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Cytoplasmic streaming and gravity sensing in *Chara* internodal cells

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Abstract. Since the nineteenth century, the merits of two alternate models for explaining the mechanism of plant gravity perception have been discussed. The gravitational pressure model states that plant cells perceive gravity by sensing their relative buoyancy to that of the surrounding medium, whereas the more popular starch-statolith model states that intracellular sedimenting particles act as gravity sensors. Vertically-oriented *Chara* internodal cells exhibit a gravity dependent polarity of cytoplasmic streaming such that the downardly-directed stream moves ca. 10% faster than the upwardly-directed stream. This polarity of cytoplasmic streaming is not simply a consequence of gravity acting directly on the cytoplasm but is rather under physiological control. When *Chara* internodal cells are placed in a medium more dense than themselves, the gravity-induced polarity of cytoplasmic streaming is reversed. This phenomenon cannot be explained by a model which relies on intracellular sedimenting particles as gravity sensors but is consistent with the gravitational pressure model for gravity sensing. We propose that gravity causes the internodal cells to settle within the confines of the extracellular matrix resulting in a tension between the plasma membrane and the extracellular matrix at the top of the cell and a compression between the plasma membrane and the extracellular matrix at the bottom of the cell. These stresses are proposed to act upon peptides which span the plasma membrane/extracellular matrix interface at the ends of the cells and which subsequently activate Ca^{2+} channels which in turn may induce a polarity of cytoplasmic streaming.

Key words: *Chara* (internodal cell) – Cytoplasmic streaming – Graviperception

Abbreviations: RGDS = tetrapeptide Arg-Gly-Asp-Ser; YIGSR = pentapeptide Tyr-Ile-Gly-Ser-Arg

Historical introduction to plant gravity sensing

Thomas Knight (1806) with his ingenious experiments using a water-powered centrifuge built by himself and his gardener, demonstrated that the phenomena of roots growing down and shoots growing up were indeed the result of plants sensing and responding to a gravity signal. The selective advantage to plants of having a mechanism to sense gravity seems clear: the roots are positively gravitropic and thus grow down into the soil where they function as organs of water and mineral ion absorption, and anchorage; the shoots, being negatively gravitropic, grow up – allowing the leaves to capture energy from the sun. How is it that plants perceive this important gravity signal? This is one issue we have been investigating over the past few years, and one I would like to address today.

Since Knight's report, the question of how plants actually perceive this gravity signal has been a matter of debate. Wilhelm Hofmeister (1867) suggested that plant cells perceive gravity by sensing their relative buoyancy in the surrounding medium or air. Pfeffer (1881) made a similar proposal, suggesting that plant cells perceive gravity by sensing a differential pressure, induced by the cytoplasm, at the top and bottom of the cell. Czapek (1898, 1901) concurred with this view.

Dehnecke (1880) reported the existence of sedimenting starch grains, and Berthold (1886) suggested that gravity perception in plants is dependent on the passive sinking of heavy components of the cytoplasm. Noll (1892) predicted the existence of sedimenting statoliths, which would be involved with gravity sensing, based on analogy with gravity sensing in crustaceans. The dominant theory for plant gravity perception, the starch-statolith theory, took a firm form at the turn of the century when Němec (1900) reported the existence of sedimenting starch grains in the columella cells of root caps, the presumed site of gravity perception in roots, and Haberlandt (1900) reported sedimenting starch grains in gravity-sensitive organs in shoots. This theory, which remains the preferred explanation for plant

gravity sensing today, suggests that intracellular sedimenting particles (statoliths) – typically starch-filled amyloplasts – act as gravity sensors (Darwin 1903; Audus 1962, 1979; Thimann 1977; Sack 1991; Sievers et al. 1991; Barlow 1995).

