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Auxin fluxes in the root apex co-regulate gravitropism and lateral root initiation

Running title: Co-regulation of root gravitropism and branching by auxin transport

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6 figures

6 supplementary figures

ABSTRACT

Root architecture plays an important role in water and nutrient acquisition and in the ability of the plant to adapt to the soil. Lateral root development is the main determinant of the shape of the root system and is controlled by external factors such as nutrient concentration. Here we show that lateral root initiation and root gravitropism, two processes that are regulated by auxin, are co-regulated in *Arabidopsis*. We generated a mathematical model that can predict the effects of gravistimulations on lateral root initiation density and suggests that lateral root initiation is controlled by an inhibitory fields mechanism. Moreover, gene transactivation experiments suggest a mechanism involving a single auxin transport route for both responses. Finally, co-regulation may offer a selective advantage by optimising soil exploration as supported by a simple quantitative analysis.

Keywords: AUX1, auxin transport, AXR3, GAL4, pericycle, root meristem.

INTRODUCTION

Exploration and exploitation of soil resources by plants depend on the development of the root system. Lateral root formation, which occurs throughout the life of the plant, is a main determinant of the shape of the root system and of its ability to adapt to a heterogeneous and changing environment (Malamy, 2005; Hodge, 2006).

The events leading to lateral root formation have been well described in *Arabidopsis thaliana* (Casimiro *et al.*, 2003; De Smet *et al.*, 2006). Lateral root development starts with asymmetric cell divisions in two adjacent pericycle cells, a process referred to as lateral root initiation (Malamy and Benfey, 1997; Dubrovsky *et al.*, 2000; De Smet *et al.*, 2006). Only pericycle cells that are in contact with the xylem poles are competent for lateral root initiation (Dubrovsky *et al.*, 2001). Lateral root formation takes place according to an acropetal gradient with lateral root initiation occurring in the differentiation zone of the root close to the root apex (Dubrovsky *et al.*, 2000, 2006; De Smet *et al.*, 2006). Subsequently, initiation can no longer occur between existing primordia (Dubrovsky *et al.*, 2006). In addition, lateral root initiation has a strong tendency toward alternation between the two xylem poles (Dubrovsky *et al.*, 2006). After initiation, the lateral root primordium goes through a series of well-characterised cell divisions that give rise to a root meristem (Malamy and Benfey, 1997; Casimiro *et al.*, 2003). The lateral root primordium then emerges from the parent root mostly by cell elongation (Malamy and Benfey, 1997).

Little is known about the mechanisms that control root branching. However, it is known that lateral root initiation, the establishment of the meristem, and lateral

root emergence are regulated independently. The plant hormone auxin plays a central role in lateral root development. It is the key signal that controls lateral root initiation (Casimiro *et al.*, 2003; De Smet *et al.*, 2006). Auxin is also involved in the growth and organisation of lateral root primordia (Benková *et al.*, 2003; Casimiro *et al.*, 2003) and in the emergence of lateral roots from the parent root (Laskowski *et al.*, 2006).

This work is part of a project combining mathematical and *in silico* modelling with experimental biology to better understand the mechanisms of root branching in *Arabidopsis*. Since lateral root initiation in *Arabidopsis* only occurs close to the root tip and since auxin is the key signal that controls this process, we decided to focus our efforts on auxin fluxes in the root apex. Auxin fluxes have already been studied in the apical root meristem (Blilou *et al.*, 2005) but little is known about the fluxes that are responsible for lateral root initiation. Interestingly, data suggests a link between root waving, which depends on gravitropism/thigmotropism, and lateral root initiation (De Smet *et al.*, 2007; Fortin *et al.*, 1989). Reorientation of primary root growth according to the gravity vector (gravitropism) depends on auxin fluxes in the root apical meristem, which have already been well described (Ottenschläger *et al.*, 2003; Swarup *et al.*, 2005).

Here we show that a gravistimulus quickly leads to lateral root initiation at the site of reorientation of root growth. We used gravistimulation to analyse the pattern of lateral root initiation. Our results indicate that lateral root initiation is rather plastic and that it is not strictly controlled by an internal rhythm. However, the existence of a minimum and a maximum time between two successive lateral root initiations demonstrate that there is a form of endogenous

control. We used our data to generate a mathematical model that can predict the effects of gravistimulations on lateral root initiation density. Moreover, we observed that the auxin flux responsible for lateral root initiation goes through the same route as the auxin responsible for gravistropism thus explaining the co-regulation of these two processes. Finally, mathematical modelling suggests that the co-regulation of root bending and branching optimize soil exploration by the root system.

MATERIAL AND METHODS

Plant Material and Growth

Wild type (Col-0) seeds were obtained from the NASC. *Pro$_{CYCB1}$:GUS* (Col-0 background) seeds were provided by Dr P. Doerner (University of Edinburgh, UK). J0951, M0013, UAS-axr3 lines in wild-type (Col-0) background and J0951, M0013, UAS-AUX1 lines in *aux1-22* mutant background were kindly provided by Dr. R. Swarup (University of Nottingham, UK). Plants were grown on vertical plates as previously described (Laplaze *et al.*, 2005). Plates were then subjected to 90° gravistimulations. For additional details on the periodical gravistimulation, see Figure S1. All gravistimulation and transactivation experiments were repeated twice independently.

Root lengths were measured from scans of the roots with the UTHSCSA ImageTool open-source software, available at http://ddsdx.uthscsa.edu/dig/itdesc.html. Lateral root development stages were

scored using an optical microscope according to Malamy and Benfey (1997). Data were analysed using the Excel statistical package.

Microscopy

Seedlings were collected and incubated in a solution containing 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$, 0.05% (v/v) Triton X-100, 0.05% (v/v) DMF, 0.02% (v/v) EDTA, and 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid and incubated at 37°C for several hours. Seedlings were then cleared in 70% (v/v) ethanol for 24 hours, before being immersed for 2 hours in 10% (v/v) glycerol 50% (v/v) ethanol; 2h in 30% (v/v) glycerol 30% (v/v) ethanol; 2h in 50% (v/v) glycerol. Seedlings were mounted in 50% (v/v) glycerol and visualised using a DMRB microscope (Leica).

Design of a mechanistic model of lateral root initiation

The mechanistic model of lateral root initiation we introduced (Fig. 3A) was formalized and transcribed in the python programming language as a logical algorithm (Fig S4). Parameter T1 (spontaneous initiation threshold) was estimated directly from the observed data as the mean time between two successive initiations in the control. The two other parameters T2 (induced initiation threshold) and G (cost of gravistimulation) were inferred from observed data, using Python-scripts to explore the parameters-space. Over 1800 parameter combinations of T2 and G were tested. The parameter combination corresponding to the best fit of lateral root initiation densities to the observed

values was selected for subsequent model prediction. The Python stand-alone module is available from the authors.

Lateral dissymmetry of soil exploitation along the primary root

As primary roots do not grow straight, successive bends induce geometric dissymmetry between the inner and outer parts of a root turn. We quantified the effect of such dissymmetry in terms of the availability of local resources using simple mathematical modelling. As *Arabidopsis thaliana* lateral root initiation takes place in a plane defined by the two protoxylem strands, this analysis was made in a 2-dimensional space. In addition, we made a number of simplification hypotheses. The number of root hairs (n) is considered equal between each side of a root turn. As a consequence, due to the differential growth of epidermal cells under gravistimulation, the density of root hairs on the external side is lower than on the internal side (Fig. 5B). We consider that each root hair harvests a fixed pool of resource (a) and that resources diffuse passively in the soil (i.e. resources flow toward the root as they become locally depleted). According to these assumptions, overall soil exploitation, defined as the volume of resources harvested per time unit, is equivalent on both sides of the root. Working in a 2-dimensionnal space, here we consider the corresponding exploited surfaces, s_1 on the inner side and s_2 on the outer side (Fig. 5C). We also assume that each root turn corresponds to a portion of a circle of radius ρ. Considering an infinitesimal portion of root turn defined by the angle $d\alpha$, soil exploitation takes place over dl_1 (inner side) and dl_2 (outer side; Fig. 5B). If (b) represents the thickness of the root (assumed to be constant in the zone concerned), we have:

(1) $dl_1 = (\rho - b / 2) \, . \, d\alpha$

(2) $dl_2 = (\rho + b / 2) \, . \, d\alpha$

The surface of soil exploited on each side can be written as:

(3) $s_1 \propto dl_1 \, . \, h_1$

(4) $s_2 \propto dl_2 \, . \, h_2$

where $\propto$ stands for proportional and h_1 and h_2 are the respective depth of exploitation on each side (not to be confounded with root hair length – see Fig. 5C). Under our hypotheses, these surfaces are proportional to the number of root hairs (n) and their harvesting power (a). These parameters being the same on each side of the root, we have:

(5) $s_1 = s_2 = s \propto n \, . \, a$

From (3), (4) and (5) we obtain:

(6) $dl_1 \, . \, h_1 = dl_2 \, . \, h_2$

And from (1), (2) and (6) we get:

(7) $h_1 / h_2 = (\rho + b / 2) / (\rho - b / 2)$

This equation gives the ratio between the depth of exploration on each side as a function of ρ (Fig. S6A). If ρ tends toward infinity, i.e. the root becomes completly straight (infinite curve radius), then the ratio h_1/h_2 tends to 1. This corresponds to an equal depth of exploration on each side of straight roots. By contrast, if ρ tends to ($b/2$), h1 becomes much greater than h2. The equation (7) is not valid for ρ inferior to ($b/2$) as this is a biological impossibility (root turn with an inner side of negative length dl_1).

Using pictures of gravistimulated root turn and waving roots, we were able to estimate various values for ρ (Fig. S6B, C). These values correspond to a ratio h_1/h_2 varying between 1.4 and 3 (Fig. S6D). Extrapolating these results to the

whole root and in three dimensions leads to an asymmetric profile of soil exploration (Fig. 5A, D) and corresponding resource depletion (Fig. 5E).

Root hair length analysis

Wild type (Col-0) seeds were grown on vertical plates as previously described (Laplaze *et al.*, 2005). Plates were then subjected to three 90° gravistimulations at 12h time intervals, starting 30h after germination. Pictures of the plants were obtained using a MZFLIII (Leica) dissecting microscope equipped with a digital camera. Root hair length was measured with the UTHSCSA ImageTool open-source software. Data were analysed using the Excel statistical package.

RESULTS

Gravistimulation leads to local lateral root initiation

Recent studies indicate that lateral root formation is correlated with root waving in an AUX1-dependent way (De Smet *et al.*, 2007). In order to test whether gravitropism and lateral root initiation are co-regulated, we tested the effect of gravistimuli on lateral root initiation. Transgenic *Arabidopsis* plants carrying a Pro_{CYCB1}*:GUS* marker for cell division were grown on vertical plates for 30 hours after germination and then subjected to a gravistimulus (90° rotation) every 12h for 3.5 days. Two different patterns of gravistimulation were used leading to stair- or crenel-shaped root growth (see Fig. S1). Plants were then left to grow for an extra 60h before testing for GUS activity. Roots were then cleared and

lateral root initiation, i.e. the presence of a lateral root primordium from the first asymmetric cell divisions in the pericycle (stage I) on, was scored under a light microscope.

We observed that lateral root initiation occurred in more than 90% of the gravistimulated zones where the root apex was reorientating its growth toward the new gravity vector (thereafter called turns; Fig. 1A, B). By contrast, only a limited number of lateral root initiations were observed between turns (<10%; Fig. 1A, B). This cannot be explained by the relative length of the gravistimulated zone versus the non-gravistimulated zone because the straight (non-gravistimulated) zone was longer than the curved (gravistimulated) zone (data not shown). Moreover, we observed that it took four hours in our growth conditions for all root apexes to reorient their growth direction after a 90° gravistimulus (data not shown) in agreement with previous studies (Swarup *et al.*, 2005). In our experiment, we therefore had about four hours of gravistimulated growth followed by about eight hours of non-gravistimulated root growth. If lateral root initiation occurs randomly or regularly, we would expect about 2/3 of the LRP to occur in the non-gravistimulated zone. We therefore conclude that lateral root initiation is induced in response to gravitropic root bending.

We next analysed the timing of lateral root initiation following a gravistimulus. Six batches of Pro_{CYCB1}*:GUS* plants were grown for 30h after germination on vertical Petri dishes then subjected to a 90° gravistimulus every six hours with a one hour delay between each batch. This was done for 24h and plants were then harvested and stained for GUS activity. This enabled us to observe gravistimulated zones every hour from 0 to 25h after stimulation. The

occurrence and stage of development of lateral root primordia at root turns were scored (Malamy and Benfey, 1997). The first occurrence of stage I lateral root primordia was found seven hours after gravistimulation (Fig. 1C). All the gravistimulated zones showed lateral root initiation 13 hours after gravistimulation (Fig. 1C). Stage II and III of lateral root development occurred six and 12 hours after lateral root initiation respectively (Fig. 1D). Our data therefore indicate that lateral root initiation occurs rapidly after gravistimulation.

We observed that lateral root primordia always appeared on the external side of the bend (100%, n=2677 LRP observed; Fig. S2). Previous studies observed a left-right alternation of lateral root formation (De Smet *et al.*, 2007). This was indeed the case in stair-shaped roots. In contrast, the crenel-shaped roots had two initiations on one side followed by two initiations on the other side (Fig. S2). In this case we observed that lateral root initiation occurred twice along the same protoxylem pole (data not shown). This indicates that lateral root initiation is not constrained to a left-right alternation but that lateral root primordia always appear on the external part of a gravistimulus-initiated root bend. This is in agreement with previous results showing that emerged lateral roots occur preferentially on the convex side of a curved root (Fortin *et al.*, 1989).

The rhythm of lateral root initiation is modified by external clues

We showed that lateral root initiation can be initiated by gravistimuli applied every 12 hours. Studies by De Smet et al. (2007) suggest that lateral root initiation sites are predetermined by an endogenous rhythm with a period of about 15 hours. In order to test whether lateral root initiation was strictly

controlled by an internal rhythm, we used the experimental design previously described applying gravistimuli every 1, 3, 6, 12 or 24 hours (Fig. 2A, Fig. S3A). Similar results were obtained for stair- and crenel-shaped roots (Fig. 2 and Fig. S3 respectively). For periods of 6, 12 and 24 hours between gravistimuli, lateral root initiation was found in more than 90% of root turns (gravistimulated zones; Fig. 2B). This value was reduced to about 50% for roots gravistimulated every three hours (Fig. 2B). For roots stimulated every hour, the roots did not have enough time to reorientate their growth and we were therefore unable to measure the percentage of turns showing lateral root initiation. Lateral root initiation occurred between turns only in roots subjected to gravistimulation at 12 hour (less than 10%) or 24h intervals (more than 35%; Fig. 2C). This confirms that lateral root initiation is induced by gravistimulation independently of the period between stimulations. As previously observed, lateral root primordia always formed on the external part of the bend.

We then determined the effect of the gravistimuli on the density of lateral root initiation. We first observed that gravistimuli had no significant effect on the growth of the primary root (Fig. 2D). Moreover within gravistimulated roots the gravistimulated and non-gravistimulated segments displayed similar root growth (Fig. 2D). We then observed that gravistimulation changed lateral root density with an optimum for gravistimulation at 6-hour intervals (Fig. 2E). Taken together our results indicate that lateral root initiation is not strictly controlled by an internal biological rhythm and that the rhythm of lateral root initiation can vary according to environmental clues such as gravity or touch. However, we also show that, in our experimental conditions, two subsequent lateral root initiations cannot occur at too short intervals. Indeed, when the interval between

two successive gravistimulations was equal to or less than three hours, the percentage of turns with LRI dropped and LRP density returned to non stimulated level. Moreover, our data also suggest that, on the contrary, two lateral root initiations cannot be separated by too long a time interval. Accordingly, lateral root initiations between turns increased with the time between gravistimulations and LRP density cannot be reduced below a minimal level that is close to non-stimulation conditions. We conclude from our experiments and previous data (De Smet *et al.*, 2007) that there is an endogenous regulatory system controlling lateral root initiation that is responsible for regular lateral root initiation in a homogeneous medium. However this regulatory system is influenced by external clues such as gravitropism.

