Autonomous Gravity Perception and Responses of Single Plant Cells

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ABSTRACT

Although multicellular plants are favored experimental subjects for most studies of gravitational responses in plants, there are single cells that can both sense and respond to gravity, independent of any input from other cells. This review focuses on what is known about these single-cell phenomena. Highlighted are studies in algae and moss that relate to the statolith hypothesis, studies in Euglena on gravitaxis, and studies in fern spores on gravity-induced calcium signaling and gene expression changes.

INTRODUCTION

Multicellular plants are favored experimental subjects for most studies of gravitational responses in plants, and the most studied of these responses is gravitropism (Morita, 2010). In multicellular plant gravitropisms, such as the downward curvature of roots and upward curvature of shoots, diverse tissue interactions are involved. Specifically in roots, chemical communication between the cap and the elongation zone, including the transport of the growth hormone auxin, appears to be crucial for the gravity signal to be transduced into downward growth (Harrison et al., 2007). Of course, in all these instances the initial response to gravity must occur in individual cells, even though, as in the case of root-cap cells, the gravisensing cells themselves may not show a growth change. However, there are single cells that can both sense and respond to gravity, independent of any input from other cells. This review will focus on what is known about these single-cell phenomena.

The simplicity of single-cell systems is advantageous for studies of the early signaling steps that link the gravity stimulus to biochemical or growth changes. Do the results of these studies have relevance or value for similar studies in multicellular systems? Gravity-induced membrane potential changes observed in unattached single cells are also observed in cells of multicellular plants (Weisenseel and Meyer, 1997). More specifically, gravity-induced rapid changes in calcium fluxes observed in single cells (Chatterjee et al., 2000; Salmi et al., 2011) are also observed as early steps in individual cells of multicellular systems (Fasano et al., 2002; Plieth and Trewavas, 2002; Toyota et al., 2008). Even at the level of gene-expression changes, there are significant similarities in the effects of gravity on single-cell and multicellular systems (Salmi et al., 2005). Although certainly there are major differences in how gravitational stimuli are transduced in independent, single cells compared to multicellular tissues, there appears to be a fundamental conservation of mechanisms in gravity responses that is maintained in all responsive plant cells. This argues for the significant value of single-cell systems as models for understanding basic processes of gravity sensing and responses.

Statolith Model of Gravity Sensing: Single-cell Results

While perhaps not applicable to all plant species, the statolith model of gravity sensing in multicellular plants is well established. The model is based on the observation that certain organelles move downward (sediment) when the position of the cell is changed. In Arabidopsis columella cells the statoliths are dense starch-containing amyloplasts. Reduced-starch mutants have both decreased statolith sedimentation ability (MacCleery and Kiss, 1999) and reduced gravitropic responses (Kiss et al., 1996). These and subsequent studies favor the hypothesis that sedimenting organelles help initiate gravity sensing in multicellular tissues (Strohm et al., in press). Evidence that sedimenting organelles initiate gravity responses in single cells has also been published in
multiple reports, most of which used cells of moss and algae as experimental subjects.

In gametophytes of the moss *Ceratodon purpureus*, amyloplast sedimentation results in negative gravitropic curvature of the protonemata, which act autonomously in their response to gravity. Conveniently, there is a wrong way response (wwr) mutant which exhibits positive gravitropic growth. The kinetics and directions of growth of these wwr mutant plants is exactly opposite that of the wild type. The mutated gene product in wwr plants seems to function downstream of amyloplast sedimentation since the position of sedimenting amyloplasts is unaltered in the mutant plants after gravity reorientation (Wagner et al., 1997). Kuznetsov et al. (1999) utilized strong magnetic fields to manipulate amyloplasts independent of the gravity vector to cement the connection between statolith movement and gravity response (Figure 1, A-D). In two instances the protonemata were rotated in a clinostat while exposed to a high-gradient magnetic field (HGMF) while in the third case they were not rotated. The clinostat served to nullify the sedimenting effects of gravity, and indeed the untreated protonemata showed neither sedimentation nor curvature (Figure 1D). However, the amyloplasts under clinorotation would sediment with an applied magnetic vector. The wild-type protonemata, as predicted, would grow away from the gravity vector forces (opposite the direction of HGMF-induced sedimentation) as seen in Figure 1A. This was true regardless of the shape of the applied magnetic field. The wwr mutants showed the expected inverted growth pattern in relation to the HGMF displacement of their amyloplasts (Figure 1B). The inclusion of wwr mutant plants in this study demonstrates the importance of amyloplast sedimentation location in this system, regardless of differences in the signaling process downstream of that sedimentation. The researchers also noted a positive correlation between both field intensity and amyloplast size and the degree of the curvature response (Kuznetsov et al., 1999). They also noted that the responses support the statolith hypothesis over the pressure model. While these data directly tie the location of sedimenting statoliths to the detection of the vector of gravity, they do little to define the nature of this receptor mechanism. Still, this dovetails neatly with their previous work in *Arabidopsis* roots showing that magnetic field alterations to amyloplast sedimentation alter gravitropic curvature of the roots in a similar fashion (Kuznetsov and Hasenstein, 1996).

