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Angle dependence as a unifying feature of root graviresponse modules

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Abstract
Gravitropism, the patterning of post-embryonic growth in relation to the gravity vector, allows plants to optimise the use of limited and non-homogenous resources in their immediate environment. Since the current model of root gravitropism has not been able to integrate all aspects of the response (perception, response, and behaviour), research on gravitropism has been dominated by different theories attempting to conceptualise each aspect individually.

In this work, we sought to reevaluate all the main components of the root graviresponse through the lens of angle-dependence. We show angle-dependence in Cholodny-Went-based auxin asymmetry and growth response, which we tracked back to angle-dependent variation in PIN asymmetry and statolith sedimentation in the columella. Thanks to this approach, we were able to suggest distinct roles for PINs and columella cell tiers, and a potential function for auxin vertical flux through the columella. Our findings provide a unifying framework to further explore the mechanisms that regulate angle-dependent gravitropic response, with major implications of time-dependent features of root graviresponse.

Significance Statement
Gravitropism, the patterning of post-embryonic growth in relation to the gravity vector, allows plants to optimise the use of limited and non-homogenous resources in their immediate environment. In this work, we reevaluated all the main components of the root graviresponse through the lens of angle-dependence. All the separate theories for root graviresponse are, therefore, likely conceptualization of the same mechanism and can be integrated in a cohesive model. Our findings provide a fundamental framework to further explore the mechanisms that regulate

angle-dependent gravitropic response.

Introduction

Plants have evolved the ability to adapt their post-embryonic growth to the non-homogenous distribution of resources in their environment through developmental responses called tropisms. Gravitropism is the ability of the plant to use the gravity field as a developmental cue to guide plant architecture (Del Bianco and Kepinski, 2018). Root architecture is particularly important for the efficient uptake of water and nutrients below ground. The manipulation of root architecture to improve water and nutrient uptake and, enhance, carbon sequestration, is becoming an attractive strategy to tackle the modern challenges associated with a changing climate (Lynch et al., 2013, 2022). Crucial to the establishment of root architecture is non-vertical growth of lateral roots, which is tightly regulated by developmental pathways and external stimuli (Roychoudhry et al., 2023; 2017). The capability to vary the growth angle of lateral roots requires the ability of these roots to discern different angles and elicit an angle-dependent response.

As described by the Starch-statolith theory (Sack, 1991), gravity is sensed thanks to dense amyloplasts (statoliths) in the specialized statocyte cells, which in the root are in the columella. When a root is displaced from its growth angle, statolith sedimentation to the new lower face of the cell (Iversen and Rommelhoff, 1978; Sack et al., 1985) triggers a signal transduction cascade that leads to the re-localization of auxin efflux proteins of the PIN-FORMED (PIN) protein family (Luschnig and Friml, 2024). In line with the Cholodny-Went theory, PIN polarization to the new lower side of the statocyte shifts the lateral efflux of auxin towards the lower side of the organ, where auxin inhibits cell expansion, causing bending (Friml et al., 2002, Li, Gallei & Friml, 2022).

Kinematic studies of gravitropism have been an intensive field of study for more than a century (reviewed in Moulia and Fournier, 2009). Initially, the Sine Law suggested that the magnitude of the gravitropic response was proportional to the component of the gravity vector that is perpendicular to the main axis of the organ (Sachs, 1888). However, the Sine Law is unable to reproduce the behaviour of plant roots gravistimulated at angles exceeding 90^{o}. Indeed, freely responding roots normally display maximum bend rates between 120-130^{o}, and not 90^{o} as a Sine Law behaviour would imply, which led to the proposal of the Modified Sine Law (Audus 1964). Despite these caveats, early studies demonstrated that there is angle-dependence in the plant graviresponse. Evidence from the starchless mutant *pgm1* has suggested that, in roots, the angle-dependent gravitropic behaviour and the formation of an auxin asymmetry (Wolverton et al., 2011; Band et al., 2012) rely on the presence of sedimenting statoliths. However, all aspects of the graviresponse have never been subjected to an integrated study to decipher their angle-dependent features.

