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Plant Cells on Earth and in Space

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Two quite different types of plant cells are analysed with regard to transduction of the gravity stimulus: (i) Unicellular rhizoids and protonemata of characean green algae; these are tube-like, tip-growing cells which respond to the direction of gravity. (ii) Columella cells located in the center of the root cap of higher plants; these cells (statocytes) perceive gravity. The two cell types contain heavy particles or organelles (statoliths) which sediment in the field of gravity, thereby inducing the graviresponse. Both cell types were studied under microgravity conditions (10^{-4} *g*) in sounding rockets or spacelabs. From video microscopy of living *Chara* cells and different experiments with both cell types it was concluded that the position of statoliths depends on the balance of two forces, i.e. the gravitational force and the counteracting force mediated by actin microfilaments. The actomyosin system may be the missing link between the gravity-dependent movement of statoliths and the gravity receptor(s); it may also function as an amplifier.

In contrast, to animals, which use extracellular particles, plant cells perceive gravity by intracellular mechanisms. Particles or organelles (statoliths) with a specific density higher than that of the surrounding cytoplasm, are displaced physically by gravity (susception) thereby influencing gravireceptors. In a stimulus-transduction process, an internal signal is created physiologically (perception) and transmitted to the site of response. This response may occur within the same cell (as in characean rhizoids and protonemata; Fig. 1) or at a distant site (as in roots; Fig. 2).

The Unicellular Characean Rhizoid and Protonema

Introduction

The unicellular, gravity-sensing rhizoids and protonemata of characean green algae are model systems for investigating gravitropic tip growth. The positively gravitropic (downward growing) rhizoids are tube-like cells with a highly polarized cytoplasmic zonation and growth limited strictly to the apical cell pole (Fig. 3). The basal zone contains a single large vacuole that is surrounded by two opposing layers of rotational endoplasmic streaming and stationary cortical cytoplasm. The 300-µm long tip region can be subdivided into a subapical and an apical zone, both characterized by a relatively stationary cytoplasm devoid of vacuoles and in which cytoplasmic streaming does not occur. The subapical zone contains randomly distributed organelles including mitochondria, proplastids, dictyosomes and the large nucleus at its basal end. Extensive endoplasmic-reticulum (ER) cisternae are mainly axially oriented. The apical zone extends from the outermost tip to 35-40 µm into the cell and incorporates the exocytosis control center, the Spitzenkörper, which consists of an accumulation of secretory vesicles surrounding a unique ER aggregate. Another conspicuous feature of the apical zone is the presence of up to 50 statoliths, which cluster at a fairly constant distance from the apical cell wall. The statoliths of rhizoids and protonemata are prominent membrane-bound vesicles of up to 2 µm in diameter. They do not contain starch, as is the case in higher plant amyloplast-statoliths, but embody a matrix of carbohydrates and proteins (Wang-Cahill and Kiss, 1995) in which $BaSO_4$-crystals are arranged in a radial pattern (Schröter et al., 1975); traces of strontium were also detected by X-ray analysis (Sievers and Schmitz, 1982). Centrifugation experiments unambiguously proved their function as statoliths (Buder, 1961). Cytological studies were backed up with mathematical analysis (Hejnowicz and Sievers, 1971) to produce a model for the gravitropic response mechanism of rhizoids in which statolith sedimentation causes a local impediment to exocytosis, resulting in differential flank growth in the apical zone (Sievers, 1971; Sievers and Schröter, 1971; Sievers et al., 1979; Sievers and Schnepf, 1981).

This was the first conclusive model for a complete gravitropic response chain. When negatively gravitropic (upward growing) characean protonemata were first used in photomorphogenetic and gravitropic investigations by Hodick (1993), it immediately became obvious that together, rhizoids and protonemata would provide an ideal experimental system for comparative studies on gravity-oriented tip growth. Rhizoids and protonemata are indistinguishable in terms of cell shape and polar

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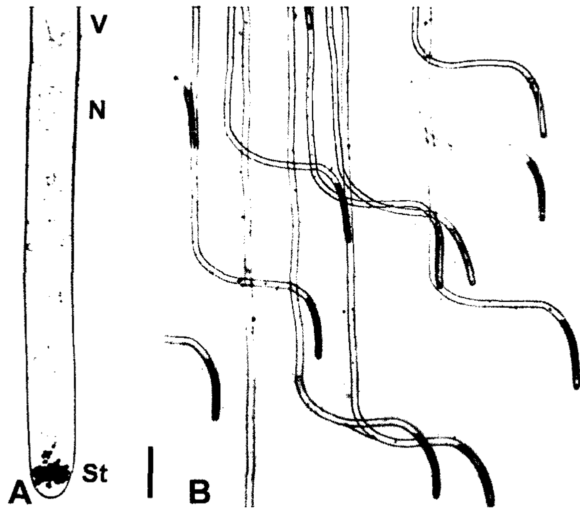

Fig. 1. A, Unicellular *Chara* rhizoid in normal vertical orientation with the basal vacuolar region (V) and the apical region with the nucleus (N) and the statoliths (St) above the apical cell wall. Scale bar=30 µm. (After Sievers et al., 1991b.) B, Positive gravitropic curvature of Chara rhizoids tilted two times by 90° (After Sievers and Schröter, 1971).

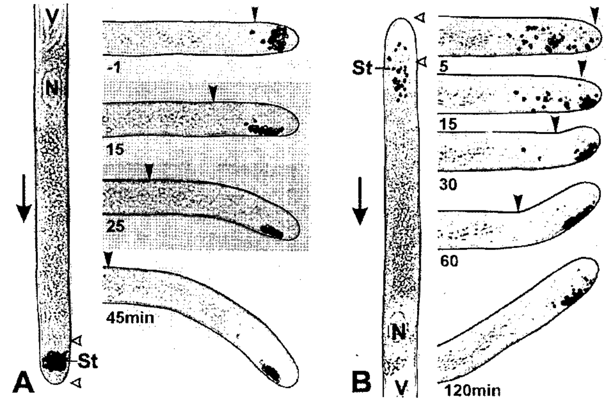

Fig. 3. Gravitropic responses of rhizoids (A) and protonemata (B) of *Chara globularis*. Only the apical zones (between the white arrowheads), the subapical zones (containing the nucleus (N) and most of the stationary cytoplasm) and a small part of the basal zones (characterized by the large vacuole (V) surrounded by rotational endoplasmic streaming) is shown. A, The positive gravitropic response of rhizoids is initiated by gravity-induced sedimentation of the statoliths (St) onto the lower cell flank that results in differential flank growth and a continuous bending of the tip downwards (bending by bowing). B, The negative gravitropic response of protonemata is initiated by the statoliths (St) which invade the apical dome and sediment asymmetrically near the growth center at the tip. This is followed by a drastical shift of the cell tip upwards (bending by bulging). Arrows=direction of gravity; black arrowheads indicate the same point in each micrograph. Diameter of the cells: 30 µm (A Modified after Braun, 1996b; B modified after Hodick, 1994).

cytoplasmic organization but show opposite gravitropic responses (Fig. 3).

There is increasing evidence from studies of the last decade that the actin cytoskeleton plays an essential role in many of the specialized features of characean rhizoids and protonemata. These characteristic features include tip growth, statolith positioning and gravitropic orientation.

Arrangement of the actin cytoskeleton

The arrangement of the actin cytoskeleton is very similar in rhizoids and protonemata and reflects the cells' polar cytoplasmic organization. The conspicuous and rapid cytoplasmic streaming in the basal zone relys on thick, interconnected actin cables that are easily visualized with rhodamine phalloidin (Tewinkel et al., 1989; Sievers et al., 1991a; Braun, 1997) and immunolabelling (Braun and Wasteneys, 1998a, b).