As long ago as 1904, Francis Darwin, while proclaiming his support for the statolith theory, cautioned against its habitual invocation for explaining graviperception in all plants. "The whole incident is an instance of what my father says somewhere about the difficulty of analysing a belief. I find it impossible to help believing in the statolith theory, though I own not being able to give good account of the faith that is in me.... As part of the general question of distribution, it must be clearly pointed out that in a large number of plants, such as the Algae and Fungi, no statoliths are known to exist, though their complete absence has not been proved. Here we must either believe in Noll's minute and hitherto unseen statoliths or in a different mechanism, such as hydrostatic pressure (Darwin 1904)." Since Darwin's time others have reported results which question the universality of statoliths as gravity sensors. Ewart (1903) reported that the large statolith-free cells of characean algae exhibit a gravity-dependent polarity of cytoplasmic streaming. Gravity-induced polarities of cytoplasmic streaming are also found in higher-plant cells lacking statoliths (Ewart 1903; Bottelier 1934). Gravitropism in mosses (von Bismarck 1959; Jenkins et al. 1986) and fungi (Pilet 1956; Dennison 1961, 1964; Dennison and Shropshire 1984; Varjú et al. 1961) takes place in the absence of statoliths; and excised tissues of higher plants exhibit gravity-dependent differentiation and growth without the presence of statoliths (Gersani and Sachs 1990). In addition, starch-deficient mutants of *Arabidopsis* sense and respond to gravity in the absence of starch and without discernible amyloplast sedimentation (Caspar and Pickard 1989; Kiss et al. 1989).

Gravity-caused polarity of cytoplasmic streaming in internodal cells of characean algae

As already mentioned, Ewart (1903) reported that single internodal cells of characean algae exhibit a gravity-caused polarity of cytoplasmic streaming. Polar ratios caused by gravity have also been reported in characean algae by Hayashi (1957), Hejnowicz et al. (1985), Wayne et al. (1990) and Buchen et al. (1991). *Chara* internodal cells are attractive for studying gravity perception because cytoplasmic streaming is easily observed and can be accurately measured. Measurements of the velocities of the upwardly- and downwardly-directed streams provide a rapid non-invasive assay of a gravity response. Since both gravity perception and response must occur in characean internodal cells, a gravity response reflects as closely as possible gravity perception.

The large internodal cells of the characean algae exhibit a highly organized and rapid rotational cytoplasmic streaming (Corti 1774). Streaming is an actomyosin-mediated phenomenon, the motive force for which is generated by the movement of myosin along actin cables, which are located at the ectoplasm/endoplasm interface just interior to the layer of chloroplasts embedded in the ectoplasm (Kamiya 1959). The nuclei are often larger than the mean width of the cytoplasm. Thus, as they stream along they push into the tonoplast, causing it to undulate. This action of the nuclei, analogous to a peristaltic pump, causes the contents the vacuole to stream as well. Since the motive force for cytoplasmic streaming is located just interior to the chloroplasts, the streaming velocity is most rapid there and decreases towards the center of the cell. Thus we confine our observations to the thin layer of streaming cytoplasm immediately interior to the chloroplasts (Staves et al. 1995). To measure the velocity of cytoplasmic streaming, we determine the time required for small, 1–5 μm particles to move 250 μm.

In agreement with Ewart (1903) we find that in horizontal internodal cells of characean algae, the cytoplasm streams right and left at the same rate. In vertical cells however, there is a polarity of cytoplasmic streaming such that the downwardly-directed stream moves about 10% faster than the upwardly-directed stream (Wayne et al. 1990; Staves et al. 1995). We refer to the velocity of the downwardly-directed stream divided by the velocity of the upwardly-directed stream as the polar ratio (Wayne et al. 1990). Thus a vertical internodal cell normally has a polar ratio of about 1.10. The polarity of cytoplasmic streaming is dependent on the orientation of the cell with respect to the vector of gravity. We find that when a cell is placed on the stage of a horizontal microscope and rotated through 360°, the polar ratios observed describe a cosine function with maxima at 90 and 270 degrees (Wayne et al. 1990).

Does this gravity-induced polarity of cytoplasmic streaming reflect gravity sensing (and by extension, a physiological response), or is it simply a physical manifestation of gravity? This question was answered serendipitously when we found that ligation of the internodal cells, at any site, causes cytoplasmic streaming to proceed up and down at the same rate. Since ligation abolished the gravity response without affecting other known rheological responses, it seemed plausible that ligation was inhibiting the gravity response by isolating the two cell ends from each other. The importance of the cell ends for gravity sensing was confirmed when we found that UV irradiation of the cell ends inhibits the gravity response while irradiation of cell middle has no effect (Wayne et al. 1990).