The effect of gravistimulations suggests a mechanism of inhibitory fields controlling root branching

These first results on gravistimulation showed a global consistent rationale that we attempted to capture quantitatively through the design of a simple mechanistic model. This model was based on an auxin budget system (Fig. 3A) and aimed to explain the effects of gravistimulations on lateral root initiation. When a root grows unperturbed it initiates new lateral root primordia regularly. We model this phenomenon as the progressive filling of an exploitable auxin pool. The filling is assumed to take place at a constant rate (P). When the quantity of auxin in the pool is greater than the threshold value T1, lateral root initiation occurs and the auxin pool is entirely consumed. This mechanism

controls spontaneous initiation (Fig. 3A, white arrows). We estimated the threshold value T1 to be equivalent to 12 hours of auxin production/accumulation in our conditions, as initiation density in our control corresponds to a 12 hours period between lateral root initiations.

When a 90° gravistimulation is applied, it either enhances the perception of auxin at the future initiation sites, or locally concentrate auxin at these points by changing auxin distribution without changing the global auxin quantity in the root. Both hypotheses are strictly equivalent at an abstract level, and can be expressed in the model by introducing a new threshold. We thus distinguish in our model the spontaneous lateral root initiation threshold T1 and the lower threshold T2 corresponding to gravistimulation-induced initiation. In addition, each gravistimulation induces an auxin consumption (G) from the auxin pool. Two cases must then be distinguished: either the remaining auxin level is higher than T2, or it is lower. In the first case, a lateral root initiation occurs and the auxin pool is flushed (Fig. 3A, grey arrows). In the second case, no initiation occurs, and the system runs its course (Fig. 3A, black arrows).

We designed a computer algorithm implementing the above mechanistic model controlling lateral root initiation in time as described in figure 3A. This model takes as an input parameter T1, estimated from observed data (T1=12h, which corresponds to the mean time between two successive initiations in the control), and a gravistimulation pattern, corresponding to a series of time intervals between gravistimulations on a given individual. The algorithm returns the predicted sequence of lateral root initiations over the time length of the gravistimulation pattern, depending on the value of T2 and G. To estimate the values of these two parameters, we proceeded to an extensive exploration of the

parameter space and compared the number of lateral root initiations of the returned initiation patterns to the observed number of lateral root initiations of gravistimulated roots. The values of T2 and G giving the best fit were T2 ~ 0.4 T1 and G ~ 0.05 T1. The output of the model obtained using those values closely follows the observed number of lateral root initiations (Fig. 3B).

In order to validate the model, we designed a new experiment to evaluate its predictive power. We selected 6 new gravistimulation patterns (Fig. S5) not previously tested, with either regular or irregular spacing between gravistimulations. Based on direct pattern observation, it was not possible to guess the total number of lateral root initiations that would be produced. Those patterns were applied on Pro_{CYCB1}*:GUS* seedlings for 48h, according to the previously described protocol of gravistimulation. The total number of lateral root initiations for the various seedlings groups were scored and compared to the total number of lateral root initiations predicted by the model (Fig. 3C). This experiment was repeated twice independently.

We found that the total number of lateral root initiations is not governed by the number of gravistimulations (Fig. 3D). The quantitative model was able to predict with accuracy the total number of LRI for each pattern, over a large range of total number of lateral root initiations without loss of accuracy (Fig. 3C,E) thus showing that the total number of lateral root initiations is actually a function of the structure of the gravistimulation pattern. Similarly to the inhibitory field models for the shoot apical meristem (Douady and Couder, 1996; Smith *et al.*, 2006), the proposed model suggests that lateral root initiations are submitted to inhibition fields (here represented by auxin consumption) that control their patterning.

Common auxin fluxes regulate gravitropism and lateral root initiation

Gravitropism and lateral root initiation are both regulated by auxin (Casimiro *et al.*, 2001; Swarup *et al.*, 2005). Gravity is perceived in the central part of the root cap and gravitropism relies on an AUX1-dependent acropetal auxin flux from the root apex through the lateral root cap and the elongating root epidermis where it induces changes in cell elongation (Ottenschläger *et al.*, 2003; Swarup *et al.*, 2005). AUX1 encodes a high-affinity auxin influx carrier (Yang *et al.*, 2006). On the other hand, very little is known about the auxin fluxes that are responsible for lateral root initiation in the root pericycle. However, the *aux1* mutant is perturbed in both lateral root initiation and root gravitropism and recent studies suggest a common auxin transport pathway for gravitropism and lateral root initiation (De Smet *et al.*, 2007).

Since we found that gravitropism and lateral root initiation are co-regulated we tested whether both processes were dependent on the same auxin transport route. We used a transactivation strategy to complement the *aux1* mutant in different tissues at the root apex as described by De Smet *et al.* (2007) and to test the effect on lateral root initiation. Plants expressing *UAS:AUX1* under the control of the GAL4 enhancer trap lines M0013 (root cap) or J0951 (root cap and expanding root epidermis) in an *aux1-22* mutant background (Swarup *et al.*, 2005) were grown for 10 days on vertical plates. They were then harvested and scored for gravitropism and lateral root primordia density. Our results on lateral root initiation (Fig. 4A) were similar to those obtained by De Smet *et al.* (2007)

on lateral root density. We therefore conclude that the auxin necessary for lateral root initiation and gravitropic root growth has to be transported through the same route in the lateral root cap and the elongating root epidermis.

We next tested whether auxin needs to be perceived in the tissues through which it flows for lateral root initiation. We transactivated a dominant negative version of the AXR3 protein (axr3-1) that was previously shown to inhibit auxin response in different root tissues (Swarup *et al.*, 2005) and tested the effects on gravitropism and lateral root initiation. F1 plants were grown for 10 days on vertical plates before analysis. Our results on gravitropism were similar to those of Swarup *et al.* (2005). When axr3-1 was transactivated in the root cap, using ET line M0013, it had no effect on gravitropism or lateral root initiation (Fig. 4B). When axr3-1 was transactivated in both the root cap and the root epidermis using enhancer trap line J0951, it abolished the gravitropic response of the root but did not perturb lateral root initiation (Fig. 4B). Thus our results suggest that in contrast to gravitropism, auxin does not need to be perceived in the root epidermis in order to direct lateral root formation.

Does co-regulation of gravitropism and lateral root initiation optimise soil exploration?

Our results indicated that lateral root initiation and gravitropism/thigmotropism are, at least in part, co-regulated. We next wondered if co-regulation could have some selective advantage. We used simple geometrical considerations to evaluate the potential effect of co-regulation on resource exploitation (Fig. 5). We estimated the volume of soil explored by a

root (see Material and Methods for details) using three simplifying assumptions: 1) the volume of soil exploited by a given root segment is proportional to the number of root hairs, 2) resources (water and nutrients) diffuse in the soil according to their concentration gradient, and 3) all root hairs have the same absorption potential. Since gravitropism/thigmotropism is due to changes in cell elongation in the root epidermis, the number of root hairs is the same on the internal as on the external side of a curved root, and root hair density (per root length) is lower on the external side (Fig. 5B). This means a greater depth of soil is exploited on the internal side (h1, Fig. 5C) than on the external side (h2). Extrapolating these results to the whole root and in three dimensions leads to an asymmetric profile of soil exploration (Fig. 5A,D). This suggests that lateral root formation on the outer parts of the turns may optimise soil exploitation (Fig. 5E). We considered in our model that root hair length was identical on both side of the bend. On the other hand, auxin is known to increase root hair length (Pitts *et al.*, 1998) and auxin preferentially accumulates on the lower side of roots during gravitropic curvature. Accordingly, we found that root hairs were significantly longer on the inside and shorter on the outside of a bend than control root hairs (Fig. S6E,F). This will therefore increase the depletion effect that observed in our model on the inside of the bend.

DISCUSSION

Our study shows that gravistimuli induce lateral root initiation. Lateral root formation in gravistimulation experiments is not due to bending itself because the root of the *aux1* mutant or J0951>>axr3 plants showed many turns without

increasing lateral root initiation. The co-regulation of lateral root initiation and root gravitropism explains why there is such a good correlation between root waving and lateral root initiation (De Smet et al., 2007). This is also in agreement with the fact that many mutants are perturbed in both processes. Simple mathematical modelling suggests that this co-regulation of gravitropism/thigmotropism and lateral root initiation leading to formation of lateral root primordia on the external side of a bend might offer some selective advantage by optimising soil exploration.

