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1 **New insights into root gravitropic signalling.**

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21 Short statement:

22 This review addresses the current understanding of the gravitropic root
23 bending response of *Arabidopsis thaliana*, chronologically summarizes
24 the process and identifies challenges for further research.

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Abstract

An important feature of plants is the ability to adapt their growth towards or away from external stimuli such as light, water, temperature and gravity. These responsive plant growth movements are called tropisms and they contribute to the plant's survival and reproduction. Roots modulate their growth towards gravity to exploit the soil for water and nutrient uptake, and to provide anchorage. The physiological process of root gravitropism comprises gravity perception, signal transmission, growth response and the reestablishment of normal growth. Gravity perception is best explained by the starch statolith hypothesis that states that dense-starch filled amyloplasts or statoliths within columella cells sediment in the direction of gravity resulting in the generation of a signal that causes asymmetric growth. Though little is known about the gravity receptor(s), the role of auxin linking gravity sensing to the response is well established. Auxin influx and efflux carriers facilitate creation of a differential auxin gradient between the upper and lower side of gravistimulated roots. This asymmetric auxin gradient causes differential growth responses in the graviresponding tissue of the elongation zone leading to root curvature. Cell biological and mathematical modelling approaches suggest that the root gravitropic response begins within minutes of a gravity stimulus triggering genomic and non-genomic responses. This review discusses recent advances in our understanding of root gravitropism in *Arabidopsis thaliana* and identifies current challenges and future perspectives.

Key words: *Arabidopsis thaliana*, auxin, calcium, differential growth, gravitropic response, root growth, tropism

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Root gravitropism

Plants adapt to environmental signals such as light, water, temperature and gravity by modifying their growth. Directional growth responses are termed tropisms. The first detailed studies of tropisms were performed by Charles Darwin and his son Christopher, who described that etiolated grass coleoptiles grew towards the source of light when they were illuminated from one side (Darwin, 1880). Plant roots provide another example of a tropism as they respond to changes in orientation (Fig. 1). Upon seed germination, roots immediately orient themselves to grow downwards along the gravity vector in the soil, in order to secure water, nutrients and anchorage (Bailey *et al.*, 2002).

In roots, gravity is perceived in the columella cells in the root cap but the gravitropic response takes place in the elongation zone cells. Thus, there is a clear physical separation between the site of gravity perception and the site of gravitropic response (Fig. 2). Root gravitropism can be divided into three spatially and temporally distinct phases: gravity perception, transmission of the gravitropic signal and ultimately the growth response itself (Swarup and Bennett, 2009).

This review will primarily focus on our current understanding of root gravitropism in the model plant *Arabidopsis thaliana*, reviewing the gravitropic response chronologically.

1. Gravity perception (Fig. 2)

The root cap is the primary site of gravity perception in higher plants, as early experiments involving surgical removal of the root cap resulted in agravitropic roots (Barlow, 1974; Blancaflor and Masson, 2003; Morita and Tasaka, 2004). The root cap is comprised of central columella cells that are surrounded by lateral root cap cells (Dolan *et al.*, 1993). More sophisticated experiments including laser ablation (Blancaflor *et al.*, 1998), genetic ablation (Tsugeki and Fedoroff, 1999) or heavy-ion microbeam irradiation (Tanaka *et al.*, 2002) of individual root cap cells showed that within the entire root cap the columella cells are the site of gravity perception. In *Arabidopsis thaliana*, there are normally four stories of columella cells termed S1 to S4 cell layers of which S1 and S2 appear

96 to be most important for root gravitropism (Blancaflor *et al.*, 1998).
97 Columella cells contain starch-filled amyloplasts and as per the starch-
98 statolith hypothesis (Haberlandt *et al.*, 1900; Němec, 1900) sedimentation
99 of amyloplasts triggers a signal transduction cascade that results in
100 generation of a signal that is then transmitted to the graviresponsive
101 tissues of the elongation zone to achieve gravitropic bending (Leitz *et al.*,
102 2009; Band *et al.*, 2012a; Baldwin *et al.*, 2013; Blancaflor, 2013; Swarup
103 *et al.*, 2013).

104
105 Despite the support for the starch-statolith hypothesis, alternative models
106 for gravitropic sensing have been suggested. The protoplast pressure
107 hypothesis is based on changes in pressure exerted by the cytoplasm on
108 the plasma membrane in response to a gravity stimulus (Wayne *et al.*,
109 1990; Wayne and Staves, 1996), and also explains the rather weak
110 agravitropic phenotype of starchless mutants (Kiss and Sack, 1989; Kiss
111 *et al.*, 1996; MacCleery and Kiss, 1999; Fitzelle and Kiss, 2001; Band *et al.*,
112 2012a). Barlow (1995) suggested that over the period of evolution
113 plants may have evolved multiple gravity sensing mechanisms, a view
114 shared by Perbal (1999), who suggests that both protoplast pressure and
115 starch-statolith hypotheses may operate side-by-side. In addition,
116 Wolverton (2002a,b) proposes that there might be additional minor gravity
117 sensing sites in the root cap outside the columella cells.

118 Following gravity perception in the central columella cells, there is a
119 change in the cytosolic pH of root cap cells from 7.2 to 7.6 in wild type
120 roots but not in the starchless *pgm* mutants (Fasano *et al.*, 2001). This
121 observation directly links these pH changes to statolith sedimentation
122 (Scott and Allen, 1999; Fasano *et al.*, 2001; Hou *et al.*, 2004), which is
123 supported by the finding that acidifying or alkalinising agents can alter the
124 gravitropic response. Also agravitropic *arg1* mutants have a defect in the
125 pH changes following gravity perception(Boonsirichai *et al.*, 2003).