If the polarity of cytoplasmic streaming were the result of gravity acting directly on particles within the cytoplasm, then neither cell ligation nor UV irradiation should have an effect on the gravity response. Since we find that both of these treatments abolish the polarity of cytoplasmic streaming, we conclude that this polarity is not a direct physical consequence of gravity acting on intracellular particles, or a consequence of the difference between the density of the endoplasm and that of the cell sap, but rather is under physiological control. Thus we consider that gravity *induces* the polarity of cytoplasmic

streaming by triggering a chain of physiological events. Further, these results indicate that both intact ends of the cell are required to achieve the normal gravity response.

We have established that: vertically-oriented *Chara* internodal cells exhibit a gravity-induced polarity of cytoplasmic streaming; this polarity of cytoplasmic streaming is under physiological control; and both ends of the cell are needed to achieve this response to gravity. *Chara* internodal cells contain no visible sedimenting particles. How then do they perceive the gravity signal? Perhaps the entire protoplast acts as a gravity sensor as suggested by Hofmeister, Czapek and Pfeffer. Will gravity acting on the protoplast provide enough energy to be physiologically meaningful?

The force resulting from the effect of gravity on the protoplast can be calculated from the equation for static buoyancy:

$$F = gV(\rho_m - \rho_p)$$

where: g = the acceleration due to gravity, 9.8 $m \cdot s^{-1}$ V = the volume of the protoplast, $(\rho_m - \rho_p)$ = the difference between the density of the external medium and that of the protoplast.

For a 3-cm long cell with a volume of 2.4×10^{-8} m^3, and a density of 1015 $kg \cdot m^{-3}$, in Artificial Pond Water ($\rho = 1000$ $kg \cdot m^{-3}$) the protoplast will settle with a force of about 3.5×10^{-6} N.

If we assume that the protoplast falls about 1 nm (equal to about a 10% compression of the plasma membrane, or 10 times the crystal ionic radius of a Ca^{2+} ion) we can calculate the potential energy made available for gravity sensing using the formula for potential energy:

Potential energy = (force)(distance).

Thus the settling protoplast in the above example would make 3.5×10^{-15} J available for gravity sensing. Since this is about 10^6 times greater than thermal noise and represents enough energy to open about 10^5 mechano-sensitive Ca^{2+} channels (Howard et al. 1988), we believe that it is within the realm of physical possibility that the protoplast can act as the gravity sensor.

The gravitational pressure model for gravity sensing

We proposed the gravitational pressure model for gravity sensing as a result of our experiments on the gravity-induced polarity of cytoplasmic streaming in the internodal cells of characean algae (Wayne et al. 1990). In this model the entire protoplast, not intracellular particles, acts as the gravity sensor, and the ability of a cell to perceive gravity depends on its static buoyancy. Thus when the cell is in a medium less dense than itself, gravity may cause the protoplast to settle within the cell wall, or extracellular matrix, resulting in a differential tension and compression between the plasma membrane and the extracellular matrix at the top and bottom of the cell, respectively. These differential pressures may activate the gravireceptors which are located at the top and bottom of the cells (Wayne et al. 1990; Wayne and Staves 1996). We use the terms "tension" and "compression" for convenience and to underscore the effects at the plasma membrane/extracellular matrix interface resulting from the vectoral forces induced by gravity. Since the pressure generated by turgor is much larger than that generated by gravity, gravity will add to the turgor-induced pressure at the bottom of the cell and diminish the turgor-induced pressure at the top of the cell. Although the gravity-induced pressure is small, its vectoral nature allows it to be discerned over the background of turgor pressure, which is equal on all parts of the membrane. The question remains: can the small, vectoral, gravity-caused pressure be perceived over the much larger turgor pressure (about a $1{:}10^5$ signal-to-noise ratio)? There are many examples of organisms sensing small signals over large background, for example: the phototactic response in *Dunaliella salina* (Wayne et al. 1991), thermotaxis of the pseudoplasmodium of *Dictyostelium discoideum* (Poff and Skokut 1977), thermotropism of maize roots (Fortin and Poff 1990), and the report by Takahashi and Scott (1991) of hydrotropism of maize and pea roots (see also a discussion of this subject in Staves et al. 1992). Since sensing, according to the gravitational pressure model, depends on the difference between the density of the protoplast and that of the surrounding medium, the gravitational pressure model is philosophically allied with the early theories for gravity sensing put forward by Hofmeister (1867), Czapek (1898, 1901) as well as that proposed by Pickard and Thimann (1966).