Given the contrasting and incomplete evidence available in the literature, we sought to reevaluate angle dependence in the main components of the root graviresponse. Using tools for highly sensitive, quantitative reporting of auxin gradients, we found that gravitropism in Arabidopsis is angle-dependent and governed by quantifiable auxin gradients from stimulation angles as low as 30°, and that PIN3 and PIN7 play nonredundant additive roles in angle-dependent gravitropism. Taken together, our work provides a new mechanistic framework for the formulation of a unifying theory of root gravitropism.

Results

Root angle-dependent gravitropic response across angles

To assess the root gravitropic behaviour, Arabidopsis seedlings were reoriented at different angles (30°, 60°, 90°, 120°, 150°, and 170°; n > 76) and imaged every 30 minutes, for 6 hours (Fig. 1A). The magnitude of the gravitropic response was then expressed as average bend rate in the first hour after reorientation (Fig. 1B). The bend rate gradually increased between 30° and 120° reorientation angles and then decreased between 150° and 170°, with ~120° eliciting maximal response. These data are consistent with the observation that the angle-dependent behaviour of freely responding roots does not follow a standard Sine Law (Iino et al., 1996; Mullen et al., 2000; Galland, 2002; Chauvet et al., 2019). For this reason, we focused on investigating the dynamics of root gravitropic behaviour over the transition from above the horizontal (>90°) to the vertical (0°).

To confirm that the increase in differential growth rate was driven by a biophysical growth response, we quantified the percent reduction in the length of the lower side of the root (see S7 for method) as a parameter for curvature (Fig. 1C). This analysis revealed that the decrease in the length of the lower side significantly increased with reorientation angle, confirming that angle-dependence is a sign of differential magnitude in the response and not, for example, in a greater persistence of the response though time. Since auxin has been shown to inhibit primary root growth in a dose-dependent manner (Fendrych et al., 2018), these data suggest that the angle-dependent gravitropic response may be due to differential accumulation of auxin.

Angle-dependence emerges from auxin asymmetry and a lack of redundancy between PINs

To study the relationship between stimulation angle, bending response, and auxin gradients, we used the ratiometric *R2D2* auxin reporter, which, combined with vertical imaging, allows for sensitive quantitative inference of auxin levels (Liao et al., 2015). In this reporter, auxin accumulation is manifested as the reduction of yellow signal relative to the red signal. Due to the inherent differences in *R2D2* signal between epidermal trichoblast and atrichoblast (Fig. S1A, B), we compared fluorescence ratios between the same cell type (Fig. S1C). Using this method, we found that quantifiable auxin gradients were present at stimulation angles as low as 30°, and that auxin gradients were correlated with stimulation angles between 30° and 120° (Fig. 1D). Taken together, these data support the idea that angle-dependent auxin gradients dictate the magnitude of root gravitropic response.

To clarify the role of columella cells in determining the angle-dependent auxin asymmetry, we first performed detailed kinematic studies of PIN polarisation in Arabidopsis primary roots. *PIN3* and *PIN7* are expressed in the columella (Friml et al., 2002a; Blilou et al., 2005) and are essential for root graviresponse. Using vertical-stage confocal microscopy, we analysed the PIN expression pattern and polarization in the columella cell membranes in the *pPIN3::PIN3:GFP* and *pPIN7::PIN7:GFP* translational lines (Fig. 2). In our experimental conditions, we found that *PIN3* was expressed strongly in the upper columella cell tiers, while *PIN7* was expressed strongly in the lower two columella cell tiers (Fig. 2A,D). After reorientation at different angles, both PIN3 and PIN7 polarized in the direction of gravity 30 minutes after reorientation in an angle-dependent manner (Fig. 2B, E). Interestingly, while PIN3 showed prominent polarization at lower angles (Fig. 2B), PIN7 showed asymmetric distribution only at angles above 60° (Fig. 2E). These results are consistent with previously published data for PIN3, but not PIN7 polarization at 45° (Klein-Vehn et al., 2010; Roychoudhry et at, 2023), although this discrepancy is likely due to difference in growth conditions and bioimaging protocol. Both PINs did not show any angle-dependent internalisation and/or polarisation away from the distal columella cell membranes (Fig. S3). Overall, these data suggest a potential differential contribution of PIN3 and PIN7 to gravitropic response at different stimulation angles.