126
127 Much research and speculations have focused on how a physical signal
128 (statolith sedimentation) can be translated into a moving physiological
129 signal. It has been proposed that that an interaction between the
130 sedimenting amyloplasts and proteins belonging to the endoplasmic
131 reticulum or the plasma membrane may be involved in the
132 graviperception. This hypothesis is based on observations in rhizoids of

133 the green algae *Chara*. As the rhizoids are single cells, the gravity
134 perception and its response both occur in the same cell. In parabolic
135 flights weightless *Chara* statoliths (barium sulfate crystals) could still
136 sense gravity as long as their statoliths remained in contact with plasma
137 membrane sensitive sites (Limbach *et al.*, 2005). It was therefore
138 suggested that not the pressure (exerted by the statoliths) but the contact
139 of the statolith with some membrane-bound receptors triggered the gravity
140 perception (Braun and Limbach, 2006). The fact that root gravitropism
141 defects of starchless mutants can be rescued in hypergravity conditions
142 (Fitzelle and Kiss, 2001) indicates that similar mechanisms may operate in
143 higher plants too. This idea is further supported by the finding that TOC
144 (translocon of outer membrane of chloroplasts) complex-related proteins
145 may be part of a receptor-ligand system (Stanga *et al.*, 2009). The TOC
146 complex is important for the delivery of nuclear-encoded proteins to the
147 chloroplasts and presumably amyloplasts. In a screen for enhancers of
148 *arg1*, *mar1* and *mar2* mutations were identified. MAR1 and MAR2 encode
149 components of the TOC complex. Mutations in TOC132 enhances the
150 gravitropic defects of a starchless mutant (Strohm *et al.*, 2014),
151 suggesting that plastids, besides functioning as statoliths, may also be
152 part of a signal transduction mechanism, facilitating interaction between a
153 ligand protein at the outer amyloplast envelope and a receptor-protein on
154 the endoplasmic reticulum or plasma membrane (Stanga *et al.*, 2009).

155 Another view is that stretch-activated mechanosensitive ion channels
156 may be involved in graviperception (Sievers *et al.*, 1991; Perbal and Driss-
157 Ecole, 2003). These ion channels may be activated by the force exerted
158 by sedimenting amyloplasts on the plasma membrane and/or
159 endoplasmic reticulum. Electron tomography experiments that show that
160 sedimenting amyloplasts bend and distort the endoplasmic reticulum at
161 the point of contact may support this view (Leitz *et al.*, 2009). Although
162 inhibitors of mechanosensitive ion channels inhibit gravitropism (Caldwell
163 *et al.*, 1998), genetic studies so far have failed to pinpoint their role in
164 gravitropism (Nakagawa *et al.*, 2007; Haswell *et al.*, 2008). It can,
165 however, not be ruled out that yet unidentified plant-specific
166 mechanosensitive ion channels are involved in root gravitropism.

167 There has been a lot of controversy on the role of the actin cytoskeleton in
168 root gravitropism and the results were often contradictory (Blancaflor and
169 Hasenstein, 1997; Staves, 1997; Yamamoto and Kiss, 2002; Friedman *et*

170 *al.*, 2003; Hou *et al.*, 2003, 2004; Palmieri and Kiss, 2005; Mancuso *et al.*,
171 2006). In recent years, it is generally accepted that actin negatively
172 regulates root gravitropism (Blancaflor, 2013; Swarup *et al.*, 2013).
173 However, Blancaflor (2013) proposes a fine-tuning role of actin in root
174 gravitropism and argues that actin may positively regulate gravitropism as
175 breaking the actin connection will result in faster sedimentation of the
176 amyloplasts and hence more compressive forces on the cortical
177 endoplasmic reticulum, and thus enhanced gravitropic curvature.

178 The finding that some of the key proteins involved in the early stages
179 of gravity perception, such as ARG1 (Boonsirichai *et al.*, 2003) and the
180 pea homolog of the MAR2-part of the TOC complex, (Jouhet and Gray,
181 2009), can both bind to actin provides a direct link between statolith
182 sedimentation and the actin cytoskeleton. The finding that the chloroplast
183 outer envelope protein CHUP1 (CHLOROPLAST UNUSUAL
184 POSITIONING1) binds to actin (Oikawa *et al.*, 2003; Schmidt von Braun
185 and Schleiff, 2008) further supports this link. If similar proteins could be
186 present in the outer amyloplast membrane as well they could potentially
187 act as ligand for some plasma membrane or endoplasmic reticulum
188 receptor for gravitropic signalling thus linking actin to gravitropic signalling.

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191 **2. Gravity signal transduction (Fig. 3)**

192

193 Gravity is perceived in the columella cells, but the actual graviresponse is
194 achieved in the root elongation zone. To explain this, Cholodny (1927)
195 and Went (1926) proposed their famous hypothesis that, following gravity
196 perception, a signal is generated and transduced from the sedimenting
197 amyloplasts to the cells in the root elongation zone, the actual site of the
198 graviresponse. Pharmacological, genetic and cell biology-based
199 approaches have shown that this signal is auxin. The use of auxin
200 response reporters DR5, IAA2 and auxin sensor DII 28 (Brunoud *et al.*,
201 2012) shows a differential accumulation of auxin between the lower and
202 upper side of a gravistimulated root (Ottenschläger *et al.*, 2003; Swarup *et al.*
203 *al.*, 2009; Band *et al.*, 2012a).

204 Indole-3-acetic acid (IAA), the major form of auxin in higher plants is a
205 weak acid and, at the intracellular pH, exists in its membrane
206 impermeable (IAA⁻) form. However, at the more acidic apoplastic pH, IAA

207 will exist in both IAAH (membrane permeable) and IAA⁻ forms. Auxin
208 moves from cell to cell in a directional manner and this polarity of auxin
209 movement is well explained by the chemiosmotic hypothesis (Rubery and
210 Sheldrake, 1974; Raven, 1975) that not only proposed the existence of
211 specialised auxin carriers but also hypothesised that the asymmetric
212 localisation of auxin transporters provides the basis for the directionality of
213 auxin movement. Genetic and cell biology approaches later confirmed
214 that auxin transport is carrier-mediated and facilitated by specific auxin
215 influx and efflux carriers (Bennett *et al.*, 1996, Gälweiler *et al.*, 1998;
216 Swarup *et al.*, 2014).