Magnetic fields are not the only way of experimentally manipulating statolith position in single cells. *Chara* algae contain large (diameter up to 30 µm), tube-like rhizoid cells that exhibit positive gravitropism (grow downward), and protonemata that exhibit negative gravitropism (grow upward), offering an elegant system for examining both gravitropic responses in single cells within the same species. Both of these gravity-sensing cell types contain structures that have been reported to serve the role of statoliths: i.e., vesicles filled with barium sulfate crystals (BaSO₄) that sediment in the direction of the gravity vector. The rhizoids and protonemata of characean algae have been manipulated using “optical tweezer” laser trapping. Laser-assisted displacement of 2-3 statoliths against the side of a horizontally oriented rhizoid for at least 5 minutes was sufficient to cause curvature towards that side (Figure 1, A’-D’). Similarly, the same conditions in a protonema lead to negative-gravitropic-like curvature away from the site of the repositioned statoliths (Braun, 2002). The manipulation of statoliths into various regions of *Chara* cells was an especially valuable approach due to two key observations. First, *Chara* cells appear to have a limited, belt-like region where gravity perception takes place (Braun, 2002) in both protonemata and rhizoids. Other gravi-responsive plant cell types are able to detect a change in orientation in any direction. Second, the actual movement of the statoliths does not appear to be the key to responsiveness (Braun, 2002). Rather, a cell requires contact, or pressure, from the statolith at the regions of sensitivity.

Findings using the *Chara* model system to study gravity perception in plant cells were recently reviewed by Braun and Limbach (2006). As they reiterate, it is clear that the position of the statolith near the plasma membrane within the region of gravity perception of the rhizoid or protonema is integral to the cell’s gravity response and to its change in direction of growth. However, this positional requirement could be acting through simple contact or by secondary conditions of the contact (e.g. applied pressure). While the contact model has strong evidence, the pressure model cannot be discounted by the data thus far.

In the first mechanism, the “gravity receptor” of *Chara* cells could act as a classical hormone, lock-and-key type receptor that specifically detects the location of these statoliths and either directly or indirectly opens ion channels. This model has some of the strongest support in *Chara* rhizoids. Limbach and colleagues observed that individual statoliths lose contact with the cell cortex in as little as 2 s after inverting *C. globularis* rhizoids. Inverting for repeated intervals as short as 10 s could impact gravitropic growth (Limbach et al., 2005). Further, the quantitative decrease in a rhizoid’s angle of
curvature appears to directly relate to the total time of inversion, and logically, therefore, to the total time interval statoliths were not in contact with the cell cortex. Perhaps the most compelling data supporting the contact model was that a decrease in gravitational forces achieved during parabolic flight experiments did not alter rhizoid curvature in any statistically significant manner (Limbach et al., 2005). The rhizoids were pre-stimulated by being rotated horizontally for 10 minutes before the first parabola, meaning that the statoliths would already have sedimented and achieved cortex contact. Further, 2 g conditions achieved through centrifugation did not affect rhizoid curvature in any detectable manner (Limbach et al., 2005). Therefore it appears that graviperception in Chara rhizoids depends almost entirely on physical contact between organelles.