Our data suggest that the regulatory system responsible for lateral root initiation is sensitive to external clues perceived at the root apex such as gravity. Indeed we were able to change root architecture simply by applying gravistimulations at different intervals. Our data also point out to internal characteristics of the regulatory system such as the minimum/maximum time between two successive initiations. We used these results to create a mathematical model that can explain and predict the effects of gravistimulations on lateral root initiation density. Our model suggests that by creating a asymmetric distribution of auxin in the apex using gravistimulations, one is able to reduce the amount of auxin necessary for lateral root initiation. Interestingly this simple mechanistic model suggests that lateral root initiation is controlled by inhibition fields (auxin consumption) in the root apex like lateral organ formation in the shoot apical meristem (Douady and Couder, 1996; Smith *et al.*, 2006).

Because it is impossible to predict the position of lateral root initiation and because initiation is a relatively rapid process, little is known about the cellular events that precede it, i.e. the very first division that occurs during lateral root development. Our results indicate that it is possible to use gravistimuli to induce

lateral root initiation locally with almost 100% success. Such a system can thus be used to monitor the course of cellular events that occur before lateral root initiation. It offers an alternative approach to auxin-based lateral root induction systems (Himanen *et al.*, 2002) to study cellular processes such as nucleus movement or changes in cellular trafficking or in the organisation of the cytoskeleton that might prepare the first cell division i.e. lateral root initiation.

Finally, our experimental data suggest a mechanism for co-regulation of gravitropism and lateral root initiation (Fig. 6). Auxin, the key signal that controls both processes, is produced in leaf primordia and transported to the root via the vascular basipetal flow (Friml *et al.*, 2006). Root meristems and lateral root primordia can also produce auxin (Ljung *et al.*, 2005). An auxin maximum is generated in the root columella (Sabatini *et al.*, 1999) and auxin is redistributed in the meristem from the columella in a PIN3-dependent way. Upon gravistimulation, PIN3 is retargeted to the lower face of columella cells thus creating an asymmetric auxin distribution (Friml *et al.*, 2002). Auxin is transported from the root tip through the lateral root cap and in the elongating root epidermis in an AUX1/PIN2-dependent way thus generating an acropetal auxin flux (Swarup *et al.*, 2005). Auxin perception in the epidermis is then responsible for root gravitropism by changing the relative elongation of epidermal cells (Swarup *et al.*, 2005). Our transactivation experiments together with previous results (De Smet *et al.*, 2007) indicate that the same acropetal flux is responsible for lateral root initiation further up the root. This is consistent with previous data indicating that acropetal auxin transport from the root tip is responsible for lateral root initiation (Casimiro *et al.*, 2001; Bhalerao *et al.*, 2002). Moreover, our axr3 transactivation data suggest that while gravitropism

requires AUX/IAA-dependent auxin perception in the root epidermis, lateral root initiation does not. This suggests that the root epidermis only acts as a passive auxin transport route in lateral root initiation. Since the dynamic changes in PIN protein cellular localisation in response to changes in auxin concentration in the root depend on the AUX/IAA-ARF pathway (Sauer *et al.*, 2006), this suggests that lateral root initiation does not require such auxin-dependent PIN relocalisation at least in the epidermis.

Later stages of lateral root development depend on basipetal auxin transport from the shoot (Casimiro *et al.*, 2001; Bhalerao *et al.*, 2002) until lateral root primordia become independent of external auxin between stage III and V (Laskowski *et al.*, 1995) when auxin synthesis may start (Ljung *et al.*, 2005). As a consequence the position of lateral root primordia is partially controlled by gravitropism/thigmotropism but the later development of these primordia is independent of these two processes and may be regulated by other factors such as water or nutrient availability (Malamy, 2005). How an asymmetric auxin distribution in the epidermis leads to lateral root initiation in the pericycle is still unknown. Interestingly, during gravitropism the auxin maximum occurs on the internal side of the bend while lateral root initiation occurs on the external side. We are currently building an *in silico* model based on this and previous studies (Blilou *et al.*, 2005; Swarup *et al.*, 2005) to try to understand how the redistribution of auxin in the root apex controls root branching.

SUPPLEMENTARY DATA

Fig. S1. Gravistimulation protocols. Seedlings were grown on vertical plates and gravistimulated by a periodic (period T) 90° rotation of the growth plates. Two different rotation protocols were used to generate either crenel-shaped or stair-shaped roots. Roots subjected to these protocols were grown under stimulation for 3.5 days and with no stimulation for an additional 2.5 days before harvesting.

Fig. S2. Localisation of lateral root initiation in a gravistimulated root. A Pro_{CYCB1}::GUS seedling was subjected to crenel gravistimulation at 12-h intervals. Lateral root initiations were localised and their development scored.

Fig. S3. Influence of varying gravistimulation on lateral root initiation density (crenel-shaped roots). (A) Vertically grown Pro_{CYCB1}::GUS seedlings were left to grow (control; n=20) or were subjected to gravistimulation at intervals of 1 h (n = 24), 3 h (n = 20), 6 h (n = 21), 12 h (n = 21) or 24 h (n= 24) over a period of 3.5 days (1), then left to grow for 2.5 days without stimulation (2). Bars = 1 cm. (B) Occurrence of LRI in root turns. (C) Occurrence of lateral root initiation between root turns. Due to the particular configuration of roots subjected to gravistimulation at 1-h and 3-h intervals (respectively presenting no visible turns and only turns), some values were not determined (na = not applicable). (D) Effect of gravistimulation on root growth. Length of the gravistimulated root segments (first 5 days of growth) and non-gravistimulated root segments (last 2.5 days of growth) were also determined. (E) Lateral root initiation densities were determined in gravistimulated and non-gravistimulated root segments. Different letters indicate significantly different results as tested by a Student T-test ($P < 0.01$).

Fig. S4. The RootInit algorithm corresponding to the mechanistic model. The pseudo-code is expressing the mechanisms described in Fig. 3A in discrete time.

Fig. S5. Gravistimulation patterns used for the evaluation of our model. Six previously non-tested gravistimulation patterns were applied to seedlings over a 48h period starting 30 hours after germination. Gravistimulation are indicated by black dots. The total number of gravistimulation for each pattern varies between 10 and 25. After the last gravistimulus, seedlings were left to grow undisturbed for 24h before harvest and observation.

Fig. S6. Curve radius, depth of exploration and root hair length. (A) Curve of the function $h_1 / h_2 = (\rho + b / 2) / (\rho - b / 2)$. (see figure 4 for additional details on the parameters) (B) Curve radius estimated for a portion of a gravistimulated root (90° re-orientation). b and ρ are the thickness and the curve radius of the chosen root portion respectively. (C) Curve radius estimated for various root turns of a waving root. (D) Ratio of exploration depths (h_1 / h_2) for various values of (ρ). (E) Direct visualisation of root hair on both sides of a root turn. (F) Root hair length was measured on both sides of root turns (n=20) and straight roots. Different letters indicate significantly different results as tested by a Student T-test ($P < 0.01$).

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FIGURE LEGENDS

Fig. 1. Influence of gravistimulation on lateral root initiation (LRI). (A) Segmentation of the root between gravistimulated (turn) and non-gravistimulated (straight) zones used for determination of the position of LRI. (B) Percentage of lateral root initiations in the gravistimulated and non-gravistimulated zone of crenel-shaped (n = 23) and stair-shaped (n = 24) roots gravistimulated at 12-hour intervals (see supplementary figure 1 for additional details on the gravistimulation protocol). (C) Kinetic of lateral root initiation after gravistimulation. Six batches of roots (n = 40) were gravistimulated every 6 hours over a 24-hour period before harvest and GUS staining, with a one-hour shift between each batch. This led to the formation of root turns aged from 0 to 25 hours, which were scored for lateral root initiation. (D) Kinetic of LRP development after gravistimulation. Arrows indicate the period of time over which the different developmental stages were observed.

Fig. 2. Influence of the gravistimulation interval on lateral root initiation density. (A) Vertically grown *Pro$_{CYCB1}$:GUS* seedlings were left to grow (control; n=20) or were subjected to gravistimulation at intervals of 1 h (n = 24), 3 h (n = 20), 6 h (n = 21), 12 h (n = 21) or 24 h (n= 24) over a period of 3.5 days (1), then left to grow for 2.5 days without stimulation (2). Bars = 1 cm. (B) Occurrence of lateral root initiation in root turns. (C) Occurrence of lateral root initiation between root turns. Due to the particular configuration of roots subjected to gravistimulation at intervals of 1 h and 3 h (respectively presenting no visible turns and only turns), some values were not determined (na = not applicable). (D) Effect of gravistimulation on root growth. The length of the gravistimulated root segments (first 5 days of growth) and non-gravistimulated root segments (last 2.5

days of growth) was also determined. (E) Lateral root initiation densities were determined in the gravistimulated and non-gravistimulated root segments. Different letters indicate significantly different results as tested by a Student T-test ($P < 0.01$).