217 The auxin influx carriers are encoded by a small gene family comprised of
218 four members (AUX1 and Like AUX1 or LAX genes). AUX1 is expressed
219 in columella, lateral root cap and epidermal cells, tissues that are involved
220 in gravity perception, transmission and response, respectively (Swarup *et al.*
221 *et al.*, 2001, 2004, 2005; 2014). Mutation in the AUX1 gene results in
222 severely agravitropic roots, pointing to the importance of AUX1 in root
223 gravitropism (Bennett *et al.*, 1996, Swarup and Peret, 2012; Swarup *et al.*,
224 2014). Using a transactivation-based approach, Swarup *et al.* (2005)
225 mapped the tissues required to transport auxin during a root gravitropic
226 response. They showed that auxin moves via the lateral root cap cells to
227 the cells in the elongation zone, which can serve as direct evidence for
228 auxin being the primary gravitropic signal and linking the site of gravity
229 perception to the graviresponse.

230 Though AUX1 is important for root gravitropism, the directionality of auxin
231 movement is provided by asymmetric localizations of PINFORMED (PIN)
232 family of auxin efflux carriers (Grunewald and Friml, 2010). Several efflux
233 carrier proteins have been localized in the root apex (Grunewald and
234 Friml, 2010; Swarup *et al.*, 2104). From these, PIN3, PIN4 and PIN7 are
235 localized in the columella cells, whereas PIN2 is expressed in the lateral
236 root cap, epidermal and cortical cells (Müller *et al.*, 1998; Luschnig *et al.*,
237 1998; Friml *et al.*, 2002a,b). Friml *et al.* (2002b) showed that upon a
238 gravitropic stimulus PIN3 is relocated **within 2 minutes** to the lateral face
239 of the columella cells, providing a very elegant mechanism for the creation
240 of an auxin gradient between the upper and the lower side of a
241 gravistimulated root (Friml *et al.*, 2000b). However, *pin3* mutants show
242 only a weak gravitropism defect (Friml *et al.*, 2002b). Kleine-Vehn *et al.*

243 (2010) showed that PIN7 is also asymmetrically localized in columella
244 cells in response to a gravity stimulus and *pin3pin7* double mutants are
245 significantly more agravitropic than *pin3* or *pin7* single mutants, strongly
246 suggesting some degree of redundancy. Recent studies, using a novel
247 auxin sensor, DII-VENUS in combination with a mathematical modelling
248 approach, indicate that auxin asymmetry between the lower and upper
249 side of the root can develop within minutes after a gravity stimulus (Band
250 *et al.*, 2012a). Thus, reorientation of PIN3 and PIN7 in the plasma
251 membrane of columella cells facilitates the establishment of a lateral auxin
252 gradient across the root cap upon gravistimulation. Once the auxin
253 asymmetry is initiated by PIN3 and PIN7, auxin is transported by
254 AUX1/PIN2 in a shootward direction through the lateral root cap to the
255 epidermal cells in the elongation zone.

256 The directionality of *this* auxin movement is provided by the asymmetric
257 localization of PIN2. In the lateral root cap, PIN2 is localized on the
258 shootward face of the cells, thus channeling auxin into the elongation
259 zone. Interestingly, in the distal elongation zone, PIN2 is localized on the
260 *shootward* face of epidermal cells but on the *rootward* face of the cortical
261 cells, which creates an auxin reflux loop that has been shown to be
262 important for root gravitropism (Blilou *et al.*, 2005).

263 Genetic and pharmacological approaches show that PIN abundance and
264 localization at the plasma membrane is carefully regulated and controlled.
265 Treatments with the vesicular trafficking inhibitor Brefeldin A (BFA) affect
266 PIN recycling and cause severe auxin-related developmental defects
267 (Geldner *et al.*, 2004). Treatment with trafficking modulator TENin1 (TE1)
268 causes accumulation of several plasma membrane proteins, including
269 PIN2, in pre-vacuolar compartments, which results in agravitropic roots
270 (Paudyal *et al.*, 2014). The small secretory peptide GOLVEN (GLV) has
271 also been shown to affect auxin transport by regulating the distribution of
272 PIN2 in the plasma membrane (Whitford *et al.*, 2012). Even auxin itself
273 appears to play a key role in regulating PIN2 plasma membrane
274 abundance. Abas *et al.* (2006) showed that auxin promotes its own efflux
275 by regulating PIN2 turnover. They observed an increased rate of PIN2
276 endocytosis and degradation on the upper side of a gravistimulated root
277 compared to the lower side. This differential accumulation of PIN2
278 between lower and upper sides of a gravistimulated root appears to be

279 crucial for maintaining the differential auxin asymmetry initiated by PIN3
280 as a single missense amino acid substitution in the *wav6-52* allele of PIN2
281 makes PIN2 resistant to degradation, resulting in agravitropic roots.
282 Besides auxin, gibberellic acid (GA) can also regulate the plasma
283 membrane distribution of PIN proteins. Löffke *et al.* (2013) showed that
284 high GA can result in more PIN proteins in the plasma membrane,
285 whereas low GA results in the internalization of PIN proteins.

286 In addition, it is well documented that posttranslational modifications, such
287 as (de)phosphorylation, also regulate polar PIN localization and therefore
288 are involved in the graviresponse. For example, the protein-
289 serine/threonine kinase PINOID (PID) regulates root gravitropism by
290 affecting PIN2 plasma membrane localisation (Sukumar *et al.*, 2009;
291 Huang *et al.*, 2010). Changes in the phosphorylation status of PIN3 has
292 also been reported to affect several PIN3-regulated processes, including
293 root gravitropism (Ganguly *et al.*, 2012). Besides, D6 family of protein
294 kinases (D6 PROTEIN KINASE; D6PK) may also regulate gravitropism by
295 regulating the phosphorylation status of PINs (Barbosa *et al.*, 2014).
296 D6PKs are membrane-bound protein kinases and auxin can promote
297 internalisation of D6PKs from the plasma membrane to the
298 endomembranes providing further evidence that abundance of PINs at the
299 plasma membrane is regulated by post-translational modifications such as
300 phosphorylation (Barbosa *et al.*, 2014).