At the apical end of the basal zone, the actin cables fan out to form a dense meshwork of fine, mainly axially oriented microfilament (MF) bundles. This extensive actin meshwork penetrates the subapical and apical zones and envelopes the large nucleus and numerous organelles. In the apex, more extensively bundled actin MFs form a less dense meshwork and focus in a central actin array, which is symmetrically positioned close to the cell tip (Fig. 4B). This spherical actin array colocalizes with the ER aggregate (Sievers et al., 1991a; Braun and Wasteneys, 1998a) first described by Bartnik and Sievers (1988). Thus, the organization of the actin cytoskeleton appears more complex in characean rhizoids and protonemata than that in

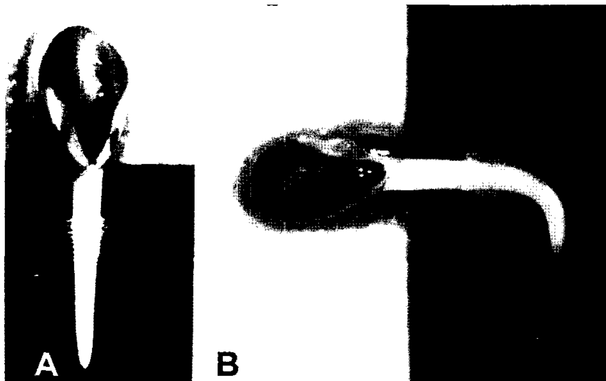

Fig. 2. A, Primary cress root 24 h after imbibition of the seed showing positively gravitropic growth perfectly oriented according to the gravity vector. B, Positive gravitropic reorientation of the same root 2 h after tilting in horizontal position (Originals by H. Behrens).

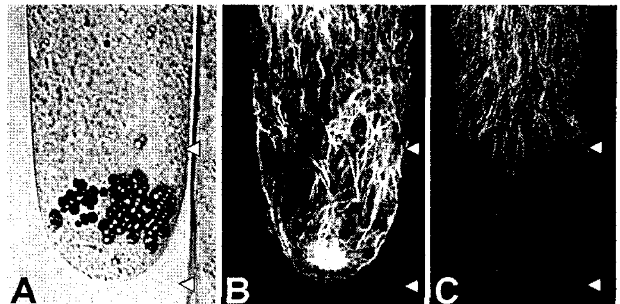

Fig. 4. Apical and part of the subapical zone of a Chara rhizoid. A, Brightfield image showing the complex of statoliths located 10-30 µm above the apical cell wall. B, The rhodamine-phalloidin labeling of the cell (shown in A) documents a fine meshwork of mainly axially oriented actin MFs which appear more loosely arranged in the apical zone (region between the two arrowheads) and form a dense spherical actin array close to the growth center at the tip. C, Anti-tubulin immunoflurescence image showing an extensive meshwork of MTs in the subapical zone and the absence of MTs from the apical zone. Diameter of the cells: 30 µm (Originals by M. Braun).

many other tip-growing cells such as root hairs (Miller et al., 1999; Braun et al., 1999a), pollen tubes (Miller et al., 1996; Cai G. et al., 1997), moss protonemata (Walker and Sack, 1995; Meske et al., 1996), fern protonemata (Kadota et al., 1999) and fungal hyphae (Jackson and Heath, 1990; Levina et al., 1994).

Actin-microtubule interactions and polar organization

The interplay between microtubules (MTs) and the actin cytoskeleton is an important feature of rhizoid and protonema structure and function. Immunfluorescence and in vivo observations of MTs in living cells by injection of fluorescent tubulin have documented a clear distribution pattern for MTs under steady state, gravistimulated, chemically and otherwise perturbed conditions (Braun and Sievers, 1994; Braun and Wasteneys, 1998a). The arrangement of MTs is different from that of the actin cytoskeleton but MTs are also distributed in accordance with the polarized cytoplasmic organization of these cells.

In the subapical region, MTs and actin MFs colocalize in a dense network of scattered but predominantly axially oriented elements (Braun and Sievers, 1994; Braun and Wasteneys, 1998a). Microinjection of fluorescently tagged tubulin showed that the subapical MTs originate from the basal side of the nuclear envelope, indicating the nuclear envelope's MT-organizing function. MTs appear to flare out into the subapical zone, but, unlike actin filaments, end abruptly 35-40 μm from the tip (Fig. 4C). MTs thus appear to be completely excluded from the apex of growing cells, and this exclusion seems to be important for both gravisensing and the gravitropic response. Terminating tip growth by mechanically manipulating the cells or by illuminating protonemata causes MTs to extend into the apical zone where they impair the actin-mediated movements and gravity-directed statolith sedimentation (Braun and Wasteneys, 1998a, b). Conversely, depolymerization of MTs by oryzalin treatment dramatically alters the cell's polar organization and disturbs the arrangement of actin MFs in the apical and subapical, but not the basal regions. The vacuole moves closer to the tip and streaming takes place in what was once stationary cytoplasm, coincident with the extension of thick actin bundles into this region. Gravitropic tip growth does not continue once the apical cytoplasm is converted into streaming endoplasm (Braun and Sievers, 1994). In contrast, disruption of the actin cytoskeleton with cytochalasin D inhibits cytoplasmic streaming (and effectively arrests tip growth), without changing the polar cytoplasmic organization or the arrangement of MTs in the basal and subapical zone (Braun and Sievers, 1994). Based on these results, it is concluded that MTs play a crucial role in the maintenance of the polar cytoplasmic zonation, a precondition for tip growth, and intimately interact with the functional arrangement of the subapical actin cytoskeleton. Similar conclusions are emerging from recent studies on tip-growing higher plant cells (Bibikova et al., 1997). The motile processes, however, i.e. cytoplasmic streaming, transport of organelles, positioning of statoliths and delivery of vesicles, are generated by the acto-myosin system.

Role of actin in tip growth

The process of polar growth has been demonstrated to be dependent on the complexly organized apical actin cytoskeleton. Cytochalasin-induced disruption of actin inhibits vesicle trafficking and exocytosis and causes the disappearance of the prominent ER aggregate near the apex (Bartnik and Sievers, 1988; Bartnik et al., 1990), resulting in termination of tip growth (Hejnowicz and Sievers, 1981; Braun & Sievers, 1993). The spherical aggregate of ER membranes in the apex represents the structural center of the vesicle-rich Spitzenkörper, whose putative function is to regulate vesicle guidance and exocytosis. The ER aggregate co-localizes with the dense apical actin array (Sievers et al., 1991a; Braun and Wasteneys, 1998a, b) and recent immunological evidence (Braun, submitted) suggests a protein with similar function and some homology to animal spectrin is specifically involved in organizing this ER-actin aggregate. This actin-spectrin interaction may play a role in anchorage and maintenance of the structural organization, providing mechanical stability to the distinct apical ER subdomain. These proteins may also provide a mechanism for the recruitment of specific membrane proteins, which are required for the characteristic functions of the ER aggregate, i.e., regulation of vesicle trafficking (Bartnik and Sievers, 1988; Bartnik et al., 1990) and control of the highly specific physiological environment at the tip.