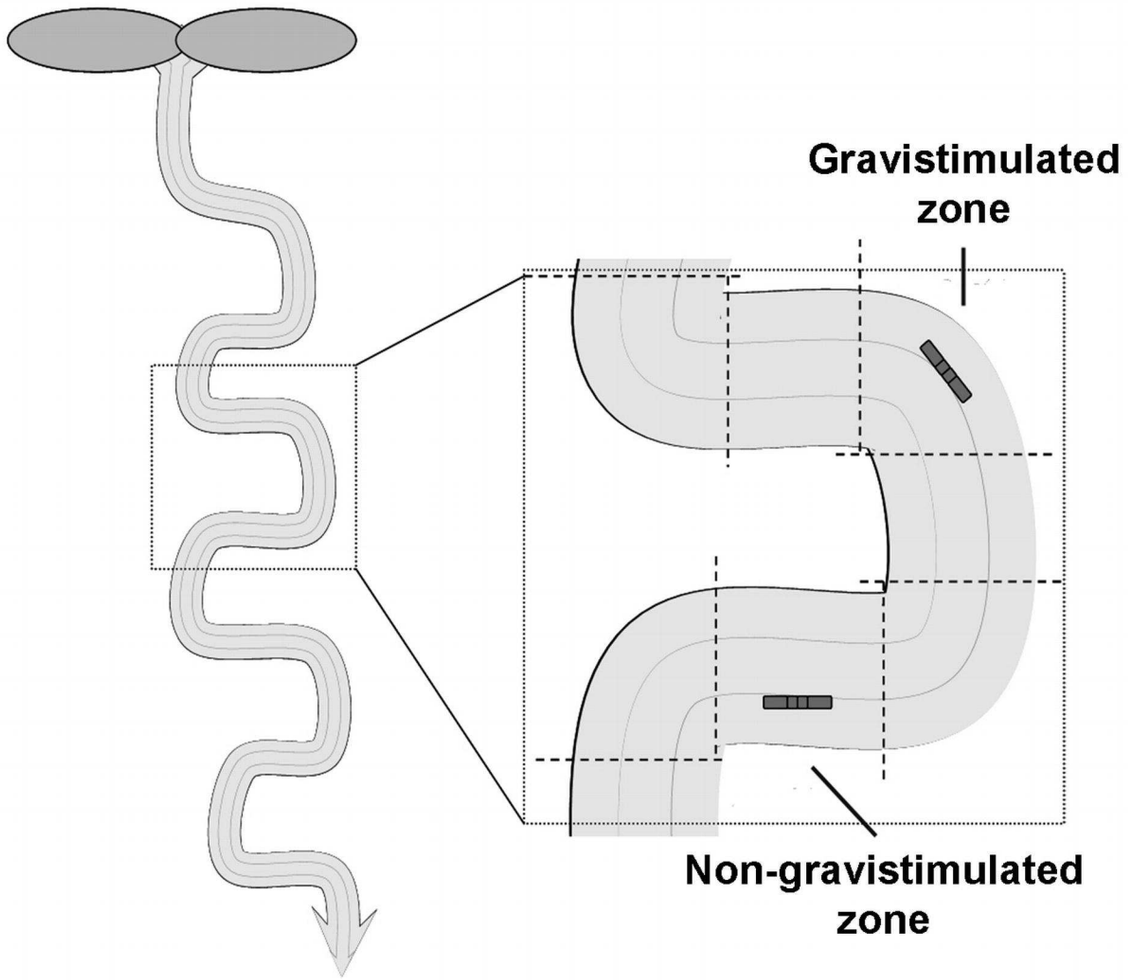
Fig. 3. Model of lateral root initiation regulation. (A) Logical circuit of the model. Auxin accumulates with a production rate P, and when its level is above the threshold T1 a lateral rot initiation (LRI) occurs. Initiations cause a flush of the auxin pool. Gravistimulations induce an auxin consumption (G) and an initiation if the remaining auxin level is higher than a second threshold T2. (B) Comparison of observed LRI densities and of the best fit output of the logical model. The parameters corresponding to the best fit were determined by extensive automated parameters space exploration. (C) Evaluation of the predictive power of the logical model. The predicted LRI densities and the LRI densities observed for each gravistimulation treatment were compared (n = 20, see figure S4 for additional details on the treatments). The control is a non-gravistimulated seedlings lot grown in the same condition as the gravistimulated seedlings (n = 20). (D) There is no relation between the number of gravistimulations and the number of LRI. Each point corresponds to one of the treatments presented in (B) or (C), as identified by the corresponding tag. TBR: time between rotations. E) Number of observed LRI as a function of the predicted number of LRI. Each point corresponds to one of the treatments presented in (B) or (C). This graph shows that the values observed match closely the predicted value.

Fig. 4. Effect of *AUX1* and *axr3* transactivation on lateral root initiation. LRP densities were determined for the AUX1 complementation crosses (A) and the axr3 transactivation (B). Different letters indicate significantly different results as tested by a Student T-test ($P < 0.01$).

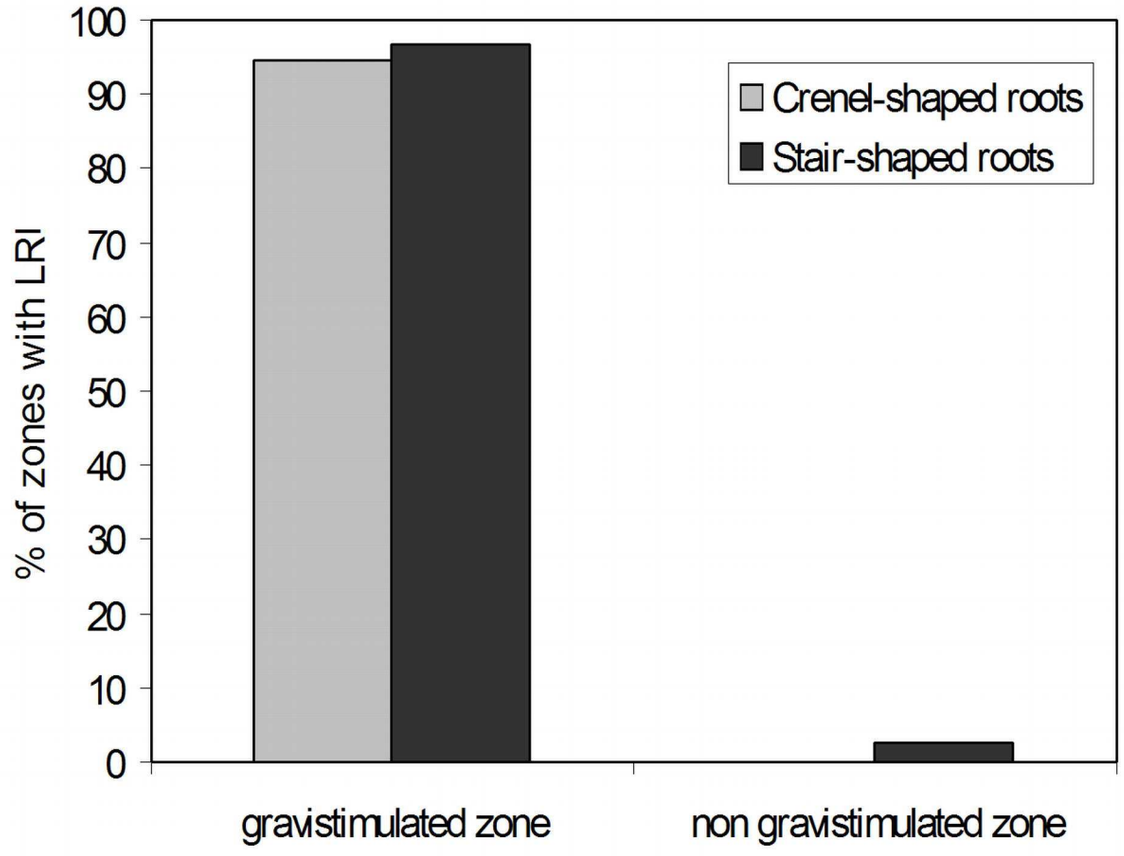
Fig. 5. Influence of root bending on resource exploitation. (A) Exploitation of soil resources by a bent root (grey zone). (B) Infinitesimal portion of root turn. Parameters are (n), number of root hairs; (ρ), curve radius of selected zone; (b), thickness of the root; ($d\alpha$), angle made by selected zone; (dl_1) and (dl_2), length of curved zone on each side of the root turn. (C) Area of soil exploited. Parameters are (s_1) and ($s2$), area of soil exploited each side of the root turn; (a), absorption strength of a single root hair ; (h_1) and (h_2), depth of soil exploited on each side of the root turn. (D) Transversal profile of soil exploitation at a root turn. (E) Corresponding depletion of resources.