Figure 1. Single-cell responses of single cells to various gravity-related manipulations. A-D: Ceratodon purpureus cells were exposed to various magnetic fields (directional force indicated by Fm) while rotated in a clinostat to reduce the effects of gravity. Arrow heads indicate amyloplasts displaced by the High Gradient Magnetic Field (HGMF). A. WT protonema after 12 h exposure to HGMF. B. wwr protonema after 6h exposure to HGMF. C. wwr protonema exposed to uniform magnetic field (non-HGMF). D. WT protonema away from HGMF. Bars = 50 µm. (Adapted from Kuznetsov et al., 1999, Copyright American Society of Plant Biologists, http://www.plantphysiol.org) A'-D': Growth changes over time of a single Chara globularis rhizoid manipulated by laser-assisted (circle) displacement (arrows) of statoliths (st). Bars = 10 µm. (Adapted with kind permission from Springer Science+Business Media: Protoplasma, 219, 2002, 150-159, Braun, M., figure 3, copyright Springer-Verlag 2002). Circular histograms: Histogram of directional swimming of Euglena gracilis as determined by motion analysis software. Upper left. Control cells. Lower right. RNAi against CaM.2, 15 days post-electroporation. Solid black arrow (theta) represents main movement direction of cells in the culture. r value represents precision of movement (0 = random, 1 = precise). (Adapted with kind permission from Springer Science+Business Media: Planta, 231, 2010, 1229-1236; Daiker, V. et al., figure 5, copyright Springer-Verlag 2010.) Line graph: Representative plot of calcium flux changes of a single Ceratopteris richardii spore in relation to changing gravity intensities from a parabolic flight. Vertical grey bars represent time of transition between hyper- and micro-g conditions. Arrows indicate time of change in calcium current (adapted with kind permission from Springer Science+Business Media: Planta, 233, 2011, 911-920; Salmi, M. et al., figure 4, copyright Springer-Verlag 2011).
These results may be related to ultrastructural observations in multicellular, statolith-dependent gravitropic responses such as in Zea mays roots. There, ER is largely absent in the central regions of the cells but enriched at the cell cortex (Yoder et al., 2001). A sedimenting statolith, then, would pass through ER-devoid regions to settle at ER-rich points at the cell membrane. It is likely not a coincidence that the ER is a main storage organelle for Ca\textsuperscript{2+}, an ion implicated in gravity perception and responses across multiple species. In animal systems, membrane contacts between the ER and PM are important for the phenomenon of store-operated calcium entry mediated by certain calcium-release activated calcium (CRAC) channels. To our knowledge, homologs of the specific animal proteins involved in this phenomenon (Orai1 and STIM1) have not been identified in plant systems, but calcium-induced calcium release has been documented with slow vacuolar (SV) channels participating in calcium release (Bewell et al., 1999). It is therefore possible that an analogous system involving contact between transmembrane proteins could be at work in gravity perception involving statoliths.

While the work of Limbach and colleagues strongly supports the contact model in C. globularis rhizoids, it does not completely rule out that statolith deformation of the plasma membrane (or ER) could result in the opening of a pressure-sensitive calcium channel that is integral to the membrane. Considering that the internal cells of Chara lack sedimenting organelles yet respond to position and differential pressures, this second mode of gravity sensing is likely to be active in this, and other, systems.

The research of Wayne, Staves, Leopold and colleagues on Nitellopsis and on Chara intermodal cells led to the hypothesis that the gravity sensor in these cells was the mass of the protoplasm rather than the movement of any specific subcellular organelle, such as a statolith (Staves, 1997; Wayne et al., 1992). The idea that pressure exerted by the entire cell was what accounts for gravity perception was tested in rice roots, and, consistent with the Chara results, high density media which would diminish the force generated by a falling protoplast but not prevent amyloplasts from falling, significantly decreased the rate of gravitropic curvature (Staves et al., 1997). Thus, results from the Wayne laboratory both in single cells and in multicellular tissues were consistent with the authors’ gravitational pressure theory of gravity sensing.