301 Besides phosphorylation, several other pathways have also been
302 proposed to regulate PIN plasma membrane abundance. For example, a
303 ROP GTPase-dependent pathway has been implicated for PIN2.
304 Mutations in ROP6-GTPase, its effector RIC1 as well as a DHR2-Dock
305 family of Rho guanine nucleotide exchange factor SPIKE1 (SPK1) affect
306 auxin-mediated suppression of PIN2 internalisation and PIN2 plasma
307 membrane localization (Lin *et al.*, 2012). Furthermore, SPK1 is required
308 for auxin induction of ROP6 activation and a Rho GTPase-based auxin
309 signalling pathway regulates PIN2 plasma membrane abundance (Lin *et*
310 *al.*, 2012). There is some evidence suggesting a role for
311 PHOSPHOLIPASE D ζ 2 in regulating PIN2 cycling, as mutations in the
312 *PHOSPHOLIPASE D ζ 2* gene or treatment with PLD-inhibitor 1-butanol
313 affect PIN2 cycling and reduce root gravitropism (Li and Xue, 2007). In
314 addition, maintenance of polar distribution of PIN at the plasma

315 membrane also seems dependent on cellulose-based connections to the
316 cell wall (Feraru *et al.*, 2011).

317 In summary, these results clearly suggest that regulation of PIN trafficking
318 is crucial for fine-tuning auxin transport and creating auxin gradients.
319 However, despite the importance of PIN2 in providing directionality of
320 auxin transport during root gravitropism, *pin2* mutants are not severely
321 agravitropic (Chen *et al.*, 1998; Luschnig *et al.*, 1998; Blakeslee *et al.*,
322 2007). Two members of the p-glycoprotein (PGP) family of auxin efflux
323 transporters, AtPGP1 and AtPGP19, are also expressed in the root
324 elongation zone (Blakeslee *et al.*, 2007). Though single or double
325 *pgp1pgp19* mutants do not have a very strong agravitropic phenotype,
326 *pin2pgp1pgp19* triple mutants are severely agravitropic (Blakeslee *et al.*,
327 2007), suggesting that both PIN and PGP families of auxin efflux carriers
328 are required for root gravitropism.

329 In contrast to PIN proteins, which provide directionality to auxin
330 movement, AUX/LAX proteins appear to be important for maintaining the
331 auxin gradient and for contributing to the pattern of auxin distribution at
332 the root tip (Band *et al.*, 2014). Despite the importance of AUX1 in root
333 gravitropism, there has been a general misconception that because
334 protonated IAA is membrane permeable, influx carriers play only a
335 supplemental role. Using confocal microscopy and fluorescent pH
336 sensors, Monshausen *et al.* (2011) recently showed that there is an
337 increase in the surface pH on the lower side of gravistimulated wild-type
338 but not in *aux1* roots. One important implication of this finding is that the
339 increase in the root apoplastic pH will result in more IAA in its ionic IAA⁻
340 form. Since this form is not membrane permeable it will require a carrier
341 (AUX1)-mediated uptake. Computer simulation studies estimate that this
342 carrier-mediated IAA uptake is 15 times greater than diffusion (Swarup *et al.*,
343 2005; Kramer and Bennett, 2006). These studies suggested that
344 epidermal-expressed AUX1 along with PIN2 minimize the effect of radial
345 diffusion while facilitating shootward auxin transport (Swarup *et al.*, 2005).

346

347 **3. Gravitropic response (Fig. 4)**

348 Genetic and pharmacological evidence suggest that the gravitropic
349 response takes place in the elongation zone. Using a transactivation-
350 based approach, Swarup *et al.* (2005) showed that the root epidermis
351 represents the primary site of the gravity response, since expression of a
352 dominant auxin response repressor *axr3*, blocking the auxin response
353 specifically in the epidermal cells, resulted in the loss of gravitropic
354 bending. This crucial role of epidermal cells is further supported by
355 computer simulation studies that indicated that a lateral auxin gradient
356 accumulates 10 to 20 fold more in the epidermis than in the underlying
357 tissues (Swarup *et al.*, 2005). Their work clearly established the
358 importance of auxin as a primary gravitropic signal that acts directly in the
359 expanding cells in the elongation zone.

360 Though the mechanistic aspects of root gravitropism are not very well
361 understood, it has been observed that auxin somehow activates cytosolic
362 Ca^{2+} waves, which in turn promotes differential apoplastic pH changes at
363 both sides of the gravistimulated root. The Ca^{2+} -dependent acidification of
364 the cell wall can increase the epidermal cell elongation rate in the upper
365 side of the root, while alkalinisation of the apoplast then reduces cell
366 elongation rate at the lower side (Ishikawa and Evans, 1993; Mullen *et al.*,
367 1998). This leads to root curvature that becomes evident **10 minutes** after
368 the onset of the gravistimulus. Because of the rapidity of the gravitropic
369 response, it has been suggested that the initial phase of the gravitropic
370 response cannot rely on newly formed proteins and therefore must be
371 non-genomic (Swarup *et al.*, 2012). Using high-resolution confocal
372 imaging, Monshausen *et al.* (2011) observed pH changes at the surface
373 of the root elongation zone cells within minutes after a gravistimulus.
374 These changes in surface pH were AUX1-dependent, but they were also
375 seen in single or multiple auxin receptor mutant backgrounds, supporting
376 a non-genomic response during early stages of root gravitropism. Gene
377 expression studies showed that peak transcript levels of several auxin-
378 inducible genes such as *IAA1*, *IAA2* and *ARF19* is seen only **15 minutes**
379 after a gravistimulus (Band *et al.*, 2012a; Brunoud *et al.*, 2012), which is
380 after the first visible signs of actual bending. Knowing that genes down-
381 stream of these early auxin-responsive genes are expressed even later,
382 this timing would also support a role for an initial non-genomic phase
383 preceding the genomic response phase. Though at present it is not well

384 understood how a non-genomic auxin response can be achieved in the
385 early stages of gravitropism, involvement of ABP1 (Auxin Binding
386 Protein1) appears to be promising. The role of ABP1 has long been a
387 subject of great debate, but in recent years it is emerging that it may play
388 a crucial role in auxin signalling. Robert *et al.* (2010) showed that ABP1
389 promotes differential growth by suppressing auxin-sensitive clathrin-
390 mediated endocytosis. Though genetic evidence for a role of ABP1 is
391 weak and complicated by the fact that ABP1 knock outs are embryo-
392 lethal, *abp1* heterozygote mutants are reported to be agravitropic (Effendi
393 *et al.*, 2011).