Fig. 6. Model of gravitropism and lateral root initiation signalling pathways. Auxin fluxes responsible for gravitropism (A) pass through the lateral root cap and are perceived in the epidermal cells via the AUX/IAA molecular pathway, eliciting auxin response (B). Auxin fluxes responsible for lateral root initiation pass through the lateral root cap and the epidermis, but do not require interaction with the AUX/IAA molecular pathway, suggesting a more direct influence on internal tissues further along the root.

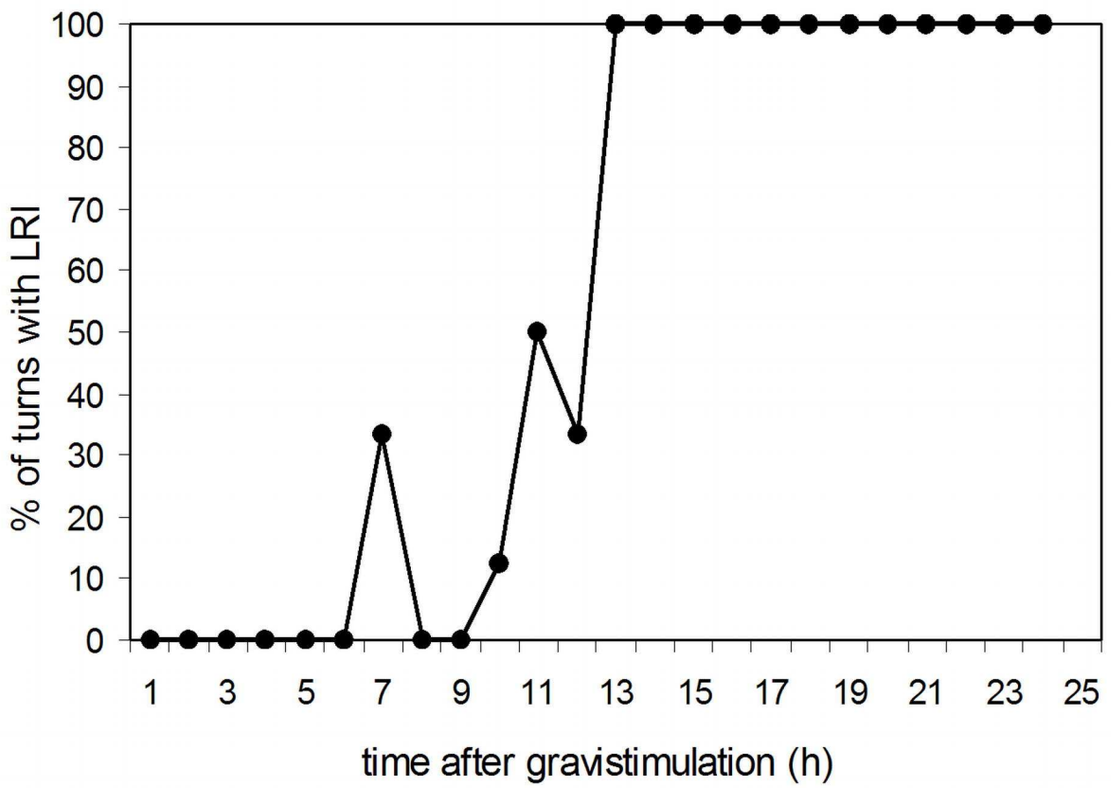
A


Gravistimulated zone
Non-gravistimulated zone

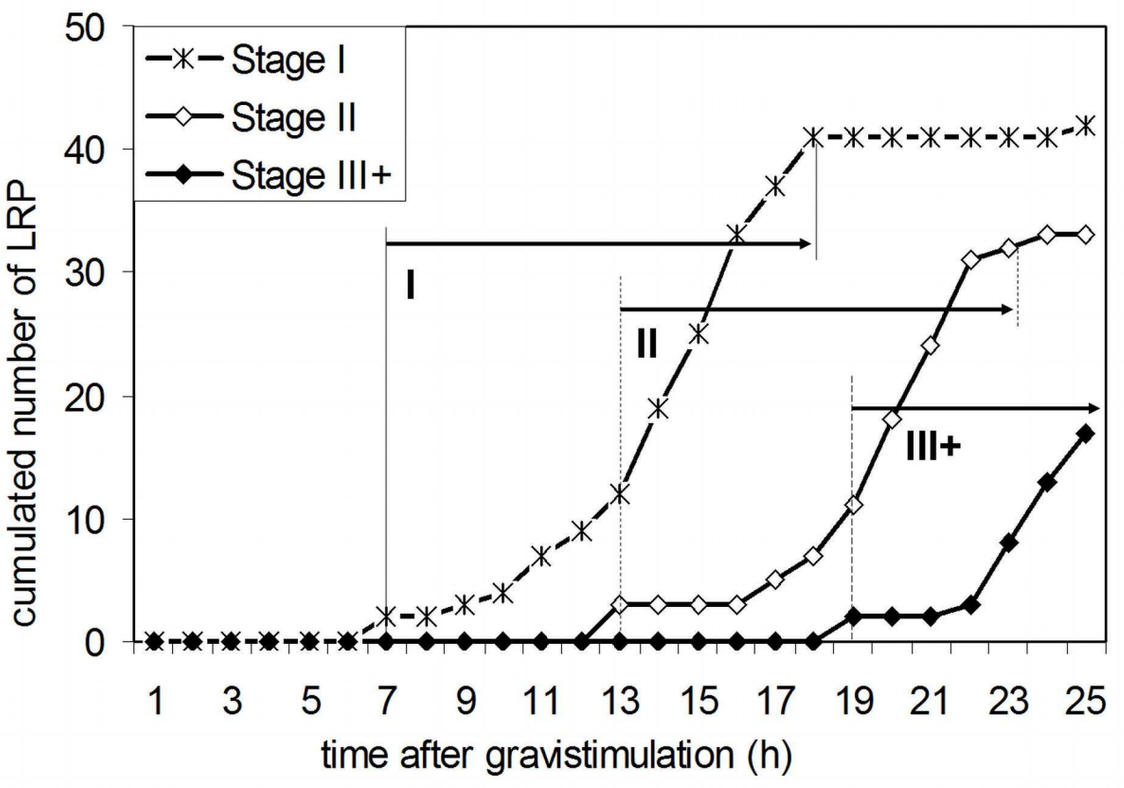
B

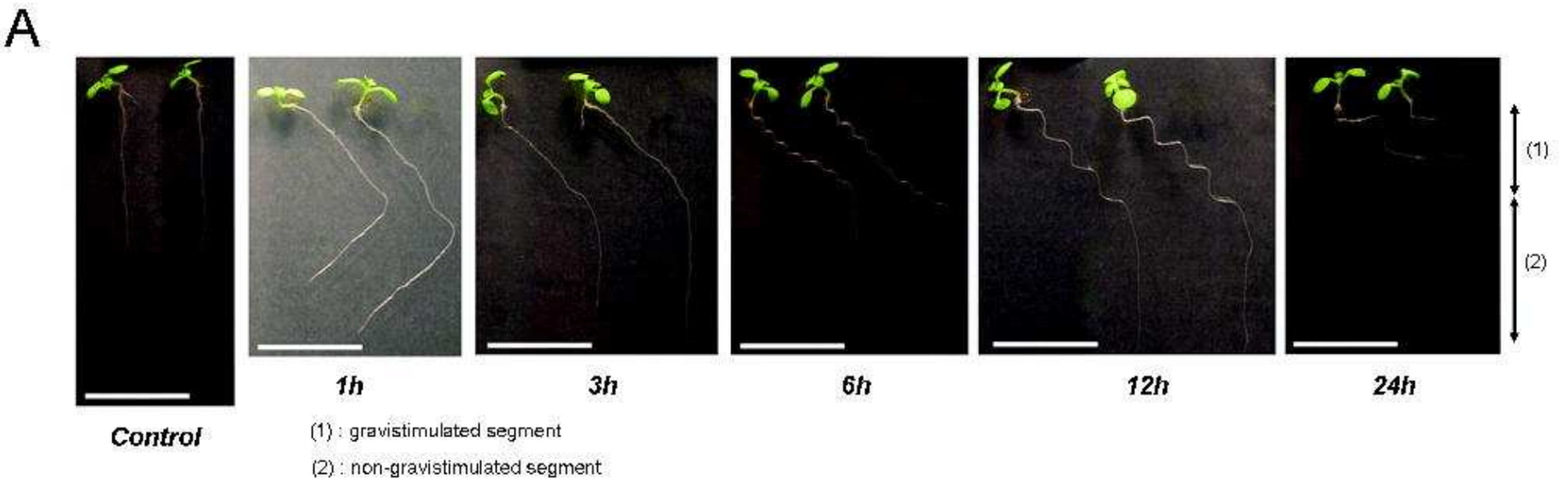

% of zones with LRI
100
90
80
70
60
50
40
30
20
10
0
Crenel-shaped roots
Stair-shaped roots
gravistimulated zone
non gravistimulated zone

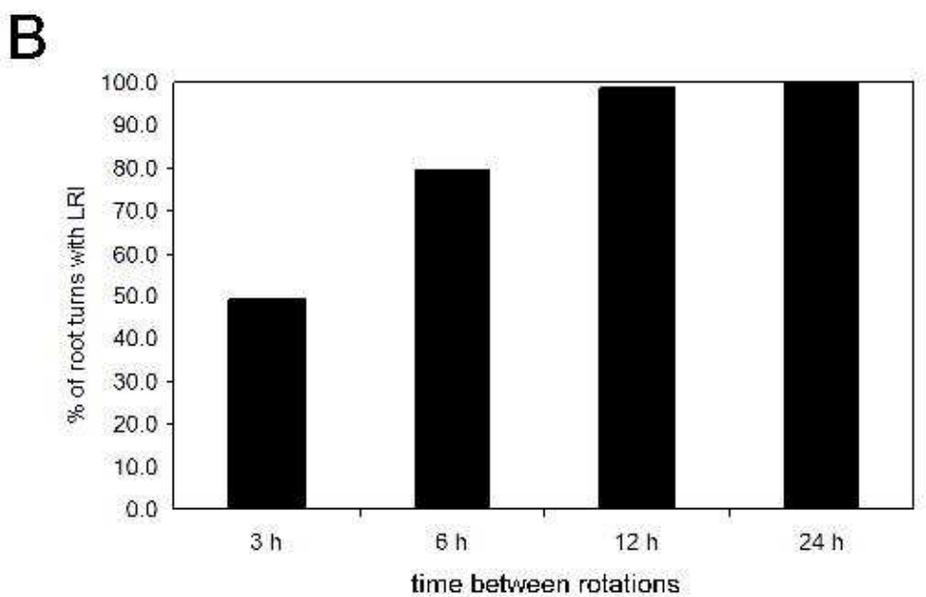
C

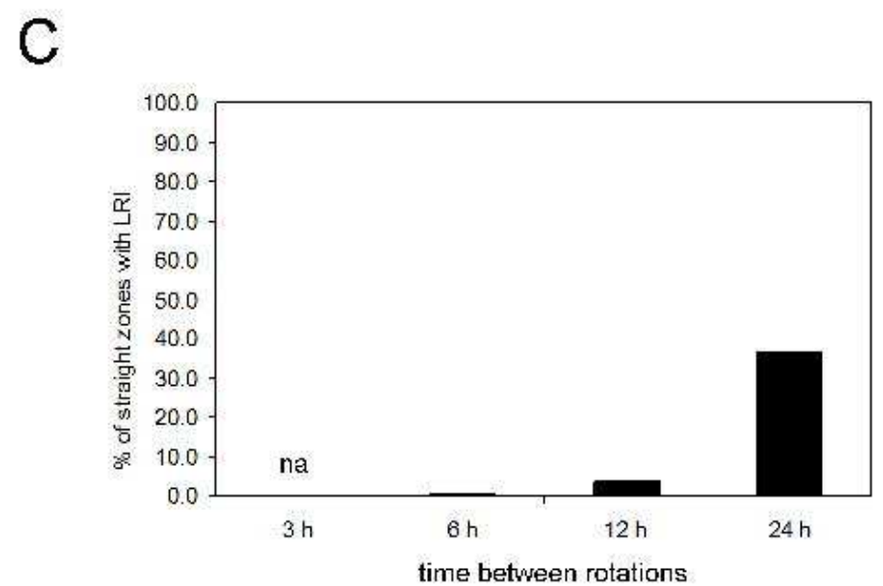

% of turns with LRI
100
90
80
70
60
50
40
30
20
10
0
1 3 5 7 9 11 13 15 17 19 21 23 25
time after gravistimulation (h)