A tip-high gradient of cytoplasmic free calcium was shown to be essential for tip growth in characean rhizoids and protonemata (Braun and Richter, 1999) in agreement with reports for many other tip-growing plant cell types (see Sanders et al., 1999 and references therein). The calcium gradient, visualized with the single-wavelength indicator Calcium Crimson, strongly colocalizes at the tip with dihydropyridine receptors, which are putative calcium channels (Braun and Richter, 1999). Both gradients dissipate when tip growth is inhibited. Interfering with the calcium gradient of rhizoids and protonemata by applying the calcium ionophore A23187 or the calcium-channel blocker gadolinium chloride causes disintegration of the ER aggregate, prevents spectrin antibody labelling and causes a major reorganization and bundling of the actin MFs. Removal of these agents is followed by a restoration of the complex actin arrangement, the reformation of the ER aggregate, the reappearance of spectrin-like epitopes and resumption of tip growth.

Actin-mediated positioning and movement of statoliths

Several observations demonstrate convincingly that

statolith positioning in characean rhizoids and protonemata is primarily controlled by the acto-myosin cytoskeleton. Myosin antibodies label the surface of statoliths (Braun, 1996a) and in the steady-state, statoliths are positioned in an actin MF-rich but MT-free zone (Braun and Wasteneys, 1998a). Cytochalasin treatments that disrupt the actin cytoskeleton impair normal responses of statoliths to gravistimulation (Hejnowicz and Sievers, 1981; Braun and Sievers, 1993). In this section, the regulation of statolith positioning and motility by the acto-myosin cytoskeleton is discussed. Despite the very similar arrangement of actin in rhizoids and protonemata, the motile properties of statoliths in these two cell types turn out to be remarkably different. These differences help explain how positive and negative gravitropic responses are mediated.

Actin-mediated positioning of statoliths is a prerequisite for gravisensing: Acropetal transport of statoliths occurs in both protonemata and rhizoids. It is more obvious and constantly active, however, in upward tip-growing protonemata in which statoliths drop in a gravity-dependent manner up to 100 µm back into the subapical zone and are sporadically retransported towards the tip. Cytochalasin prevents retransport, demonstrating actin's critical involvement in generating the net-acropetal forces on statoliths that allows them to keep pace with the growing tip (Hodick, 1994; Hodick and Sievers, 1998). In rhizoids, however, the positioning of statoliths is mediated by net-basipetal forces; gravitational forces preclude the need for active retransport as long as cells are downward pointing but if cells are repositioned in a horizontal or upward pointing direction, acropetal transport of statoliths is required to prevent slippage of statoliths into the basal region of the cell, where they are useless for graviresponses. Indeed, removing statoliths from original position by basipetal centrifugation abolishes graviresponsiveness (Buder, 1961; Sievers et al., 1991a) and graviresponsiveness is only restored once the displaced statoliths are retransported to their original position. Cytochalasin experiments demonstrated that this retransport, which can overcome basipetal accelerations of up to 70 *g* (Braun and Sievers, 1993), is mediated by actin (Sievers et al., 1991a). Statoliths retransported against basipetal centrifugal forces take longer to sediment along the gravity vector, implying that statolith-actin interactions become stronger under such conditions (Braun and Sievers, 1993). The actin cytoskeleton is thus able to adapt to altered gravitational environments in order to maintain statoliths in a strategic position.

Displacement of the Spitzenkörper by statoliths is involved in gravitropic bending of protonemata: The constitutive activity of the acropetal transport mechanism in protonemata is demonstrated when cells are gravistimulated (Sievers et al., 1996; Braun, 1997). Upon horizontal positioning of protonemata, statoliths not only sediment in the direction of gravity, but are also simultaneously transported into the apical dome where they then settle asymmetrically against the apical plasma membrane (Fig. 3; Hodick, 1994; Hodick et al., 1998). This apical positioning of statoliths close to the growth center at the outermost tip is followed by upward-directed reorientation of the protonema tip. Inverting protonemata in a tip-downward orientation results in statoliths settling into the apical dome where they leave only a small region at the outermost tip free. Random asymmetric distribution of statoliths eventually initiates reorientation of the protonema tip back to the normal upright position (Fig. 3). The apical intrusion of sedimenting statoliths in protonemata is suggested to induce the negative gravitropic reorientation of the growth direction by displacing the Spitzenko rper and, thus, a repositioning of the growth center from the outermost tip to the upper flank (Sievers et al., 1996; Braun, 1997). This mode of bending was termed bending by bulging (after Green et al., 1970).

A basipetally directed transport mechanism excludes statoliths from the growing tip of rhizoids: In contrast to protonemata, rhizoids have an additional mechanism that prevents statoliths from sedimenting against the plasma membrane of the apical dome. Statoliths are held in a dynamically stable position 10-30 µm above the tip where they continuously perform saltatory and rotational movements (for review see Sievers et al., 1996; Braun, 1997). By controlling this position, actin MFs prevent statolith sedimentation into the physically lowest cell apex, an outcome that would impede exocytosis. Cytochalasin treatments provide indirect evidence for actin's role in statolith positioning: disruption of the actin cytoskeleton causes the statoliths to drop onto the apical cell wall and thus inhibits tip growth (Hejnowicz and Sievers, 1981; Braun and Sievers, 1993). After removing cytochalasin, the statoliths are lifted back to their original position and tip-growth resumes (Hejnowicz and Sievers, 1981; Braun and Sievers, 1993). Thus, in normal, tip downward oriented rhizoids, the gravity force, which pulls the statoliths towards the tip, is compensated by an internal counteracting force mediated by the acto-myosin system (Sievers et al., 1991b; Volkmann et al., 1991).

Further evidence for the existence of this counter-gravity mechanism in rhizoids has literally come from outer space. When rhizoids were subjected to the 6-min microgravity phase ($< 10^{-4}$ *g*) of the parabolic flight of sounding rockets (TEXUS), statoliths moved basipetally and doubled their original distance from the cell tip (Fig. 5A). Treating rhizoids with cytochalasin D prior to launch caused statoliths to settle onto the apical cell wall, and they were not displaced during the microgravity phase (Fig. 5B). Removing the inhibitor resulted in a repolymerization of the actin cytoskeleton and the cells quickly recovered. This proved that the statolith lifting in cytochalasin-free rhizoids could not be

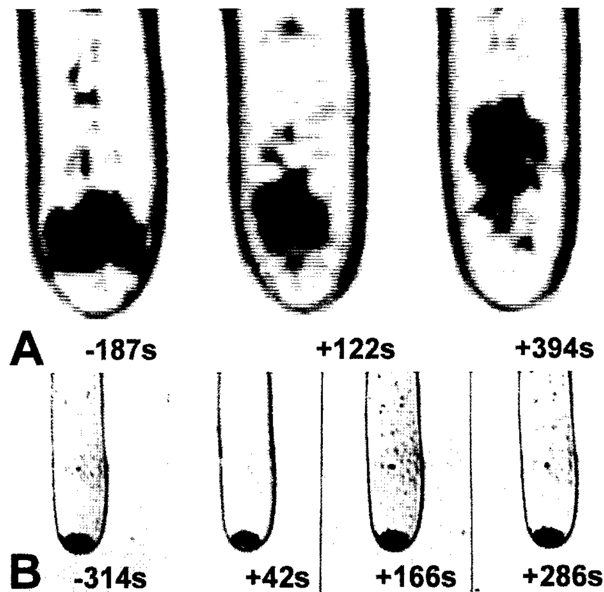

Fig. 5. Videomicroscopic images showing the behaviour of statoliths of characean rhizoids during the 6-min microgravity phase of a TEXUS-rocket flight. A, In untreated rhizoids, the statoliths nearly doubled their original distance from the tip (-187s) during the microgravity phase (+122s, +394s). B, In rhizoid in which the statoliths were settled into the physically lowest cell tip due to cytochalasin D treatment prior to launch (-314s), the statoliths were not displaced during the microgravity phase (+42s, +166s, +286s) but were lifted back to their original position after removing the inhibitor. Diameter of the cells: 30 µm (A Modified after Volkmann et al., 1991; B modified after Buchen et al., 1993).

caused by a simple physical effect, such as random positioning of weightless particles (Buchen et al., 1993). When rhizoids were positioned horizontally prior to the launch of the TEXUS rocket, the complex of sedimented statoliths was displaced basipetally rather than towards the former upper flank during microgravity (Buchen et al., 1997). Together, these experiments indicate that the position of statoliths in rhizoids is highly controlled and regulated in both axial directions, but only weakly controlled in the lateral direction (Braun, 1997). Interestingly, earlier optical tweezer experiments came to the same conclusion by measuring the laser output power required to move statoliths (Leitz et al., 1995); the force needed to move statoliths toward the apex is greater than the force to move the statoliths towards the flank. Thus, the actin cytoskeleton actively controls the position of the statoliths to accomplish graviresponsiveness and allows the rapid, gravity-directed sedimentation of statoliths in lateral directions which is crucial for the initiation of the graviresponse.