394 Besides auxin there are several other signals, such as Ca^{2+} , inositol 1,4,5-
395 triphosphate (InsP_3), NO and reactive oxygen species (ROS) that have all
396 been implicated in root gravitropism, and these may also be involved in
397 the initial non-genomic phase of root gravitropism. For example,
398 asymmetric Ca^{2+} gradients have been reported in gravistimulated roots
399 (Lee *et al.*, 1984) and the application of Ca^{2+} chelators results in a loss of
400 gravitropic sensitivity (Lee *et al.*, 1983). Using an elegant cell biological
401 approach, Monshausen *et al.* (2011) showed that application of auxin at
402 the root apex results in a shootward wave of Ca^{2+} that is not observed
403 when treated with Ca^{2+} channel blockers. They proposed that the auxin-
404 mediated increase in cytosolic Ca^{2+} could be due to activation of a Ca^{2+}
405 channel that in turn activates a plasma membrane H^+/OH^- conductance.
406 This would then result in alkalinisation of the apoplast. Changes in cell
407 wall pH have been implicated in cell expansion (Cosgrove, 1998, 2000),
408 perhaps by impacting cell wall integrity and affecting intermolecular
409 crosslinks (Brady and Fry, 1997) by cell wall remodelling proteins, such as
410 expansins and xyloglucan endotransglucosylase/hydrolases (XTHs),
411 whose actions are pH-dependent (Cosgrove, 2005; Nishitani and
412 Vissenberg, 2007; Maris *et al.*, 2009, 2011).

413 Treatment with U73122, an inhibitor of InsP_3 biosynthesis, results in
414 an attenuated gravity response (Andreeva *et al.*, 2010), indicating the
415 involvement of InsP_3 in regulating root gravitropism. Moreover,
416 concentrations of InsP_3 have been observed to oscillate and then to
417 increase in the lower side of oat, maize and *Arabidopsis thaliana* stems
418 following gravistimulation (Perera *et al.*, 1999, 2001, 2006), suggesting
419 that a similar mechanism could be active in the root.

420 Nitric oxide (NO) may also play a role in root gravitropism since it has
421 been shown to accumulate on the lower side of a gravistimulated root (Hu
422 *et al.*, 2005), which may interfere with auxin transport (Fernández-Marcos
423 *et al.*, 2011). This differential accumulation can be blocked by treatment
424 with the auxin transport inhibitor NPA, but is restored with asymmetric
425 application of auxin. Though it is not well understood how NO could
426 participate in root gravitropism, it appears that NO signalling is mediated
427 via cGMP (Hu *et al.*, 2005). It has been further suggested that NO can
428 either directly modify key proteins involved in root gravitropism via
429 nitrosylation (Durner and Klessig, 1999; Terrile *et al.*, 2012) or by
430 elevating cGMP levels (Durner *et al.*, 1998).

431 Like NO, reactive oxygen species (ROS) accumulate at the lower side
432 of gravistimulated roots (Joo *et al.*, 2001, 2005), but unlike NO, ROS
433 seem to act downstream of auxin as application of hydrogen peroxide
434 induces curvature even in roots treated with the auxin transport inhibitor
435 NPA (Joo *et al.*, 2005). De Cnodder *et al.* (2005) have reported that ROS
436 can mediate cross-linking of structural cell wall proteins during ACC-
437 induced cell elongation arrest of *Arabidopsis thaliana* root epidermal cells.
438 It is therefore tempting to speculate that similar differential cross-linking
439 events could influence cell elongation rates at the lower and upper side of
440 the root during root gravitropism.

441 In contrast to our limited understanding of the non-genomic phase of root
442 gravitropism, the genomic phase is much better understood. Mutations in
443 several auxin signalling components result in agravitropic roots and have
444 led to the identification of several key molecular players. In the cell, auxin
445 is perceived by its receptor TIR1/AFB family of F-box proteins that then
446 facilitates degradation of auxin repressor AUX/IAA proteins via the
447 ubiquitin proteasome pathway (Gray *et al.*, 2001). As a result, auxin
448 response factors (ARFs) are de-repressed and initiate expression of
449 auxin-regulated genes. In *Arabidopsis thaliana*, AUX/IAAs and ARFs are
450 part of large gene families with 29 and 23 members, respectively, and the
451 output of the TIR1/AFB pathway is dependent on the dynamic equilibrium
452 of the abundance of these two classes of proteins. Genetic studies
453 suggest that among all ARFs, ARF7 and ARF19 appear to be most
454 important for root gravitropism, and AXR2, AXR3, SLR1 and SHY2 are
455 the key AUX/IAA proteins that mediate root gravitropism. The genomic

456 auxin response likely involves the differential expression of genes at the
457 upper and lower side of the root and transcriptome analysis suggested
458 that several cell wall-related genes are under ARF7 and ARF19 regulation
459 in expanding root epidermal cells, including AtXTH18 and AtXTH19. The
460 fact that AtXTH18 and AtXTH19 encode cell wall enzymes that modify
461 hemicellulosic tethers between adjacent cellulose microfibrils, and
462 therefore can help to regulate expansion, led Swarup *et al.* (2013) to
463 speculate that '*initially through its non-genomic effect, auxin brings about*
464 *rapid differential cell expansion, whilst later on auxin regulates cell wall*
465 *biosynthesis and remodelling through its genomic effects*'.