Schwuchow et al. (2002) tested this hypothesis in single-cell protonemata of the moss C. purpureus, which bend upward when reoriented horizontally. Their results indicated that plastid-based sensing of gravity, not pressure exerted by the entire cell functioned to initiate gravitropism in these cells, a conclusion also arrived at in the HGMF study by Kuznetsov et al. (1999), noted earlier. Certainly the statolith/plastid model of gravity sensing is the more accepted one today (Blancaflor and Masson, 2003; Strohm et al., 2011); but, as noted previously, certain specific systems depend largely or singularly on organelle contact rather than pressure. Further, the participation of the whole protoplasm as a sensor, integrated by the cytoskeleton to function as a single mass, cannot be ruled out, particularly in systems lacking sedimenting organelles or particles.

Single spore cells of the fern Ceratopteris richardii offer another system for examining the statolith hypothesis. Here gravity directs the downward migration of the nucleus, which occurs about 24 h after the spore is induced to germinate by light. That the downward migration of the nucleus is, indeed, directed by gravity was demonstrated in Shuttle mission STS93, during which video microscopy revealed that the migration still occurred in microgravity, but its direction was random instead of being polarized toward one end of the cell (Roux et al., 2003). The nuclear migration toward the bottom of the cell at 1 g both localizes the site of the first cell division and the subsequent downward emergence of the rhizoid. These cytological effects of gravity on cell polarity are already set about 15 h before the nuclear migration begins, so the downward movement of the nucleus is part of the response to gravity rather than being part of the perception event (Edwards and Roux, 1994; Edwards and Roux, 1998). Most of the plastids in the spore are closely appressed around the periphery of the nucleus, and separate movements of these plastids prior to when the polarizing effects of gravity are set were not observed. Thus a clear statolith candidate has not yet been identified for the fern spore response to gravity.

**Single-cell Taxic Response to Gravity**

Unlike the other plant systems discussed here, the unicellular ciliate alga Euglena gracilis has a slightly different relationship to gravity. Since they are both aquatic and motile they exhibit a number of taxic responses in order to optimize their ability to carry out photosynthesis in a body of water. Light is scattered by water so light intensities inversely relate to depth. For proper photosynthesis E. gracilis needs to remain near the surface of a water column. The most obvious response, therefore, would be to light, and they do exhibit strong, intensity-dependent phototaxis. However, as light is not continuous throughout a 24 hr cycle, gravity is also an important...
stimulus for *E. gracilis* to remain near the surface. To that end, *E. gracilis* exhibit a negative gravitaxis response.

Spaceflight experiments have demonstrated that the direction of swimming becomes random when gravity is less than 0.16 of normal (Häder and Hemmersbach, 1997). It is also thought that gravity is mainly sensed through differential pressures (dictated by the gravity vector) on cells. This idea is supported in *E. gracilis* by experiments manipulating media density. Directional movement was impaired without affecting taxis rate by increasing the surrounding densities. Even higher densities reversed the direction of normal taxis, effectively resulting in a situation where the algae appear to be positively gravitaxic (Häder and Hemmersbach, 1997). These outcomes parallel those we see in other plant systems. So while these protozoan-like algae differ in response (movement instead of asymmetric growth), they appear to share a similar mechanism for detection of the gravity vector.

More recent work with *E. gracilis* has expanded the molecular understanding of the gravitaxic sensing and response system(s). Like other systems, calcium is thought to be a main component of the relay between perception (orientation to gravity) and response (flagellar beating). Specifically, the gravitaxis of the ciliates *Stylonychia mytilus* (Krause et al., 2010) and *Euglena gracilis* clearly involves calcium signaling (Streb et al., 2001). To investigate whether the calcium-binding protein, calmodulin, was involved in the response, Daiker et al. (2010) sequenced five distinct cDNAs of calmodulin family proteins, designated CaM.1 through CaM.5. The sequences were unique enough that specific dsRNAs could be developed against each to knock them out/down to examine the effects. RNAi against CaM.3, CaM.4, and CaM.5 had no significant impact on gravitaxis. RNAi against CaM.1 was shown to have alterations in morphology and transient alterations in gravitaxis. Early after treatment the cells exhibited random, nonprogressive (euglenoid) movement, but by 15 days post-electroporation directed, negative gravitaxis was restored. It was noted that the change in morphology did not include noticeable alterations to the flagellum itself. Suppression of CaM.2 through RNAi similarly had alterations to morphology and induction of euglenoid movement. In this case, free swimming behavior was recovered within several days to a week. Also unlike the suppression of CaM.1, RNAi against CaM.2 did not show a return to normal gravitaxis for extended periods post-electroporation (Figure 1, circular histograms). The RNAi affects appear to be maintained by these cells, as clonal *E. gracilis* out to 30 days post-electroporation still showed free swimming, but in a generally randomized vector in contrast to the strongly negative gravitaxis of the control cells.