In an attempt to simulate the effect of weightlessness on statolith positioning, rhizoids were rotated on the horizontal axis of slow and fast-rotating clinostats (Cai W. et al., 1997). Rotational velocities of 60-90 rpm resulted in a basipetal displacement of statoliths, similar to but slower than the movement of statoliths during the microgravity phase of TEXUS flights. During clinostatting, however, the cluster of statoliths became more and more dispersed and eventually moved closer towards the tip again. This apical approach was interpreted as a cytoskeleton-mediated adaptational effect, something also observed in rhizoids grown for hours in real microgravity aboard the Space Shuttle (IML-2, S/MM05; Braun, 1997). In contrast, slower rotational velocities of the classical clinostat (1-10 rpm) failed to simulate the effect of microgravity. Statoliths were displaced from the periphery towards the central cell axis and, thereby, the complex of statoliths elongated axially, extending more basally and more apically as compared to the original position. These results suggest mechanical perturbations as the major effect of the classical clinostat, but mainly endogenous actomyosin-driven movements of the statoliths induced by fast rotations, thus, properly simulating microgravity effects.

Gravisensing mechanisms develop even in the absence of gravity: *In vivo* videomicroscopy and ultrastructural analysis of rhizoids that were initiated at 1 *g* on Earth and grown for several days in microgravity (IML-2) versus rhizoids that developed and grew exclusively in microgravity aboard the SpaceLab (IML-2) and SpaceHab (S/MM05), provided evidence that the gravisensing cells follow their endogenous program for development and morphogenesis even in the absence of net-accelerations in a microgravity environment. Deprived of directional accelerations, rhizoids radiated in all directions from the nodal complexes, and developed normal cell shape, organization and the polar distribution of organelles including the ER-aggregate in the center of the vesicle-rich Spitzenkörper at the cell tip (Braun, 1997; Braun et al., 1999b). Furthermore, by using a centrifuge microscope (Friedrich et al., 1996), it was confirmed that the microgravity-grown rhizoids are still able to respond to acceleration stimuli, even when statoliths are positioned further away from the tip, so long as they remain in the MT-free zone (Braun et al., 1999b). Based on experiments performed aboard the Space Shuttle during the IML-2 mission and during the 13-min microgravity phase of the parablic flight of a MAXUS-rocket (MAXUS-3), the threshold value for mass acceleration of rhizoids can be expected to be $> 0.1\ g$ and clearly $< 0.3\ g$. These results and preliminary immunolabelling studies indicate that the organization and function of the cytoskeleton is not subjected to drastic changes in microgravity (Braun et al., 1999b). However, it also becomes evident that the actin-mediated statolith positioning is perfectly adapted to the Earths 1 *g*-environment. Therefore, gravisensitivity in microgravity is unlikely to be exactly the same as in 1 *g*-controls (Braun et al., 1999b).

Protonemata bend by bulging whereas rhizoids bend by bowing: The experiments described so far, whether performed in microgravity, with laser tweezers, clinostats, centrifuges, or incorporating actin-specific inhibitors, all

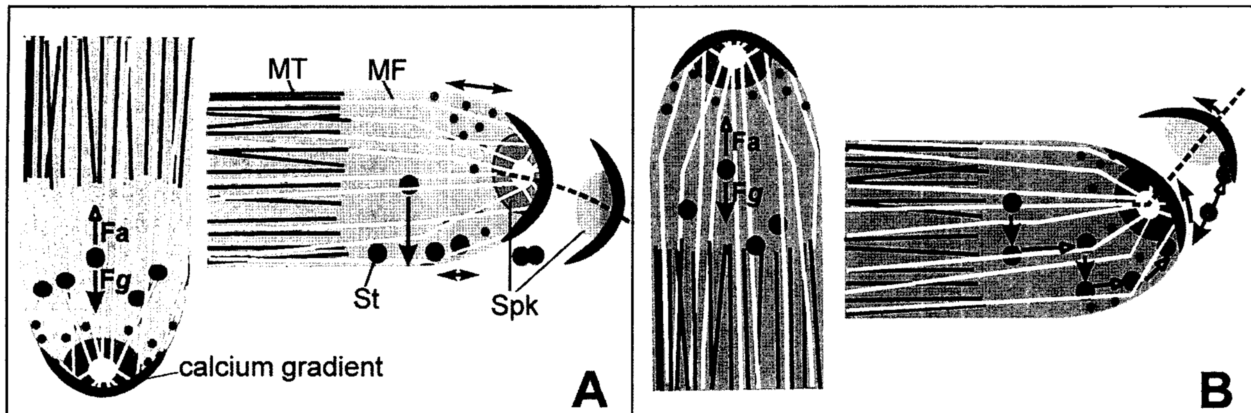

Fig. 6. Schematic drawing of the apical and part of the subapical zone of a characean rhizoid (A) and a protonema (B) in normal vertical and horizontal position (gravistimulated). A, In tip-downward growing rhizoids, the statolith (St) position results from net-basipetally acting actin-mediated forces (Fa) compensating the gravity force (*Fg*). Upon tilting, the statoliths simply follow the direction of gravity and sediment onto the lower cell flank. The subsequent curvature is induced by differential extension of the opposite cell flanks. The Spitzenkörper (Spk) and the calcium gadient always remain symmetrically located at the tip and are not directly involved in gravitropic bending. B, In upward growing protonemata, net-acropetally acting forces mediated by the actin microfilaments (MFs) compensate the basally directed gravity force. Placed in a horizontal position, statoliths settle near the growth center at the tip by following the gravity vector and by the additional forces of the acropetally acting actin MFs. This causes a drastical shift of the calcium gradient and the Spitzenkörper towards the upper flank and the new outgrowth occurs at that site. Until the protonema reaches the upright position, the statoliths are continuously pushed upward towards the growth center and fall back again. MT=microtubules (Originals by M. Braun).

point to the critical nature of the growth center in defining the properties that support positive versus negative gravitropism (Fig. 6). In protonemata, displacement of the growth center from the apex results in bending by bulging (as defined by Green et al., 1970). Actin-mediated acropetal transport of statoliths ensures that protonemata bend upward when moved out of their vertical position. In rhizoids, the transport of statoliths is net-basipetal and protects the growth center from statolith intrusion. This results in statoliths sedimenting along on the subapical flank, suppressing the growth rate along that flank (Sievers et al., 1979; Sievers and Schnepf, 1981). Differential flank growth generates bending by bowing (Green et al., 1970). Because the growth rate of the statolith-free upper flank is higher than of the lower flank, rhizoids bend downward.