If the gravitational pressure model for gravity sensing is true for *Chara* internodal cells, then changing the density of the external medium should change the gravity response. As the density of the external medium approaches that of the protoplast, the protoplast will become neutrally buoyant, thus it will no longer settle within the extracellular matrix and there will be no gravity response (polar ratio = 1.0). Increasing the density of the external medium beyond this point will cause the protoplast to become buoyant within the extracellular matrix and create a compression between the plasma membrane and the extracellular matrix at the top of the cell and a tension between the plasma membrane and the extracellular matrix at the bottom of the cell. Since this is the inverse of the normal condition, it should result in a reversal of the gravity response, i.e. the velocity of the upwardly-directed stream will be greater than that of the downwardly-directed stream (polar ratio < 1.0). This is, *mirable dictu*, exactly what we observe. As the density of the external medium is increased, the polarity of cytoplasmic streaming decreases and finally disappears when the density of the external medium is equal to that of the cell ($1015 \cdot kg^{-3}$). A further increase in the density of the external medium causes a reversal of the gravity response (Staves et al. 1997). These results are consistent with the gravitational pressure model of gravity sensing since the buoyancy of the protoplast is dependent on the difference between the density of the protoplast and the external medium.

These results also demonstrate that gravity sensing in *Chara* internodal cells is not accomplished by the "minute and hitherto unseen statoliths" mentioned by Darwin since, according to the laws of hydrostatics, the movement of intracellular particles will be unaffected by changes in the extracellular concentration of impermeant molecules. Movement of intracellular particles might be affected if the cell regulates its density to match the external medium; however we find no significant density regulation in *Chara* internodal cells (Staves et al. 1997).

Further support for the gravitational pressure model comes from the finding that hydrostatic pressure can mimic gravitational pressure and elicit a polarity of cytoplasmic streaming in *Chara* internodal cells. Normally a horizontal cell has a polar ratio of 1.0, however when a hydrostatic pressure of 490 Pa is applied to one end of a horizontal internodal cell, the stream moving away from the applied pressure moves ca. 10% faster than the stream moving in the opposite direction. This observation does not reflect a direct effect of pressure on the cytoplasm since ligation or UV irradiation of the cell ends abolishes the hydrostatic-pressure-induced polarity of cytoplasmic streaming just as these treatments abolish the gravity-induced polarity of cytoplasmic streaming (Staves et al. 1992).

Since the gravitational pressure model suggests that gravity sensing takes place at the interface between the extracellular matrix and the plasma membrane we used impermeant hydrolytic enzymes to look for candidate molecules which might connect the extracellular sensing events with the intracellular response. We found that cellulases, hemicellulases and peptidases all inhibit the gravity response in *Chara* internodal cells. Further, the tetrapeptide RGDS abolishes the polarity of cytoplasmic streaming, indicating that an integrin-like protein may be involved in gravity sensing (Wayne et al. 1992). The importance of the ends of the cells is underscored by the fact that the hydrolytic enzymes as well as RGDS were effective only when applied at the cell ends.

We find that RGDS is only effective at the site of tension between the plasma membrane and the extracellular matrix: at the top of the cell, when the density of the cell is greater than the density of the external medium; and at the bottom of the cell, when the density of the external medium is greater than that of the cell. We searched for a molecule that might inhibit the gravity response when applied to the site of compression between the plasma membrane and the extracellular matrix. We found that the pentapeptide YIGSR specifically inhibits the gravity response when applied to the bottom of the cell when the density of the cell is greater than the density of the external medium, and when it is applied to the top of the cell when the density of the external medium is greater than that of the cell. When RGDS is applied to the site of compression and YIGSR is applied to the site of tension in the same cell, the normal gravity response is observed. However, if the chamber containing the cell is inverted, the gravity response is abolished. The gravity-induced polarity of cytoplasmic streaming may be restored by inverting the chamber once more. We conclude that RGDS may specifically inhibit the tension receptor and YIGSR may inhibit the compression receptor. The two receptors are present on both ends of the cell; however, at present we are unable to determine if the same protein or different proteins function as the two receptors.