Taken in total, it appears that not only does *E. gracilis* contain calmodulins with distinct functions, but at least one of them, CaM.2, acts as a main component in the gravitaxis response. The current model positions CaM.2 as an intermediary between gravity-induced [Ca$$^{2+}$$] changes and alterations of flagellar beating via the cAMP output of a calmodulin-dependent adenyl cyclase. So while *E. gracilis* exhibits an overall different end response to the gravity vector when compared with other non-ciliate plants, it utilizes similar transduction and/or amplification steps involving calcium.

**Modulating Responses in Single Cells by Changing the g-Force**

As described above, after light induces single-cell spores of *Ceratopteris richardii* to germinate, gravity directs the polarization of these cells, including the downward migration of the nucleus. This migration occurs some 15 h after gravity has set the polarity of the cell, so there must be some gravity-induced event that occurs earlier. Because trans-cell calcium currents, driven by influx channels on one end and pumps at the other end, were known to induce cell polarization in tip-growing systems such as pollen tubes (Weisenseel et al., 1975), Chatterjee et al. (2000) used a self-referencing microelectrode to test whether such currents occurred in *C. richardii* spores, and, if so, whether gravity could influence the direction of these currents. They found that within two hours after the spores were irradiated, a calcium influx occurred at the spore bottom, and a 20-fold larger efflux emerged from the spore top. When the spore was rotated, the bottom-to-top calcium current rapidly realigned parallel to the vector of gravity.

This initial study could not resolve how quickly the rotation-induced realignment of the current occurred, but recently Salmi et al. (2011) answered this question using a silicon microfabricated sensor array that could simultaneously measure calcium currents in real time from multiple cells arranged on a microchip. Their assay showed that on earth rotating the spores on the chip reverses the trans-cell calcium current within 25 sec.

Another key finding of Salmi et al. (2011) was that a change in g-force could modulate the magnitude of the current (Figure 1, line graph). When the spores were assayed during parabolic flight on the NASA C-9 aircraft, the trans-cell current increased in hyper-g and decreased to near baseline in micro-g. The current began to rise within 2 seconds as the g-
force increased during the aircraft’s transition out of the micro-g segment of its flight. Very likely the gravity-directed trans-cell calcium current, which peaks about 9-10 h after light-induced germination (Chatterjee et al., 2000; Salmi et al., 2011) is needed for cell polarization, because at 1-g, the calcium-channel blocker nifedipine blocks the current and randomizes the direction of rhizoid emergence (Chatterjee et al., 2000, Salmi et al., 2011). In the moss Funaria, the direction of nuclear migration is also toward the site of calcium entry, and blocking calcium entry also blocks this migration (Saunders and Hepler, 1983). In contrast to the nifedipine results, the calcium-pump inhibitor eosin yellow suppressed the current, but not gravity-induced cell polarization. Taken together, the results of this study were consistent with the conclusion that gravity-induced calcium entry through channels at the bottom of the spores is one of the earliest responses of C. richardii spores to gravity, and that gravity can rapidly (probably post-translationally) modulate the activity of channels and pumps that drive a trans-cell current in these cells.

Other studies in single-cell model systems also show that gravity perception occurs in response to gravity forces less than 1 g. Data from TEXUS sounding rocket missions indicate that E. gracilis responds to as low as 0.12 g (Häder et al., 1998) and MAXUS-5 sounding rocket missions have provided evidence that Chara rhizoids perceive 0.14 g (Limbach et al., 2005). Although the precise gravity force necessary to activate gravity receptors has not been studied in the spores of C. richardii, an obvious hysteresis of de-activation was observed on NASA parabolic flight missions that may be explained by cellular perception of sub-earth gravity (in the realm of 0. 1 g) (Salmi et al., 2011). The evolutionarily conserved role of calcium, as well as the kinetics of gravity signaling in many cell types, suggests that a likely candidate for the gravity receptor in plant cells is a stretch activated calcium channel.