Centrifugation experiments provide further insights into bending mechanisms in protonemata and rhizoids. When centrifuged at an angle of 90° normal to the cell axis, protonemata showed a gradual reduction in statolith sedimentation-mediated growth curvature as accelerations increased from 1 *g*-8 *g* (Hodick and Sievers, 1998). These strong centrifugal forces press the statoliths onto the lateral cell flank so this probably inhibits their actin-mediated acropetal movement. Acropetal movement therefore appears to be essential for initiating bending by bulging, which requires lateral displacement of the growth center. This is clearly in contrast to the results of rhizoid centrifugation experiments, using similar lateral accelerations (Braun, 1996b). Positive gravitropic bending of horizontally positioned rhizoids started abruptly when lateral centrifugal forces were introduced but bending was only transient. Under continuous centrifugation, further bending by bowing was inhibited and rhizoids continued growth at a uniform angle to the acceleration vector. Lack of further bending appears to occur because the sedimented statoliths displace the Spitzenkörper and, thereby, the growth center towards the centripetal flank. The balance between bowing towards the acceleration vector (positive gravitropism) by differential flank growth and bulging away from the acceleration vector (negative gravitropism) may explain how the modified growth axis is maintained. Indeed, vertically positioned rhizoids can be induced to deviate from their axis if centrifugal forces are applied at an angle greater than 5° and less than 90° to the original axis. If the acceleration angle is less than 5°, statoliths sediment symmetrically into the apex and radial swelling of the tip but no bending occurs. At greater centrifugal angles, statoliths sediment asymmetrically, allowing both bowing and bulging to occur.

Spitzenkörper anchorage is more stable in rhizoids than in protonemata: The fact that apically sedimented statoliths impair bending of rhizoids, but promote negative gravitropism in protonemata suggests that one major difference between protonemata and rhizoids is the relative ease at which the growth center can be displaced by sedimenting statoliths. There is evidence from centrifugation experiments (Braun, 1996b; Hodick and Sievers, 1998) and from attaching particles to the surface of bending cells (Sievers et al., 1979) that the position of the growth center at the cell tip is relatively stable and that the Spitzenkörper is more tightly anchored in rhizoids than in protonemata. Asymmetric statolith sedimentation against the apical cell wall and displacement of the Spitzenkörper occurs naturally in protonemata but requires considerable centrifugal force

to happen in rhizoids. Rhizoids can be forced to respond to some extent like protonemata but only when centrifuged or by pushing statoliths aymmetrically into the apical dome with optical tweezers (Braun, unpublished results).

How does the actin cytoskeleton modulate gravitropic mechanisms?: The idea that the degree of Spitzenko rper anchorage is mediated by properties of the actin cytoskeleton and the activity of calcium-dependent, actin-binding proteins (Braun and Richter, 1999) is supported by recent experimental data. Immunofluorescence of spectrin in graviresponding cells labels the ER aggregate, the structural center of the Spitzenko rper. The symmetrical position of the spherical labelling pattern was drastically displaced towards the upper flank, the site of future outgrowth, during initiation of the graviresponse in protonemata, clearly before curvature was recognizable by the formation of a bulge on the upper flank (Braun, submitted). In contrast, the anti-spectrin-fluorescence labelling in rhizoids remained symmetrically positioned in the apical dome throughout the graviresponse, confirming that a repositioning of the ER aggregate is involved in the negative graviresponse of protonemata, but not in the positive graviresponse of rhizoids. Further evidence comes from calcium imaging, which demonstrates a drastic shift of the calcium gradient from the tip towards the upper flank during initiation of the graviresponse in protonemata, but not in rhizoids. In accordance with this observation, dihydropyridine-fluorescence, which indicates a symmetrical distribution of putative calcium channels at the tip of normal vertically growing cells, was also found to be displaced towards the upper flank only in graviresponding protonemata. These results suggest that the early asymmetric repositioning of the calcium gradient in protonemata may result from statolith-induced displacement or more likely from statolith-induced differential activation or inhibition of apical calcium channels. The asymmetric influx of Ca^{2+}, in turn, may mediate the repositioning of the Spitzenko rper and the growth center by differentially regulating the actin-anchorage or the activity of actin-associated proteins along the shifting calcium gradient. In *Fucus* zygotes and rhizoids, an asymmetric Ca^{2+} gradient establishes a labile new growth axis and the actin cytoskeleton is required to fix the new growth direction (Quatrano et al., 1991; Shaw and Quatrano, 1996). Similarly, the tendency for protonemata to reorient towards the former growth axis after a short gravistimulation indicates that the new growth axis induced by the upward shift of the Ca^{2+} gradient is rather labile and may also require actin cytoskeletal anchorage to stabilize the new growth direction (Braun and Richter, 1999).

Concluding remarks

Tip growth is certainly not simply a case of limited cell wall expansion, a relict of primitive single-celled organisms. In this chapter, it has been demonstrated that highly specialized, actin-mediated mechanisms have evolved in charophytes in response to the need for effective adaptations to specific environmental conditions. Actin's involvement in tip growth is less clearly defined in other plant cell types, including higher plant root hairs and pollen tubes. The full extent of the actin cytoskeleton or its exact organization in these cells remains the subject of considerable debate (Taylor and Hepler, 1997; Cai G. et al., 1997a). It has been argued that the absence of actin from the outermost cell tip may be essential for the process of vesicle docking and exocytosis (Miller et al., 1999) but, as the studies described in this chapter demonstrate, this certainly does not apply in the case of characean rhizoids and protonemata (and for root hairs see Braun et al., 1999a). The direction of growth in most tropistic-oriented cells, however, is determined by external gradients of chemicals or ions, attractants or repellents. Such exogenous growth cues may allow or necessitate quite a differently organized actin MF system. In root hairs for instance, a cap-like pool of G-actin and profilin has been visualized at the tip, which may mediate their specific mode of polar growth (Braun et al., 1999a). In contrast, the protonemal and caulonemal tip-growing cells of mosses exhibit an extensive, but uniform, meshwork of actin MFs. These cells can orient phototropically and gravitropically according to external physical stimuli but their tropic responses are relatively slow. Therefore, the highly polarized cytoplasmic and cytoskeletal organization, including the unique ER aggregate of characean rhizoids and protonemata may reflect their highly sensitive and responsive gravity-oriented tip growth.

The Multicellular Root Cap

Introduction

The root cap covers the outermost tip of the root. It protects the apical meristem, senses the direction of gravity and other environmental signals, and generates the rhizosphere. The central columella cells function as statocytes: they perceive the gravity stimulus.

Structural polarity in statocytes

Nucleus, amyloplasts and other organelles: The nucleus occupies a position near the proximal periclinal cell wall of the stocytes of most plants. The distance between the nucleus and proximal periclinal cell wall of cress statocytes remains constant irrespective of the increasing length of the statocytes. Since treatment with cytochalasin B (a drug known to destroy actin microfilaments) led to sedimentation of the nuclei, a role of actin microfilaments in anchoring the nucleus was suggested (Hensel, 1985; Lorenzi and Perbal, 1990).