466

467 **4. Restoration of the symmetrical auxin flow (Fig. 5)**

468 Recent studies provide some insight into important questions such as how
469 long the PIN3/PIN7 -facilitated auxin asymmetry is maintained, whether it
470 lasts throughout the whole bending period and how the normal auxin
471 gradient is restored. Using the DII-Venus auxin sensor (Brunoud *et al.*,
472 2012) in combination with mathematical modelling studies, Band *et al.*
473 (2012a) suggested that the auxin asymmetry persists for about 100
474 minutes or roughly up to the point when the root tip angle reaches 40°.
475 Cell biology studies showed that at this angle, because of the specific
476 morphology of the central columella cells, the statoliths reposition to the
477 new physiological bottom of the cell. This led Band *et al.* (2012a) to
478 propose a tipping point mechanism to explain restoration of auxin
479 symmetry. Statoliths reposition in the columella cells when the root tip
480 reaches 40°. As a consequence this triggers the restoration of PIN3/PIN7
481 location, and hence, auxin flow is no longer asymmetric.

482

483 **Open questions and future challenges**

484 Regardless of the major success in uncovering major regulatory
485 physiological and genomic processes and factors, there remain several
486 open questions regulating various stages of gravitropism:

487 1) Little is known about the nature of the gravity receptors controlling
488 gravity perception. Where are they located? Are there more than one kind

489 of gravity receptors? The role of mechanosensitive ion channels such as
490 MCA1, MCA2, MS9 and MSL10 is almost ruled out in root gravitropism
491 (Nakagawa *et al.*, 2007; Haswell *et al.*, 2008) unless plant specific novel
492 mechanosensitive or gravity-related ion channels are identified. On the
493 other hand, the receptor ligand-based hypothesis is sketchy and needs
494 further investigation. Though the role of auxin in regulating root
495 gravitropism is well established, any possible role of other phytohormones
496 during root gravitropism is not very clear and requires further
497 investigation. As mentioned earlier, GA has been shown to regulate the
498 plasma membrane distribution of PIN proteins (Löpfke *et al.*, 2013).
499 Further understanding of GA-regulated PIN localization will provide
500 greater insight into the role of GA in regulating root gravitropism.

501
502 2) With respect to the gravity signal transduction, current cell-live imaging
503 technologies are not yet fully optimized to monitor intracellular changes of
504 all signalling molecules *in vivo*. Computer-based systems coupled to
505 confocal laser scanning microscopy (CSLM) and the use of fluorescent
506 dyes have permitted the visualization of either plant organelles or
507 physiological parameters as: Ca²⁺, pH, metal ions, etc. (Pollastri *et al.*,
508 2012). However, for this group of molecules, the quantification and
509 abundance under a particular developmental or physiological process can
510 be problematic. Hence, development of sensors that can precisely
511 monitor very small or transient concentration changes in Ca²⁺, pH and
512 also other signalling molecules regulating root gravitropism (InsP₃, nitric
513 oxide, ROS) coupled to new detection methods, such as light sheet or
514 Selective Plane Illumination Microscopy (SPIM) (Costa *et al.*, 2013), are
515 very promising. Ideally, their simultaneous visualization and quantification
516 will shed light on how these signalling molecules interact or counteract
517 with one another upon gravistimulation. A spatio-temporal map of their
518 action would be very informative not only for plant tropisms, but also for
519 other biological processes.

520 3) While transcriptomic approaches have proven very valuable to shed
521 light on later events of gravitropism (Zhu *et al.*, 2002; Kimbrough *et al.*,
522 2004), due to the speed of the gravitropic response, proteomics or
523 chemical biology approaches are likely to be more useful tools to identify
524 novel regulators during early stages of gravitropism (Yamazoe *et al.*,

525 2005; Young *et al.*, 2006; Na *et al.*, 2011). Also downstream events after
526 the development of the auxin asymmetry remain unclear. How is
527 differential growth regulated? Both the intricate complexity of the cell wall
528 and the functional redundancy of the diverse cell wall remodelling
529 enzymes make the identification of key cell wall-related enzymes in
530 regulating differential growth challenging. Use of solid-state ¹³C NMR,
531 FTIR spectroscopy and Raman microscopy promises to provide a better
532 picture of the composition of the cell wall and will allow comparison of the
533 upper and the lower flank of plant roots.

534 4) Auxin symmetry is restored when the root tip angle reaches 40°, but
535 the question remains as to how the remaining bending is regulated. In
536 addition, the apex of lateral roots should respond to gravity as primary
537 roots. Yet, especially at early stages, lateral roots grow at a certain angle
538 to the primary root (Fig. 6). Whether the gravisensing machinery is
539 working and how this might be overruled remains open for debate.

540 5) In recent years mathematical modelling approaches have started to
541 provide more quantitative insight into root gravitropism (French *et al.*,
542 2009; Band *et al.*, 2012a, 2014). These approaches are enabling
543 researchers to develop experimentally testable hypothesis providing
544 deeper insight into root gravitropism (Swarup *et al.*, 2005; Laskowski *et al.*
545 *et al.*, 2008; Kondrachuk *et al.*, 2011; Band *et al.*, 2014). Since the
546 gravitropic response is an interplay between perception, transmission and
547 actual response, and involves a wide variety of key players (from ions to
548 proteins, hormones and cell wall-related molecules), the next big
549 challenge is to integrate all these factors in mathematical models and
550 develop mathematically sound and experimentally testable hypothesis.

551 Addressing these issues will be challenging but recent advances in
552 genetic, genomic and imaging technologies in combination with a systems
553 biology approach and improved mathematical models are likely to provide
554 a global more holistic understanding of gravitropism at the molecular,
555 cellular and tissue level.

556

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Figure legends

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Figure 1. Gravitropic bending of an *Arabidopsis thaliana* root

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Sequential imaging of a bending root with color-codes at different

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timepoints.

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Figure 2. Gravity perception in *Arabidopsis thaliana*

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At time point 0, roots grow vertically. After a 90° turn, the following series of events take place:

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(1) At 10 seconds, statoliths are still at the old bottom of the cell. After 3 minutes, statoliths move towards the new bottom of the cell to be uniformly distributed at 5 minutes (Leitz *et al.*, 2009)

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(2) PIN3 and PIN7 relocation is achieved 2 minutes after the gravity stimulus and in consequence, a lateral auxin gradient is generated between the upper and lower side of the root (thin and thick orange arrows respectively) (Friml *et al.*, 2002b).

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(3) Development of differential extracellular pH levels between the upper (acidic) and lower (alkaline) side of the gravistimulated root (Monshausen *et al.*, 2011).