Despite their central role in mediating the gravitropic response, plants carrying single *PIN* loss-of-function mutations display only mild phenotypes (Kleine-Vehn et al., 2010). This has been suggested to be caused by the functional redundancy between members of the PIN family. Using a constant gravitropic stimulation system (Mullen et al., 2000), we were able to closely assess the root gravitropic response in *pin3* and *pin7* single mutants. This analysis revealed that the *pin3* mutant displays an overall flattening of the typical bell-shaped response, with a decrease in the magnitude of bend rate over all the angles tested (Fig. 2C). On the other hand, *pin7* showed a normal phenotype up to 60°, with a severely impaired gravitropic response at higher angles (Fig. 2F). Analysis of the *pin3pin7* double mutant under constant stimulation revealed a pattern of response similar to that of *pin3* single mutant, but more severe (Fig. S2). This suggests that while PIN3 might play a central function in mediating an angle dependent response, PIN7 still plays a redundant as well as non-redundant, additive role in root graviresponse. In this context, it is also important to note that previous studies have shown that loss-of-function mutations in single and multiple PIN proteins lead to ectopic upregulation of other PIN proteins (Vieten et al., 2005; Omelyanchuk et al., 2016).

Among the PINs expressed in the root, *PIN4* is detected in the stem cell niche, basally in provascular and epidermal cells, and in the first tier of the columella (Friml et al., 2002b, Fig. 2G). We assessed the phenotype of the *pin4* loss-of-function mutant under constant gravitropic stimulation and found it to be similar to *pin3* (Fig. 2H). However, the analysis of the p*PIN4::PIN4:GFP* translational marker line after gravistimulation at different angles revealed that PIN4 failed to polarise significantly at any angles (Fig. 2H). Quantification of *PIN4:GFP* membrane fluorescence within distal plasma membranes of gravistimulated roots also showed no significant variations at different stimulation angles (Fig. S3). These data indicate that the *pin4* mutant phenotype may not be due to an abnormal response downstream of statolith sedimentation.

Statolith sedimentation determines angle-dependent PIN polarisation

It has been previously shown that the Arabidopsis starchless mutant *pgm1* retains a basal gravitropic response and no angle-dependence (Wolverton et al., 2011). To confirm the role of statoliths in the angle-dependent polarization of PIN proteins, the *pPIN3::PIN3:GFP, pPIN4::PIN4:GFP* and *pPIN7::PIN7:GFP* marker lines were introduced in the *pgm1* mutant background. Vertical-stage confocal microscopic analyses revealed that PIN3, PIN4 and PIN7 polarisation was abolished in a *pgm1* mutant background (Fig. S4A-C). This confirms that angle dependence is specifically mediated by statolith sedimentation and downstream PIN relocalization.

Previous studies have suggested a proportionality between stimulation angles and overall statolith sedimentation (Iversen & Larsen, 1971; Perbal & Perbal, 1976). To better assess statolith sedimentation in our system, we reoriented Arabidopsis roots expressing the fluorescent plastid marker Pt-YK (Nelson et al., 2007) at different angles. In columella cells imaged 5 mins post reorientation, in accordance with previous studies, statoliths tended to sediment in aggregates (Lietz et al., 2009). Statolith sedimentation ratio along the columella cell membrane was estimated as the length of the cell membrane in contact with statoliths, divided by the total length of the columella cell (See Fig. 3A). Quantification of statolith sedimentation in all the columella cell tiers demonstrated that sedimentation ratios increased in an angle-dependent manner up to 135^{o}, but declined at 150^{o}, as the almost vertically inverted orientation led to increased sedimentation on the upper, horizontal cell wall (Fig. 3B), consistent with a maximum bend rate at around 135^{o}. In the *pgm1* mutant, Pt-YK labelled statoliths showed an abnormal, filamentous morphology (Fig. S5A). Consistent with previous studies (MacCleery & Kiss, 1999), statolith sedimentation did not occur (in an angle-dependent manner, or otherwise) in the starchless *pgm1* mutant background even 15 min after gravistimulation (Fig. S5B).