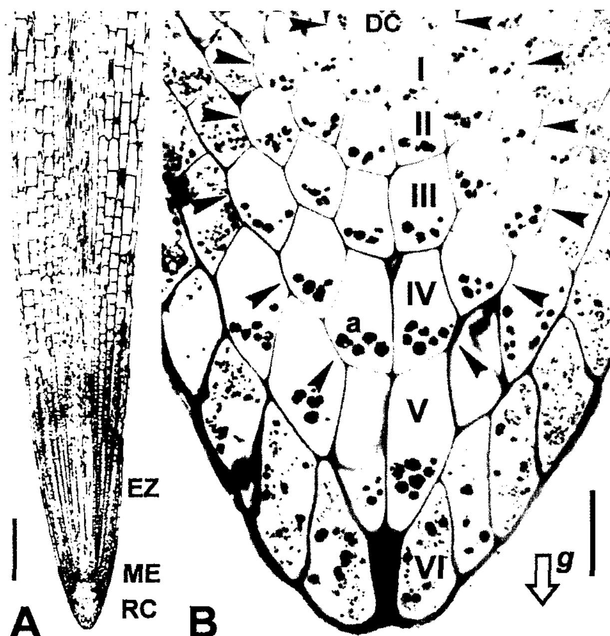

Fig. 7. A, Light micrograph of the median longitudinal section of a cress root. Graviperception is a specific function of the central cells of the root cap (RC). Graviresponse is performed by cells of the elongation zone (EZ). ME Meristematic region. Bar=200 µm. Original by R. Kruft. B, Median section of the cress root cap. The cells functioning as statocytes are located in the center of the root cap (statenchyma; outer cell walls indicated by the arrowheads) and are characterized by sedimented amyloplasts (a; statoliths). The statocytes (I-IV) derive from the dermatocalyptrogen (DC) and develop into mucilage secreting cells (V-VI) which finally separate from the root cap tissue. *g*=direction of gravity (Original by H-J. Ensikat). Scale bar=20 µm.

Proplastids of the meristematic layer(s) accumulate starch and, because of this transformation into the amyloplast stage, sediment according to gravity (for references, see Barlow et al., 1984). Amyloplasts from coleoptiles have negative zeta potentials of about -19.4 mV, as measured by rate of transport in an electric field, a feature that may also be valid for amyloplasts of root statocytes (Sack et al., 1983).

Other organelles in statocytes, such as mitochondria, dictyosomes, vacuoles, and microbodies, are distributed randomly, although their distribution is limited to the area between the proximal nucleus and the distal ER complex with the sedimented amyloplasts. Lipid bodies remain, in general, in the vicinity of ER membranes.

Endoplasmic reticulum: In root statocytes of all plants investigated so far, there exists only cortical ER (the distal ER complex included) but no 'endoplasmic' ER. The location, amount, and even the internal organization of the ER in statocytes are reported to vary depending on the species being studied. Therefore, we shall introduce the well-studied cress statocytes (Fig. 7) as a model system. In the first studies of cress root caps, Sievers and Volkmann (1972) and Volkmann (1974) noted that the number of ER layers in the distal ER complex increases with statocyte development. By means of inhibitor experiments, it was shown that ER movement within statocytes and anchorage at the distal cell pole depend on the action of the cytoskeleton. The ER in statocytes is produced by an outgrowth of the outer membrane of the nuclear envelope (Hensel, 1985). An actin microfilament-dependent process translocates the newly formed ER membranes into the cortical cytoplasm of the cells (Hensel, 1988). Thereafter, coordinated action of plasma membrane-bridged microtubules and actin microfilaments translocates the ER into the distal cell pole (Hensel, 1987). The retranslocation of ER, displaced by centrifugation from the distal cell pole, is also driven by actin microfilament action (Wendt and Sievers, 1986). The distal ER complex is anchored in its position by microtubules arranged in a crisscross layer near the plasma membrane of the distal cell edges (Hensel, 1984). Actin microfilaments, however, also contribute to this anchorage, since only 7 min of treatment with cytochalasin D (an actin microfilament-destroying drug, more potent in action than cytochalasin B) led to disintegration of the complex (Hensel, 1987). It was suggested that cortical actin microfilaments may hold the ER complex under tension; thus contributing to the regular appearance of the distal ER complex in control statocytes. Although the ER transported along the anticlinal cell walls may consist of smaller elements of the tubular type (Stephenson and Hawes, 1986; Hensel, 1987), the organization of the distal ER complex in cress was shown by freeze-etching to be cisternal (Sievers and Volkmann, 1977). Several species have not evolved an ER complex at the distal cell pole, which serves as a cushion for the sedimenting amyloplasts, although Volkmann (1974) presented some examples for distal ER complexes in plants other than cress (see also Olsen and Iversen, 1980). Nevertheless, an increase in ER during statocyte development appears to be a general feature for root caps (e.g. Juniper and Clowes, 1965; Barlow and Sargent, 1978; Stephenson and Hawes, 1986; Hensel, 1987). Values that vary from 2400 μm^2 ER area per statocyte up to more than 9000 μm^2 for maize have been reported. The ER is stable in its natural location, possibly because of the anchoring action of the cytoskeleton.

If roots grown in the normal vertical orientation are rotated for 20 h on the horizontal axis of a slow (two rotations per minute) clinostat, the polar arrangement of the organelles is destroyed. The ER appears at different sites in the statocytes, often forming whorls or aggregates. As pointed out by Hensel and Sievers (1980), this overstimulation by continuous omnilateral gravistimulation leads to the self-destruction of the statocytes. This is further indicated by a confluence of lipid bodies, the appearance of autophagosomes, the loss of amyloplast starch, and digestion of anticlinal cell walls. The effect of the self-destruction of statocytes was twofold: (i) the graviresponse of the overstimulated roots was drastically reduced and (ii) the root meristem responded to this damage by reducing

the period of the cell cycle, thereby causing a faster repair of the statenchyma (Hensel and Sievers, 1980). It should be mentioned that the unique lack of endoplasmic ER in mature statocytes in cooperation with the lack of prominent endoplasmic cytoskeletal elements facilitates the sedimentation of statoliths, thereby optimizing graviperception. The function of the remarkable amount of ER in statocytes can be interpreted in connection with the existence and the role of the Ca^{2+}-ATPase in plant ER membranes which was discovered in cress roots (Buckhout, 1983).

Cytoskeleton

Extensive tissue-specific arrangements of cortical and endoplasmic actin microfilaments and microtubules have been reported in the various cell types of roots and shoots. In statocytes, however, all labeling techniques applied so far have failed to visualize prominent endoplasmic actin microfilament bundles and microtubules (Baluška et al., 1997). Soon after termination of the mitotic division in the prospective statocytes, distinct actin microfilament bundles can no longer be visualized and microtubules are limited to dense cortical arrays (Hensel, 1984; Baluška et al., 1997). This unique cytoskeletal arrangement is considered responsible for the cell polarity, the exclusion of larger organelles (e.g. nucleus, plastids, ER membranes) from the interior of the cells and for the absence of cytoplasmic streaming. In statocytes of coleoptiles and hypocotyls, where cytoplasmic streaming interferes with sedimentation of the amyloplast statoliths, actin cables have been observed (White and Sack 1990). Gravity-directed sedimentation of the starch-containing amyloplasts in root cap statocytes may also be related to the specific cytoskeletal properties which may due to the specific calcium concentration in the cytosol.

The diffuse actin labeling found at the cell periphery and close to the sedimented amyloplasts (Baluška et al., 1997; Blancaflor and Hasenstein, 1997) suggests that actin is organized in the form of delicate meshworks of oligomers. Interestingly, actin microfilament bundles were visualized in enzymatically extracted root cap tissues (Hensel, 1988; White and Sack, 1990), which indicates a potential ability of the actin cytoskeleton to rapidly change its organization. Inhibitor experiments indicate that the translocation and the polar distribution of the ER as well as the movements of the amyloplasts are based on the actomyosin system. The motor protein myosin was immunocytochemically detected in root tips of *Allium cepa* (Parke et al., 1986) and in statocytes of cress roots (Baluška and Hasenstein, 1997) by an antibody against animal myosin which cross-reacted on Western blots with a plant polypeptide of approximately 200,000 molecular weight. Myosin immunofluorescence was found in the vicinity of the sedimented amyloplasts in statocytes. Interconnections between actin microfilaments and plastids were described and are involved in the light-dependent movement of chloroplasts (Grolig and Wagner, 1988; Grolig, 1990).