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Figure 3. Gravity signal transduction and transmission

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Auxin transport and redistribution upon a gravity stimulus

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AUX1 and PIN2 channel auxin from the shoot to the root tip (black arrows). Auxin efflux is distributed through the vascular tissue to the columella cells by PIN4 (blue arrows). PIN3 and PIN7 set up the auxin flow (green arrows), with an accumulation on the lower side of the root. PIN2 and AUX1 transport auxin through the lateral root cap to the epidermal cells in the elongation zone (orange arrows) where the actual growth response will occur.

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Figure 4. Gravity response

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Several molecules, such as pH, Ca²⁺, reactive oxygen species (ROS), nitric oxide (NO) and inositol 1,4,5-triphosphate (InsP3), serve as signalling molecules in the non-genomic phase of root bending. Following

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994 this initial phase, a change in the expression of auxin-regulated genes is
995 seen within 15 minutes.

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998 **Figure 5.** Restoration of the symmetrical auxin flow and of vertical growth

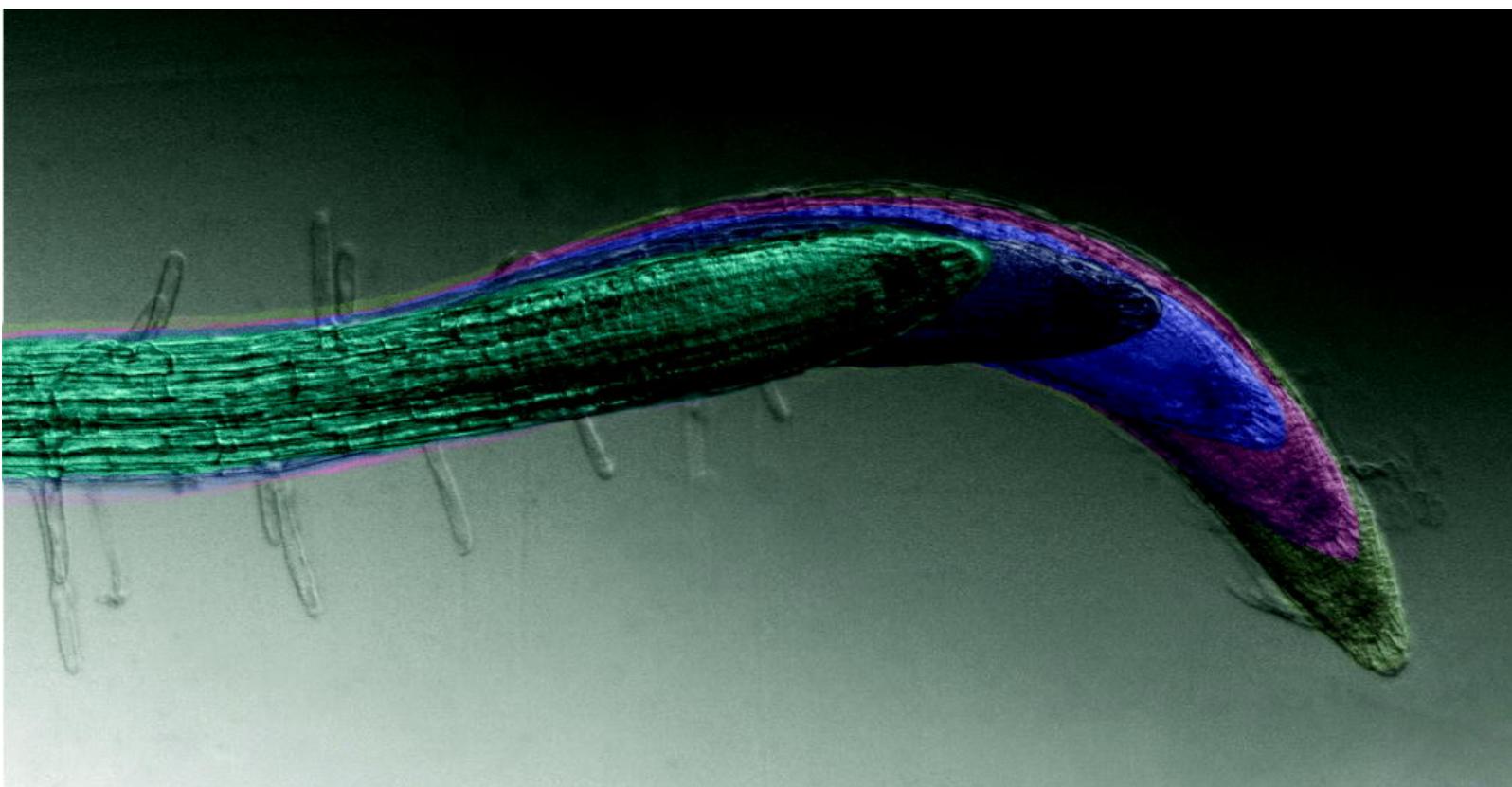
999 When the root reaches 40° (about 100 minutes after the initial
1000 gravistimulus), the auxin symmetry is restored (Band *et al.*, 2012a) and
1001 the root continues to bend until it ultimately regains growth along the
1002 gravity vector.