To further assess statolith sedimentation, we quantified, statolith sedimentation ratios along the distal columella cell membrane and found that these decreased in an angle-dependent manner (FigS6A). Further, we also quantified the velocities of sedimenting statoliths using time-lapse live cell imaging. Roots expressing YFP or CFP tagged plastid fluorescent markers (Pt-YK or Pt-CK; Nelson et al., 2007) were reoriented at different angles and imaged from 60 seconds up to 5 minutes post reorientation, when statoliths would settle on the new basal surface of the cell. Statolith sedimentation velocity increased generally in an angle-dependent manner up to 120^{o}, before declining again at 150^{o} (Fig. S6B). These data could justify the initial observation of ~120° eliciting maximal response and opens the possibility of a complex interaction between angle-dependent magnitude and time factors within the columella.

Discussion

Gravitropism has been conceptualized through three main theories: the starch-statolith theory for sensing, the Cholodny-Went model for signal transduction and growth control, and the law of angle-dependence for behaviour. Previous work on the starchless mutant *pgm1* had already suggested that the angle-dependent gravitropic behaviour of plant roots (Wolverton et al., 2011) and the formation of an auxin asymmetry (Band et al., 2012) rely on the presence of sedimenting statoliths. Here, we demonstrate that angle-dependence in Cholodny-Went-based auxin asymmetry and growth response can be traced back to angle-dependent variation in PIN protein asymmetry in the gravity-sensing columella cells and statolith sedimentation, even at low stimulation angles. Our results agree with previous work that showed that angles as small as 15° were sufficient to trigger a gravitropic bending in Arabidopsis roots (Mullen et al., 2000). By showing that all major components of the gravitropic response display angle-dependent behaviour, we provide a mechanistic framework towards the formulation of a unifying theory of root gravitropism.

In this work, we were able to uncover novel features of the root graviresponse using the more sensitive R2D2 marker and taking into account the variation in signal among epidermal cell types. Importantly, we attempted to assess angle-dependence independently of time. Previous work using the Arabidopsis DII-Venus reporter had proposed a 'tipping-point' model of root gravitropism (Band et al., 2012), where roots would reach the vertical thanks to the persistence of the auxin response, while the gradient is lost half-way through the graviresponse. The results here obtained by addressing other reorientation angles would suggest that the 'tipping-point' model might actually be highlighting time-dependent features of the graviresponse. The existence of time-dependent features would imply that the gravitropic behaviour of roots reoriented at a lower angle is different from roots that reach that same angle after being reoriented at a higher one. Constant gravitation stimulus is the only available experimental approach that can circumvent the change in stimulation angle as a root responds to gravity. Interestingly, constant stimulus studies have shown a constant response at different angles but a different angle-dependence behaviour, more similar to the Sine Law, compared to freely responding roots (Mullen et al., 2000). This indicates that a maximum bend rate above 90° could be due to time-dependent features, e.g. response speed. For example, here we show that statolith sedimentation velocity is consistent with a maximum bend-rate above 90°. Moreover, it was shown that plastid sedimentation rates are heterogeneous within the columella (Blancaflor et al., 1999). Additional layers of complexity to the resulting root gravitropic kinetics could arise from other elements of the response, like cell expansion rate and a potential proprioceptive response (Bastien et al., 2012). More work will therefore be needed to understand the contribution of possible time-dependent features of the graviresponse to the overall kinetic (Levernier, Pouliquen & Forterre, 2021).