Importance of statocytes' structural polarity

The structural polarity of statocytes (Fig. 8) appears to be a crucial precondition for graviperception. This conclusion can already be drawn from the fact that the polar differentiation is the result of an endogenous developmental program and from the fact that the lack of endoplasmic structures like ER and prominent cytoskeletal elements facilitates sedimentation of statoliths. In addition, especially the results of two experiments support this conclusion. (i) The biogenic polarity is changed to a physical stratification by root-tip-directed centrifugation (20 min at 50 2,000 *g*; Sievers and Heyder-Caspers, 1983). Within the following 8-10 min at 1 *g*, the structural polarity at the distal cell pole was re-established in most statocytes, regardless of their orientation to the gravity vector. The lag phase of graviresponse was also increased by 8-10 min in centrifuged roots as compared to controls and independent of the applied centrifugation dose. The kinetics of the response were identical to controls. That means that after stratification some reorganization of the statocyte is necessary and sufficient for graviperception. (ii) Treatment of roots with gibberellic acid and kinetin causes not only complete destarching of amyloplasts but also a total loss of structural polarity in statocytes and graviresponsiveness of roots (Busch and Sievers, 1990). Twenty-two hours after removal of the hormones, the polar arrangement of cell organelles was restored and starch was re-synthesized so that the roots again responded gravitropically.

Gravity sensing

The requirement of the root cap for graviperception by roots is well established; experimental evidence for this goes back to the pioneering root tip-removal experiments of Charles Darwin (1880). The unequivocal proof was provided by the careful decapping experiments of Juniper et al. (1966). Decapped roots did not respond to gravitropic stimulation, whereas their growth was reported to be unaltered. More recent evidence for the statocyte function of the root cap columella cells comes from laser ablation experiments (Blancaflor et al., 1998) specifying the innermost columella cells as the most important sites for graviperception.

The mechanism of graviperception is far from being understood. However, since gravity can only work on (deform or displace) masses, biological gravisensors must be equipped with receptors which are able to perceive the information resulting from the physical process of deformation or displacement known as susception. For graviperception in higher plants the starch-statolith theory published by Němec (1900) and Haberlandt (1900) is widely accepted. In gravisensitive

tissues of shoots and roots, starch-containing sedimentable amyloplasts were observed in specialized cells, the statocytes, and are believed to act as susceptors or statoliths (cf. the reviews by Volkmann and Sievers, 1979; Wilkins, 1984; Björkman, 1988; Sack, 1991, 1997; Chen et al., 1999). A good correlation was found between gravisensitivity and amyloplasts of different starch content reduced by physiological treatment or mutations (Iversen, 1969; Perbal and Rivière, 1976; Busch and Sievers, 1990; Sack, 1991; Kiss et al., 1996, 1997; Weise and Kiss, 1999). Even starchless amyloplasts could produce a high enough signal-to-noise ratio to activate the hypothetical receptor molecules. Furthermore, *Arabidopsis* mutants lacking the endodermal parenchyma in shoots and roots were reported to show no shoot but root gravitropism (Fukaki et al., 1998). Since amyloplast-containing statocytes are present in the shoot endodermis but not in the root endodermis, these mutants strongly support the proposed hypothesis.

Non-statolith theories have been discussed suggesting the possibility that amyloplast sedimentation might not be the sole mechanism of gravity sensing (Sack, 1997). However, there is evidence from high-gradient magnetic field experiments that amyloplast sedimentation in statocytes of roots and shoots is sufficient to initiate gravitropic curvature. Root curvature occurred in the direction in which the amyloplasts were displaced by the magnetic field (Kuznetsov and Hasenstein, 1996) whereas in coleoptiles and hypocotyls, amyloplast displacement initiated curvature in the direction opposite to that of the statolith movement (Kuznetsov and Hasenstein, 1997) according, respectively, to the positive and negative gravitropic responses of the organs.

Molecules involved in the mechanism of graviperception, the transduction of the physical process of sedimentation into a physiological signal, are still to be characterized. The presentation time of cress roots was reported to be 12 s (Iversen and Larsen, 1973). By intermittent stimulation, the minimum time to be perceived (perception time) by *Avena* coleoptiles and cress roots was determined to be 0.5 s (Hejnowicz et al., 1998 and references cited therein). During the perception time, the amyloplasts are displaced approx. 8 nm. As a consequence, attention should be paid to small displacements of statoliths from the initial equilibrium in order to understand how the work done by gravity on statoliths is transferred to competent cellular structures (Sievers et al., 1991b). Thus, cytoskeletal elements of the statocytes are the most likely candidates to be involved in the transduction of the statolith sedimentation into a physiological signal which is transmitted to the responding target cells in the root elongation zone (Sievers et al., 1991b). Circumstantial evidence for this model is provided by the finding that treatment of *Phleum* roots with the actin inhibitor cytochalasin D inhibits gravity-induced

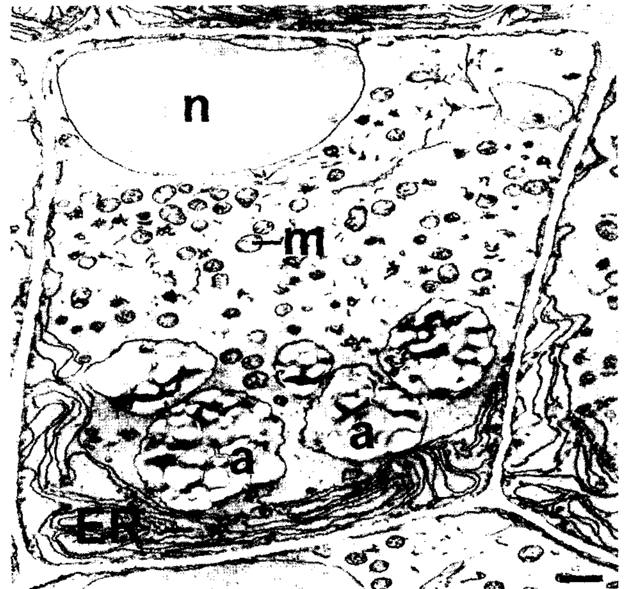

Fig. 8. Electron micrograph of a statocyte of a cress root from a position near the median plane of the root. The nucleus (n) is located at the proximal, a complex of ER at the distal cell pole. Amyloplasts (a) lie on this ER complex. Mitochondria (m) are seen in the cell center (After Busch and Sievers, 1990). Scale bar=1 µm.

pH changes at the root surface (Monshausen et al., 1996), whereas 8 min after tilting, the surface pH of gravistimulated control roots starts to decrease at the meristem and apical elongation zone of the physically upper root side (Zieschang et al., 1993; Monshausen et al., 1996; the observation of an inhibitory effect of cytochalasin D on the gravitropic curvature of *Phleum* roots is, however, in direct contrast to the results of Staves et al. [1997] who did not observe any effect of cytochalasin D on the graviresponse in roots of some other species). The unique lack of prominent actin bundles and microtubules in the statocyte interior probably facilitates unconstrained sedimentation of their statoliths (Baluška and Hasenstein, 1997; Volkmann and Baluška, 1999). A proposed delicate meshwork of oligomeric actin surrounding the statoliths and myosins localized in the vicinity of the sedimented statoliths (Baluška and Hasenstein, 1997) is the most likely basis for statolith movements and the displacement of statoliths under microgravity conditions (Fig. 9; Volkmann et al., 1991; Driss-Ecole et al., 2000) as was also shown in Chara rhizoids (cf Section I). Inhibitors affecting the polymerisation of actin (cytochalasin, phalloidin) and tubulin (colchicine, taxol) were reported to cause significant differences of the sedimentation rate of amyloplasts (Sievers et al., 1989). These results additionally support the idea that actin microfilaments or microtubules are directly or indirectly involved in statolith-mediated graviperception. Cytoskeletal filaments could be interconnected with mechano-sensitive ion channels either in the cortical ER or other membranes (Fig. 10; Falke et al., 1988; Schroeder and Hedrich, 1989; Pickard and Ding, 1992; Hwang et al., 1997).