**Gene-expression Changes That Occur in Single Cells in Microgravity**

Recently several genes have been added to the list of those involved in various steps of the higher plant gravitropism signaling cascade (Agee et al., 2010; Fortunati et al., 2008; Nakamura et al., 2011; Yang et al., 2011). Data from microarray analysis of gene expression changes induced by changes in orientation (Kimbrough et al., 2004; Moseyko et al., 2002) or, perhaps even more significantly, development in microgravity (Paul and Ferl, 2002; Salmi and Roux, 2008) provide a very useful starting point for a more targeted forward genetics approaches to identify genetic components of gravity perception signaling. Signaling components that have been implicated in gravity perception and response in several studies, in different plants, or even other organisms are an obvious target for this type of evaluation. For instance, those genes identified in the study of root Arabidopsis gravitropism (Kimbrough et al., 2004) that have likely homologs identified the spore microgravity study (Salmi et al., 2008), such as Catalase 3, Fibrillarin 2, and Protodermal factor 1, could be evaluated in the commercially available Arabidopsis insertion mutant lines. A comprehensive evaluation of knockout lines of these genes might result in the identification of new and valuable mutants in gravity perception and/or gravitropism.

It is important to integrate these high throughput data with previously identified aspects of gravity perception and gravity directed polarity development, like the well established involvement of calcium as a component of the gravity response mechanism. In C. richardii spores, numerous genes that likely encode proteins involved in calcium mediated signal transduction have been identified. A study of gene expression changes during the germination and early development of spores (Salmi et al., 2005) found several genes which likely encode calcium binding proteins that are up-regulated during early development. None of these genes have changes between microgravity and 1g development in any of the developmental time points analyzed (Salmi et al., 2008). Of course, the most important molecular changes induced by gravity to alter the polarity of growth could all be post-transcriptional, or even post-translational. There is one gene however, likely to encode a protein with a C2 domain, that is down regulated during normal spore early development, and is also up regulated in spaceflight samples at the time point coincident with its highest abundance during normal development. The C2 domain is a calcium dependent, membrane binding feature of many proteins involved in membrane trafficking. Proteins with the C2 domain have recently been implicated in the viability and elongation of tip growing pollen tubes of angiosperms and seed germination (Lee et al., 2009; Li et al., 2007; Yang et al., 2008), two plant systems highly regulated by gravity. The expression profile of this particular gene in C. richardii spores indicates that it is more abundant early on in germination, when the gravity-directed calcium current of the spore is likely creating localized regions of high cytosolic calcium (Chatterjee et al., 2000; Salmi et al., 2011). As yet there has been no test to determine whether this gene encodes a protein essential for converting an elevated localized calcium concentration into downstream cellular events to
establish polarity. In general all the data evaluating gene expression changes should be translated into systems where targeted genetic manipulations are possible in order to directly evaluate the role of candidate genes in the cellular perception and earliest responses to gravity.

CONCLUSION
As indicated throughout this review there are significant similarities between the gravity responses found in single cells and those in other plant cells that perceive gravity (i.e. angiosperm root columella “statocytes”). Of course, there are other similarities besides those highlighted in this review. For example, cytoskeletal elements significantly influence gravity responses in single cells (e.g., in the stability and movement of Chara statolith vesicles) (Braun, 2002; Limbach et al., 2005), and in multicellular tissues (Blancaflor and Masson, 2003). To the extent that the cellular responses to gravity found in single plant cells are fundamental to cellular gravity sensing and responding, one could expect to find at least some of them, such as the activation of mechanosensitive channels and rapid calcium changes, also in animal cells. Future studies will clarify the extent to which the response mechanisms of cells to gravity are evolutionarily conserved not only in single-cell and multicellular plants, but also in animals.

REFERENCES