The analysis of the PIN polarization and mutant combinations under constant stimulation presented here has revealed non-redundant, additive roles in root graviresponse for PIN3, PIN7, and PIN4. PIN3 polarisation is most prominent at lower angles, however its phenotype suggests that its function is required for the generation of an asymmetric auxin gradient across a wide range of angles. The discrepancy between free response and constant stimulus could indicate a possible time-dependent feature of PIN3 polarization. PIN7 polarisation is instead more prominent at angles exceeding 60^{o}, in accordance with its mutant phenotype under constant gravitational stimulus. Laser ablation studies have previously revealed that columella cell tiers play different roles with respect to gravity sensing (presentation time) versus graviresponse (final tropic growth response of the root). The removal of tiers I and II had the greatest effect, while ablation of tier III slows the kinetics of bending but has no effect on sensitivity suggesting that the signal was produced in tier I and II, and amplified in tier III (Blancaflor et al., 1998). Conversely, it could be concluded, from the further evidence produced in this work, that PIN3 polarization could mediate the bulk of the response from tier I and II, while PIN7 would amplify the signal at angles >90° from tier II and III. Unfortunately, the lack of R2D2 expression in the lateral root cap and the delay in DR5 response upon auxin treatment (Brunoud et al., 2012) hinder our ability of directly prove this hypothesis. Moreover, PIN expression is influenced by auxin variations in a tissue-specific manner, and the loss of even single PINs can lead to the ectopic overexpression of other PINs (Vieten et al, 2005; Omelyanchuk et al, 2016). Mathematical modelling, and more refined auxin markers and genetic approaches will therefore be needed to verify the distinct role of PINs in the root graviresponse, circumventing gene redundancy and complex regulatory pathways.

Auxin distribution within the root meristem and columella is likely important for a normal gravitropic response. PIN4 mediates the formation of an auxin sink around the quiescent centre and the input of auxin from the vasculature to the columella (Friml et al., 2002b). The loss-of-function mutation of *PIN4* causes a flattening of the auxin gradient, with a decrease in auxin response in the columella. While *pin4* has a gravitropic phenotype very similar to *pin3*, our analysis showed a lack of gravi-dependent repolarization of PIN4:GFP in both lateral and basal membranes. This suggests that the *pin4* mutant phenotype could be the symptom of an overall disturbed auxin distribution, which could affect *PIN3* and *PIN7* expression and/or the creation of upper/lower auxin gradient. Moreover, it has been suggested that PIN proteins, including PIN4, PIN3, and PIN7, form protein complex containing homo- or heterodimers (Luschnig and Friml, 2024). PIN4 could therefore act as a general regulator of cellular auxin efflux in a gravity-independent way. Interestingly, though the ablation of whole tiers abolishes curvature, laser ablation of vertical files of central columella cells does not elicit the same effect (Blancaflor et al., 1998). This could suggest that the vertical flux within the columella, albeit indirectly, is important for graviresponse. While the levels of PIN4, PIN3 and PIN7 localised to the distal plasma membrane do not vary in response to gravistimulation (Fig. S3), an impaired auxin flux to lower tiers could contribute to the phenotype of the loss-of-function mutants. In the future, an approach based on live-imaging and computational modelling will be required to study the complex effects of the overall fluxes and distribution of auxin within the root meristem during the root graviresponse.

Our statolith sedimentation analyses ties into the most recent hypothesis in plant gravitropism: the ‘position-sensor hypothesis’ (Chauvet et al., 2016; Pouliquen et al., 2017). This hypothesis, first developed in the context of shoot gravitropism, states that statocytes act as clinometers, wherein the position of the statoliths in relation to the plasma membrane induces corresponding auxin fluxes. Here, we show that PIN polarisation is proportional to the degree of statoliths sedimentation in the root gravisensing cells. This link is supported by recent studies demonstrating the role of LAZY proteins (Chen et al., 2023; Nishimura et al., 2023). It has been suggested that LAZYs translocate to the plasma membrane thanks to statolith sedimentation, where they seem to act as positional sensors for PIN polarisation and/or activation, presumably through RLD1 (Furutani et al., 2020) and D6PK-dependent mechanisms (Kulich et al., 2024). Thus, taken together, our data provide molecular support for the ‘position-sensor hypothesis’ in the root.

The work presented here shows that all the main components of the root graviresponse, from statolith sedimentation to response, are linked by angle-dependence. All the separate theories for root graviresponse (starch/statolith and Cholodny-Went) are, therefore, likely conceptualization of the same mechanism and can be integrated thanks to a cohesive angle-dependence framework (Fig 4). This conceptualization allowed us to postulate distinct roles of PINs and columella cell tiers, and the existence of time dependent features in the root graviresponse. Overall, these observations represent an important step forward in our understanding of the biology of gravitropism and towards the exploration of major outstanding questions in the field.

Methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* in a Columbia (Col-0) background were used in this study. The following lines have been previously described: *pin3*, *pin4*, *pin7*, *pin3pin7* (Friml et al., 2002a; Friml et al., 2023*)*, *pPIN3::PIN3:GFP* (Kleine-Vehn et al., 2010), *pPIN4::PIN4:GFP* (Vieten et al., 2005), *pPIN7::PIN7:GFP* (Kleine-Vehn et al., 2010), PT-YK, PT-CK (Nelson et al., 2007) and *pgm1* (Perriapuram et al., 2000).

Seeds were surface sterilized using chlorine gas (Roychoudhry et al., 2023). Following sterilization, seeds were sown in chambered slides containing solidified Arabidopsis thaliana salts (ATS) growth medium. A 1 cm well was cut into the top of each chambered slide using a sterile scalpel and 2-3 seeds were transferred in the corner between gel and glass slide. Seeds were kept at 4 °C for at least 2 days and transferred to environmental growth cabinets, with each slide positioned in a vertical orientation to maintain primary roots parallel with the gravity vector. Seeds were incubated in standard tissue culture conditions under 20 +/- 2°C, long day (16h light/ 8h dark cycle), and 400–500 µmol m^{-2} s^{-2} light conditions, for 5-7 days.

For constant gravitropic stimulus experiments, plants were grown as previously described (Mullen et al. 2000). Briefly, seeds were surface sterilized in ethanol solution (70% v/v), followed by washes in 95% ethanol. Seeds were sown on 60 mm petri dishes containing solid half-strength MS media, refrigerated for 1–5 days before being placed under continuous illumination with cool-white fluorescent lights (80 μmol m−2 s−1) for 4–5 days at 22 ± 2°C.

Reorientation assays and confocal microscopy

Vertically grown 5-day-old Col-0 seedlings were gravistimulated in infrared light and imaged at 30-minute intervals using a converted infrared camera with an 830 nm filter (Roychoudhry et al., 2022), for the analysis of gravitropic response kinetics. For curvature analysis, four hours after reorientation, whole roots were cut with the agar and carefully mounted on glass slides with 1.5 mM propidium iodide and imaged on an inverted Zeiss LSM 880 Axio Imager 2 confocal. Following one hour of acclimation, five-day-old *R2D2* seedlings were imaged through the root mid-plane using a vertical-stage confocal microscope setup (Fig. S1A). Roots were then gravistimulated at different angles (30°-120°) and imaged after 40 minutes. Relative auxin levels were calculated for each nucleus of the upper and lower root epidermis as in Roychoudhry et al. (2023). Briefly, excluding the lateral root cap, nuclear fluorescence was measured in ten consecutive epidermal cells within the two outermost flanking cell files, beginning from the root tip for each root. Experiments were performed three times with at least ten root tips for each orientation per experiment. Nuclear fluorescence intensity was measured across both GFP and mTomato channels. For each nucleus, the ratio of GFP/mTomato signal was determined. Since we found inherent differences in GFP/mTomato signal between hair and non-hair cells (Fig. S1B), we compared ratios between the same cell type (Fig. 1D).

Curvature was expressed as the delta, in %, between the length of the inner vs outer root perimeter across the length of the gravitropic bent (Fig. S7). Measurements were performed using ZEN Blue software (Zeiss) with a segmented line. Col-0 plants were gravistimulated at 0°, 45°, 90°, and 135° in the same set-up used for reorientation experiments. These angles were chosen as being representative of a smaller angle, of the horizontal, and of an angle slightly above that inducing the fastest bend rate.

For PIN protein polarization and statolith sedimentation experiments, roots were stained with propidium iodide and gently placed into an LSM800 inverted confocal microscope (Zeiss) rotary stage at 0° GSA, using a 40x (numerical aperture 1.2) ultrasound immersion objective. For *PIN:GFP* lines, a Z-series of columella cells was acquired for each root by capturing 25-55 slices at 1 μm intervals, using a 488nm laser at 4x averaging. Roots were initially fixed onto a backboard at angles ranging from 0° to 150° using a 2-axis spirit level, then left for 30 minutes to allow PIN polarization to take place. The angle of reorientation was then maintained as slides were positioned in the LSM800 confocal for image acquisition. The same images were used to quantify statolith sedimentation ratios as well as *PIN:GFP* fluorescence from the distal membrane at different angles of gravistimulation. For distal membrane data, the statolith sedimentation ratio was quantified as the ration of the distal membrane in contact with statoliths divided by the total length of the distal membrane. Distal *PIN:GFP* fluorescence was quantified as the ratio between the fluorescence at the distal membrane divided by the total fluorescence across all membranes for each columella cell. For quantification of statolith sedimentation velocity using PT-YK and PT-CK plastid marker lines, seedlings were illuminated with a 500 +/- 10nm or 405 +/- 10 nm laser, respectively, at angles ranging from 0° to 150° for 50 frames (5.8-13.4 s/frame depending on microscope performance). Scaling per pixel (0.185 µm x 0.185 µm) and resolution (512 x 512-pixel) was consistent between statolith experiments.