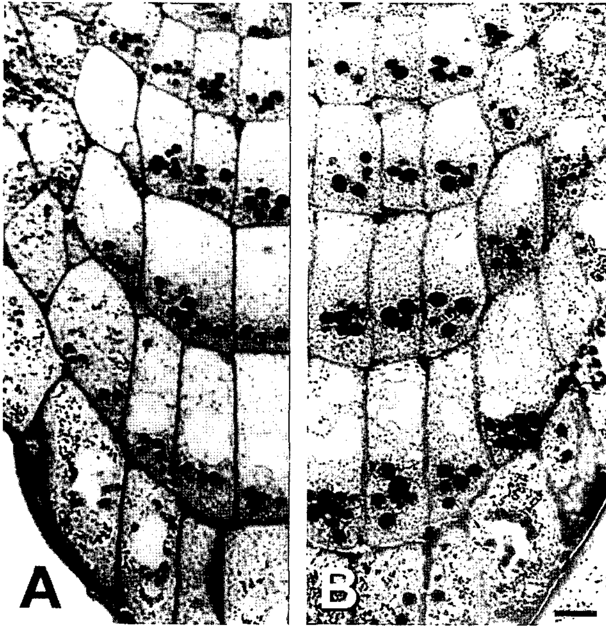

Fig. 9. Light-microscopic photographs of median longitudinal sections through the statenchyma of cress roots. A, Control root which was fixed with potassium permanganate at 1 *g*. B, Root which was fixed at the end of the 6-min microgravity phase of the parabolic flight of a rocket (TEXUS). Microgravity conditions resulted in a considerable displacement of the statolith-amyloplasts in the direction opposite to that of the gravity vector (Modified from Volkmann et al., 1991). Scale bar=10 μm.

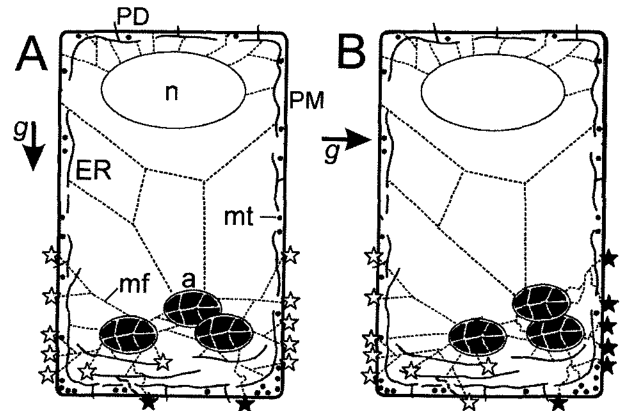

Fig. 10. Scheme of a central statocyte of a cress root illustrating the difference in the tension of the proposed oligomeric actin-network and the asymmetrical activities of ion channels, both induced by gravistimulation. A, Normal vertical orientation with most actin microfilaments in tension. B, Horizontal orientation with asymmetrically stretched and relaxed actin microfilaments due to the gravity-induced displacement of the amyloplasts. a, amyloplast (statolith); ER, endoplasmic reticulum; *g*, direction of gravity; mf, actin microfilament; mt, microtubulus; n, nucleus; PD, plasmodesma; PM, plasma membrane. Open and closed stars symbolize postulated channels in two different activity states (Modified from Sievers et al., 1991b).

Even the energy of small displacements of statoliths amplified by the cytoskeleton would allow dramatic changes in ion fluxes including calcium which appears to play a crucial role in signal transduction (Björkman, 1988; Sievers et al., 1991a; Sievers and Busch, 1992). This is in full agreement with the tensegrity model of cytoskeleton-mediated mechanotransduction (Ingber, 1993).

Further support for a participation of the cytoskeleton in graviperception comes from a genetic approach. In *Arabidopsis* mutants with altered root and hypocotyl gravitropism, a gene has been identified that affects the graviperception phase. The *ARG1* locus encodes for a 45-kDa DnaJ-like protein containing a coiled-coil domain which is typical for cytoskeleton-interacting proteins (Sedbrook et al., 1999). A cytoskeleton-membrane anchoring function seems possible because of the presence of several hydrophobic amino acids in the middle, a putative transmembrane region. *ARG1* may represent a component of the early cytoskeleton-mediated gravity signal-transduction chain. However, because of the fact that *ARG1*-protein was found to be expressed in all plant tissues and is related to a conserved signal transduction molecule, a more general function of *ARG1* in signal transduction, protein folding, or protein trafficking can not be ruled out.

Calcium and phosphoinositides possibly act as second messengers in the signal transduction pathway. Differential activity of phosphatidylinositol-4-phosphate-5-kinase was found in the lower and upper side of grass nodes after gravistimulation, suggesting the involvement of inositol-4,5-bisphosphate, a second messenger known to activate Ca^{2+} release from internal stores, in early gravitropic events (Perera et al., 1999). High concentrations of calcium were detected in statocyte amyloplasts and membranes (Chandra et al., 1982; Busch et al., 1993) and calmodulin concentrations in statocytes are higher than in all other cell types (Allan and Trewavas, 1985), though the cytoplasmic $[Ca^{2+}]$ in statocytes does not appear to be different from those found in other root tissues (Legué et al., 1997). Furthermore, the degree of the polar arrangement of organelles in statocytes and the gravisensitivity of roots were reduced or eliminated after application of blockers of stretch-activated calcium channels and inhibitors of calmodulin or Ca^{2+}-ATPase activities (Biro et al., 1982; Björkmann and Leopold, 1987; Stinemetz et al., 1987; Wendt and Sievers, 1989; Sievers and Busch 1992). Lu and Feldman (1997) discussed the involvement of a Ca^{2+}/calmodulin-dependent protein kinase in the light-dependent gravitropism of corn roots. These data indicate the involvement of calcium-calmodulin activity in the gravitropic signal-transduction pathway, but only touch-induced, not gravity-induced changes in cytosolic calcium-levels could be shown (Legué et al., 1997). In this context it is noteworthy that a root is not only gravistimulated by tilting (dynamic gravistimulation; Sievers et al., 1991b). Vertically oriented roots commonly used as controls are also permanently (statically) stimulated; in order to obtain information on the cytoplasmic $[Ca^{2+}]$ of unstimulated controls, one would therefore have to study the statocytes of roots grown in a stimulus-free microgravity environment - such as the International Space Station (Sievers, 1999).

Nevertheless, calcium seems to act as a second messenger interfering with the polar transport of auxin within the root cap, and could mediate the redirection of auxin to the physically lower root flank (Lee et al., 1984; Lee and Evans, 1985; Evans and Hasenstein, 1987).

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