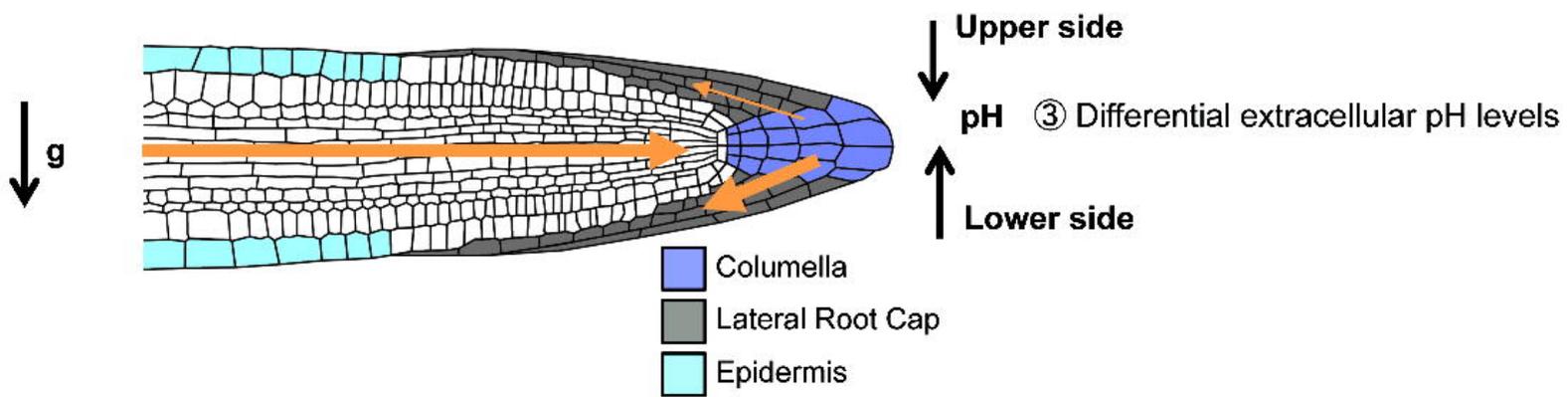
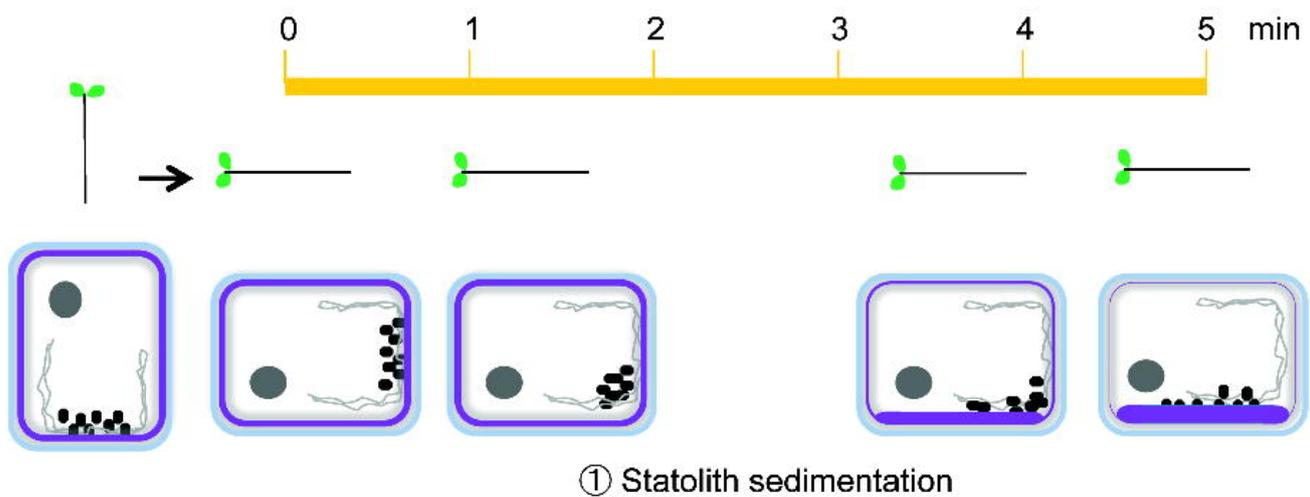
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1004 **Figure 6.** *Arabidopsis thaliana* root system

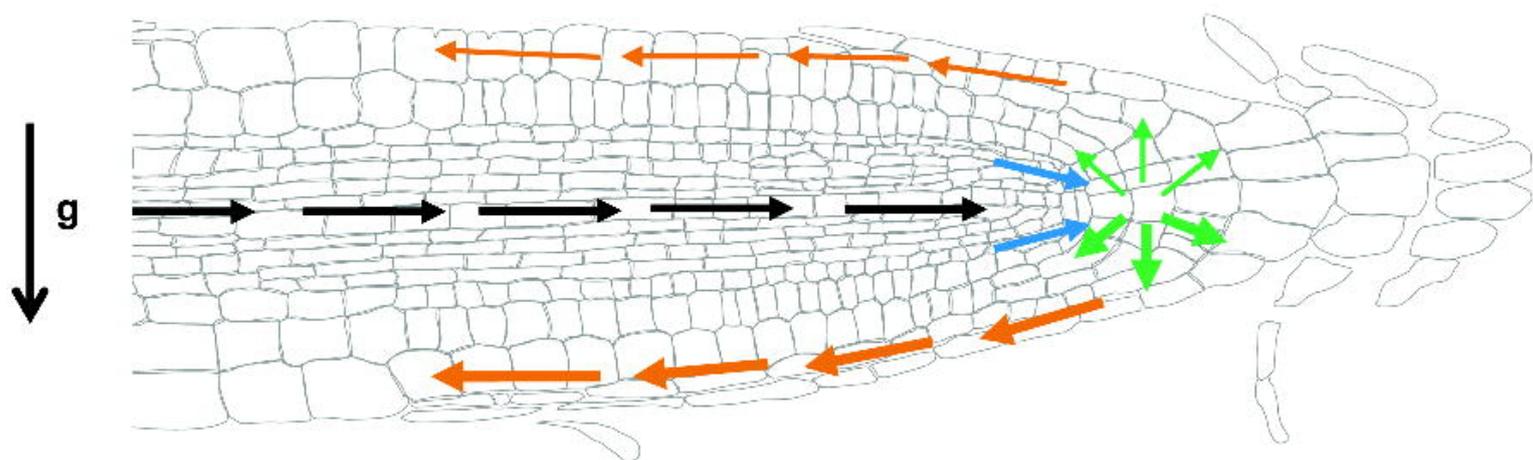
1005 Overview of the *Arabidopsis thaliana* root system redrawn from a 7-day-
1006 old seedling, showing the orientation of primary and lateral roots.

1007





② PIN3 relocation & lateral auxin gradient formation





Root gravitropism

Non-genomic phase

Genomic phase

- Signalling molecules**
- pH
 - Ca²⁺
 - ROS
 - NO
 - InsP3