To image the expression domain of PIN3/4/7::GFP, seedlings were grown on vertically oriented ATS medium plates for 5 days, then mounted in propidium iodide solution on glass slides and standard cover slips using an LSM880 inverted confocal microscope (Zeiss). Images were captured at 40X magnification using the 488 nm and 543 nm lasers for GFP and propidium iodide respectively.

Image analysis

To discern the apical-basal targeting of PIN3 and PIN7, measurements were performed using ImageJ software (National Institutes of Health). The fluorescent intensity across internal membranes of columella cells was quantified using the approach in Roychoudhry et al., 2023. Using the 'Plot profile' function in ImageJ, the *x*-axis point of maximal intensity in the PI channel was identified as the cell wall. The GFP fluorescence was measured and calculated across each cell membrane on either side of the cell wall. The log signal ratio before and after reorientation was calculated as the average of signal intensity ratios per cell for each angle. 10 replicates of 3-6 seedlings were analysed.

Statoliths were tracked using IMARIS (Bitplane Scientific Software) and ZEN Blue (Zeiss) software, with 5-10 centrally positioned statoliths per root tracked across C1-C4 tiers. Each track was set to a maximum gap size of 1.5x the diameter of an individually tracked statolith, and each track was manually checked so that track jumping within statolith aggregates did not overestimate an individual plastids movement through time. Where statoliths moved too far through the z-axis to be tracked, a minimum of 15 frames was used as the benchmark for calculating velocity. To compensate for any object drift and ensure that time-resolved velocity of individual statoliths was kept consistent between images, a cell membrane was tracked in the PI channel. The horizontal velocity (Vx) and vertical velocity (Vy) outputs provided the total statolith velocity using the trigonometric function

$$\Delta z = \frac{\sqrt{\Delta x^2 + \Delta y^2}}{t}$$

where *t* is the length of time between each frame in the series. Direct motion of statoliths could then be calculated by subtracting the resultant vector (Δz) for the plasma membrane from the Δz for the statoliths. Statistical analysis was calculated using MS Excel 365 software (Microsoft). Statolith sedimentation speeds were calculated by measuring the length of a statolith aggregate in microns and waiting until they had spread an equivalent distance following reorientation on the (new) basal membrane, using a digital calliper.

Constant gravitropic stimulus

Constant gravitropic stimulus experiments were performed using the ROTATO image analysis and feedback system, as previously described (Mullen et al. 2000). Briefly, seedling roots were positioned on a rotatable stage in the centre of the axis of rotation and custom software was used to maintain the tip segment in a fixed orientation relative to gravity through image analysis coupled to a stepper motor. As the root continued to undergo gravitropic curvature, its response kinetics were captured as the rotation required to constrain the tip at the prescribed angle.

Statistical analysis

Unless stated above, all statistical analyses were performed in SPSS (IBM). The normality of data was assessed (Kolmogorov Smirnoff test with Lilliefors correction) and a one-way ANOVA was performed with a *post hoc* Tukey's HSD test.

Authors contributions

SR and HT: Generated and analysed statolith sedimentation, statolith velocity and PIN polarisation data
KSF: Generated and analysed root bend rate and R2D2 data
IS and CW: Generated and analysed the constant stimulus data
JF: Methods and technical supervision for R2D2 data collection.
MDB and SK: Funding, conceptualisation and experimental feedback
All authors contributed to writing and editing the manuscript. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration

The authors declare that they have no competing interests.

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