cultivation (modified after Legué, 1997)

1M KNO$_3$
1M Ca(NO$_3$)$_2$ x4H$_2$O
1M (NH$_4$)$_2$HPO$_4$
0.5M MgSO$_4$ x7H$_2$O

micronutrients:
25µM KCl
25µM Fe-Na EDTA
17.5µM H$_3$BO$_3$
1µM MnSO$_4$
1µM ZnSO$_4$
0.25µM CuSO$_4$
0.25µM (NH$_4$)$_6$MoO$_4$

ERL (Spurr Low Viscosity Embedding Media, Catalog No. 17706-1, Polysciences Europe GmbH, Eppelheim, Germany)

soft medium:
4.1g ERL 4221
1.9g Diglycidyl ether of polypropylene glycol (D.E.R. 736)
5.9g Nonenyl succinic anhydride (NSA)
0.1g Dimethylaminoethanol (DMAE)

Methylene Blue/Azure II

Filter 1% Methylene Blue in A. dest and 1% Azure II in sodium tetraborate (Borax). Mix 1:1.
Before use, mix dye solution : A. dest (1:3).
2.5 Solutions and Media