Calcium in the external medium is required for the gravity response in *Chara* internodal cells (Staves et al. 1992). Calcium-channel blockers inhibit the polarity of cytoplasmic streaming in a dose-dependent fashion only when applied to the ends of the cell. Thus we conclude that the influx of Ca^{2+} across the plasma membrane at the ends of the cells is required for the gravity response.

We found that Sr^{2+} can substitute for Ca^{2+} to produce the gravity response in *Chara* internodal cells and have used it as an inexpensive, non-radioactive tracer for Ca^{2+}. We find that in horizontal cells, the Sr^{2+} influx at the ends of the cells is 20 nmol $\cdot$ m^{-2} $\cdot$ s^{-1}, while the Sr^{2+} influx at the middle of the cells is 10 nmol $\cdot$ m^{-2} $\cdot$ s^{-1}. Again, there is a physiological difference between the middle and ends of the internodal cells. When internodal cells are placed in the vertical orientation, the Sr^{2+} influx at the middle of the cell and the bottom of the cell remain virtually unchanged (10 nmol $\cdot$ m^{-2} $\cdot$ s^{-1} and 20 nmol $\cdot$ m^{-2} $\cdot$ s^{-1} respectively), whereas the Sr^{2+} influx at the top end of the cell increases to 60 nmol $\cdot$ m^{-2} $\cdot$ s^{-1}. The increased flux is always located at the site of tension: when the density of the external medium is greater than the density of the cell, the higher Sr^{2+} influx is located at the bottom end of the cell. The increased Sr^{2+} influx is inhibited by RGDS, further evidence of the close link between the polarity of Ca^{2+} influx and the polarity of cytoplasmic streaming. Cytoplasmic streaming has been demonstrated to depend on cytoplasmic Ca^{2+} concentration, with high concentrations of cytoplasmic Ca^{2+} causing inhibition (Tominaga and Tazawa 1981; Kikuyama and Tazawa 1982; Williamson and Ashley 1982; Tominaga et al. 1983). It must be emphasized that we are measuring Sr^{2+} influx across the plasma membrane but have no information on the intracellular Sr^{2+} distribution. Strontium ions may be pumped rapidly into the vacuole, such that there is only a localized increase in cytoplasmic Sr^{2+} concentration.

Recently, Ackers et al. (1994) reported that they were unable to detect a substantial polarity of cytoplasmic streaming in vertically oriented *Chara* internodal cells. They reported a polarity of 1–2%, with a streaming velocity of ca. 50 $\mu m \cdot s^{-1}$, whereas we typically find a gravity-caused polarity of cytoplasmic streaming of ca. 10%, with streaming velocities of 90–100 $\mu m \cdot s^{-1}$ (Steaves et al. 1992, 1995, 1997; Wayne et al. 1990, 1992). There are at least two possibilities which could account for these discrepancies. Firstly, we may be measuring streaming at different sites in the cell; however Hejnowicz, Ackers and Sievers (personal communication) state that the optical configuration they employed for laser-Doppler-spectroscopy allowed them to measure the velocities of all particles. Alternatively, the fact that Ackers et al. (1994) reported streaming velocities of about half of those which we routinely measure may indicate that the cells they employed for

their experiments were in different physiological condition than the cells we typically use. We find that the age of internodal cells as well as the temperature at which they are grown have a profound effect on the magnitude of the gravity-caused polarity of cytoplasmic streaming (Staves et al. 1995).

In summary, we propose that in *Chara* internodal cells, gravity sensing can be best described by the gravitational pressure model which states that the entire protoplast acts as the gravity sensor and that the cell perceives the vector of gravity by sensing the differential tension and compression between the plasma membrane and the extracellular matrix at the top and the bottom of the cell, respectively. This sensing is mediated by peptides at both ends of the cell: an RGDS-sensitive peptide at the site of tension and a YIGSR-sensitive peptide at the site of compression. The gravity-induced polarity of cytoplasmic streaming is correlated with a polarity of Ca^{2+} influx (measured as Sr^{2+} influx) such that a higher Sr^{2+} influx is measured at the site of tension between plasma membrane and the extracellular matrix.

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