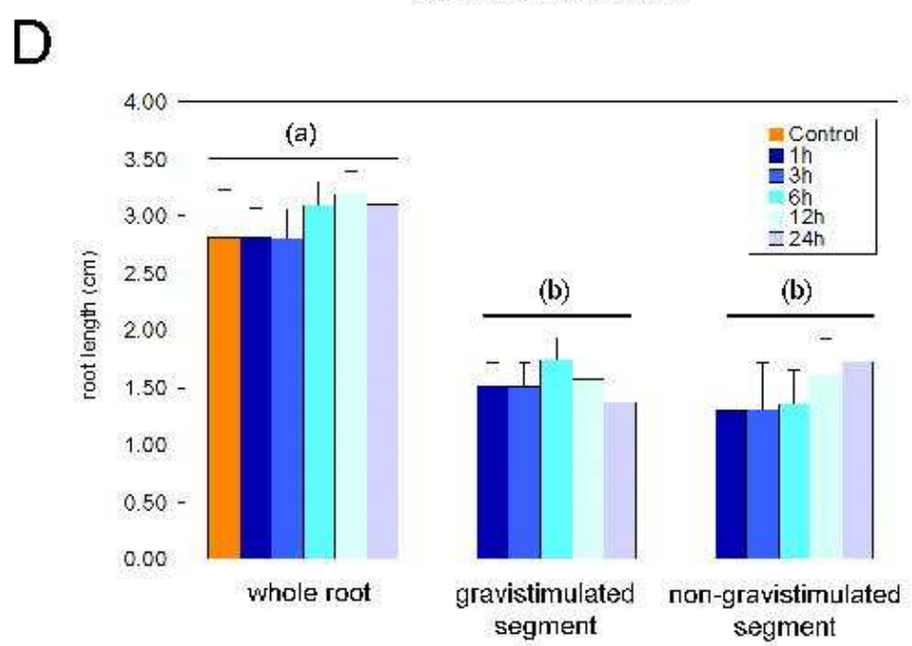
D

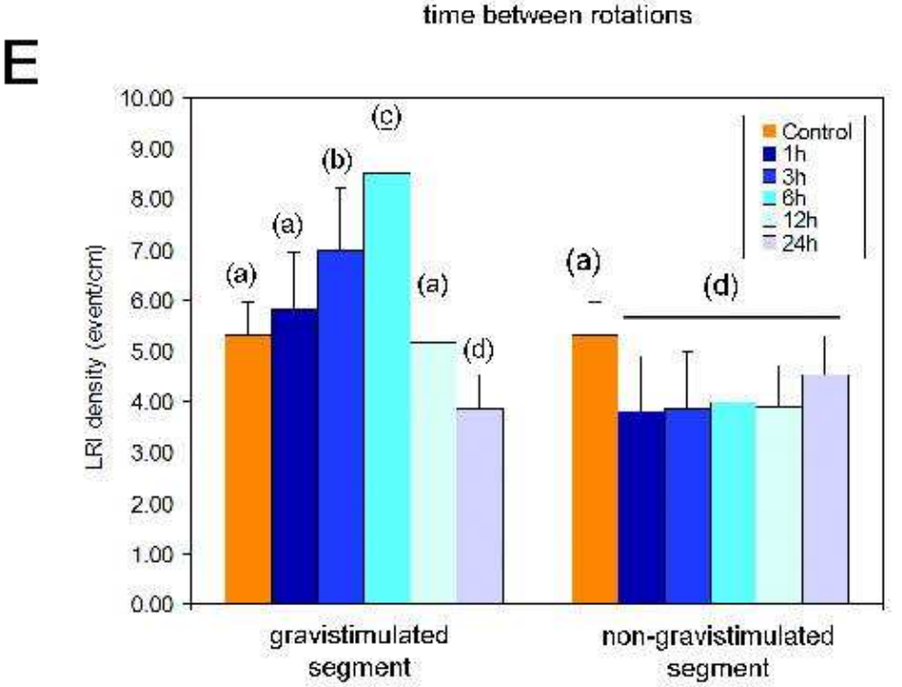

cumulated number of LRP
50
40
30
20
10
0
Stage I
Stage II
Stage III+
I
II
III+
1 3 5 7 9 11 13 15 17 19 21 23 25
time after gravistimulation (h)

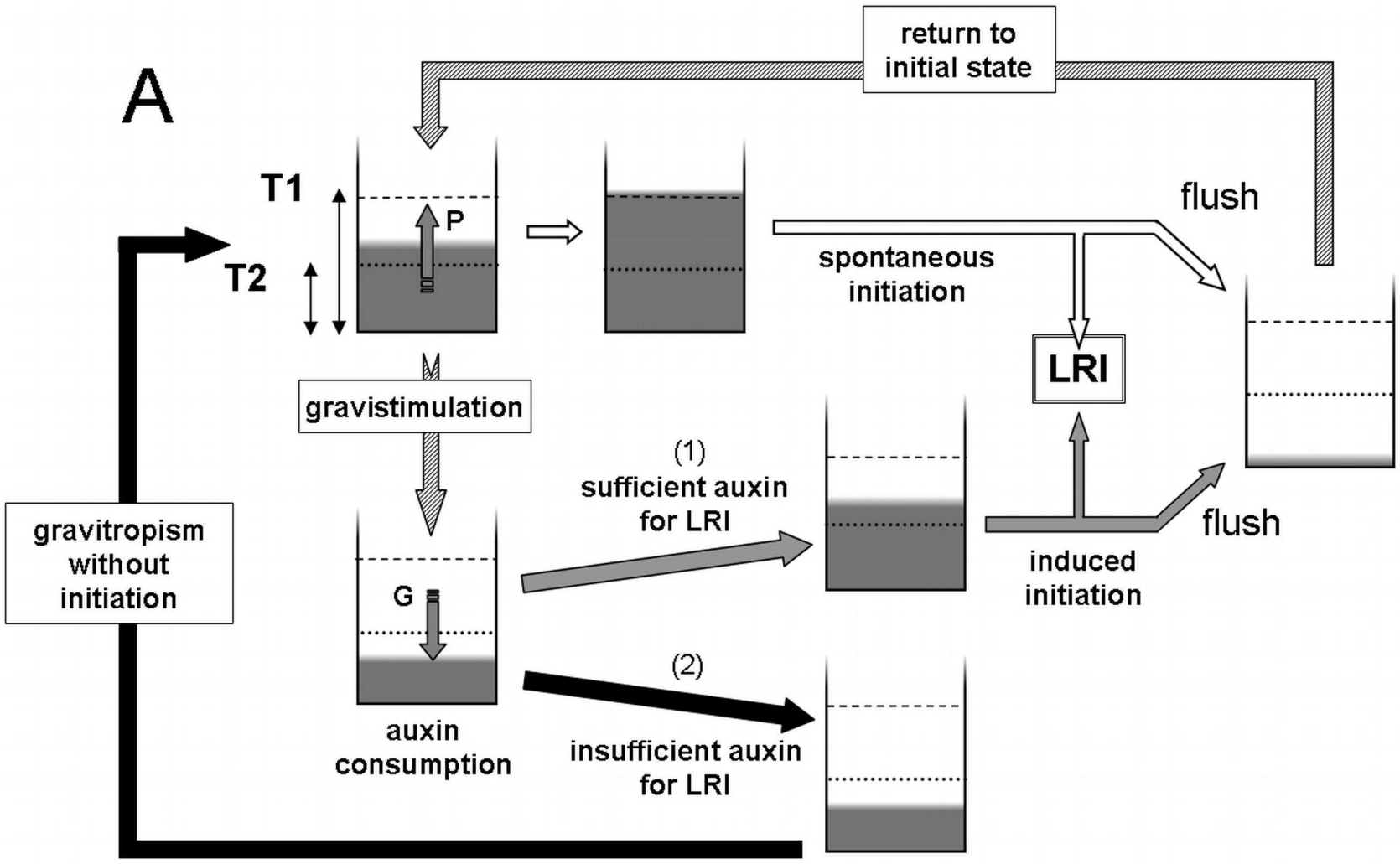

A
(1)
(2)
1h
3h
6h
12h
24h
Control
(1) : gravistimulated segment
(2) : non-gravistimulated segment

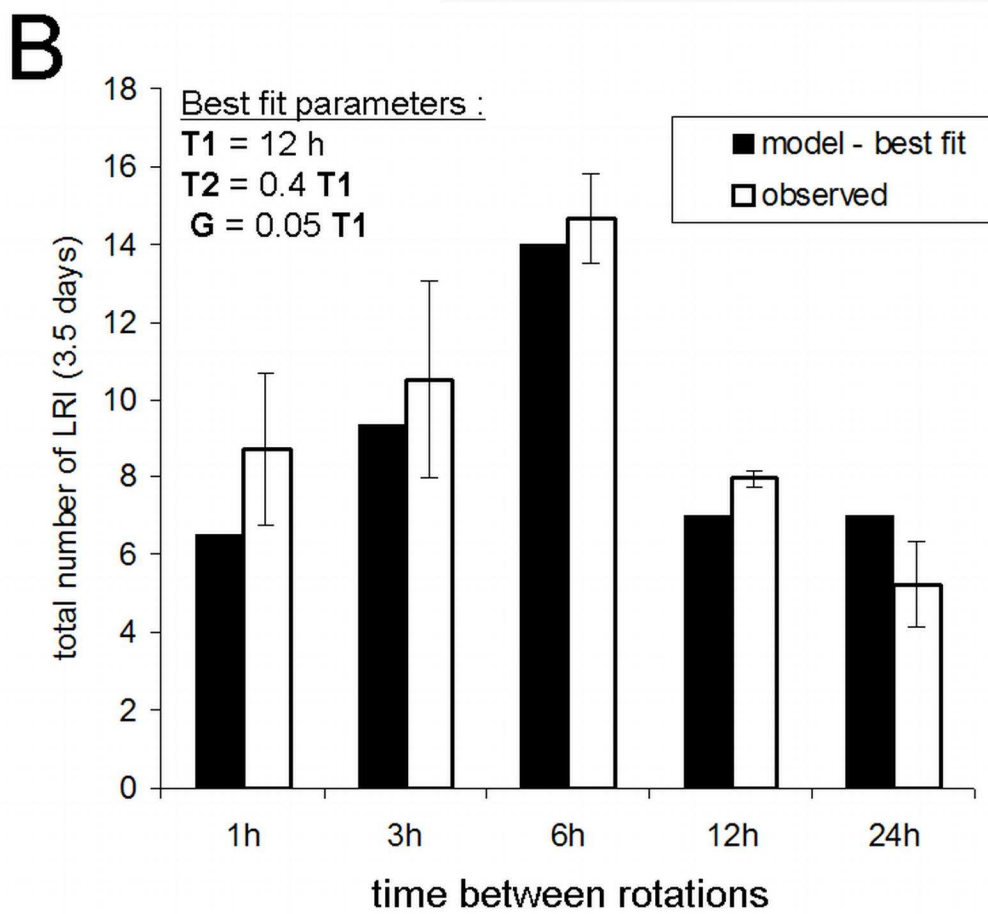

B
% of root turns with LRI
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
3 h
6 h
12 h
24 h
time between rotations

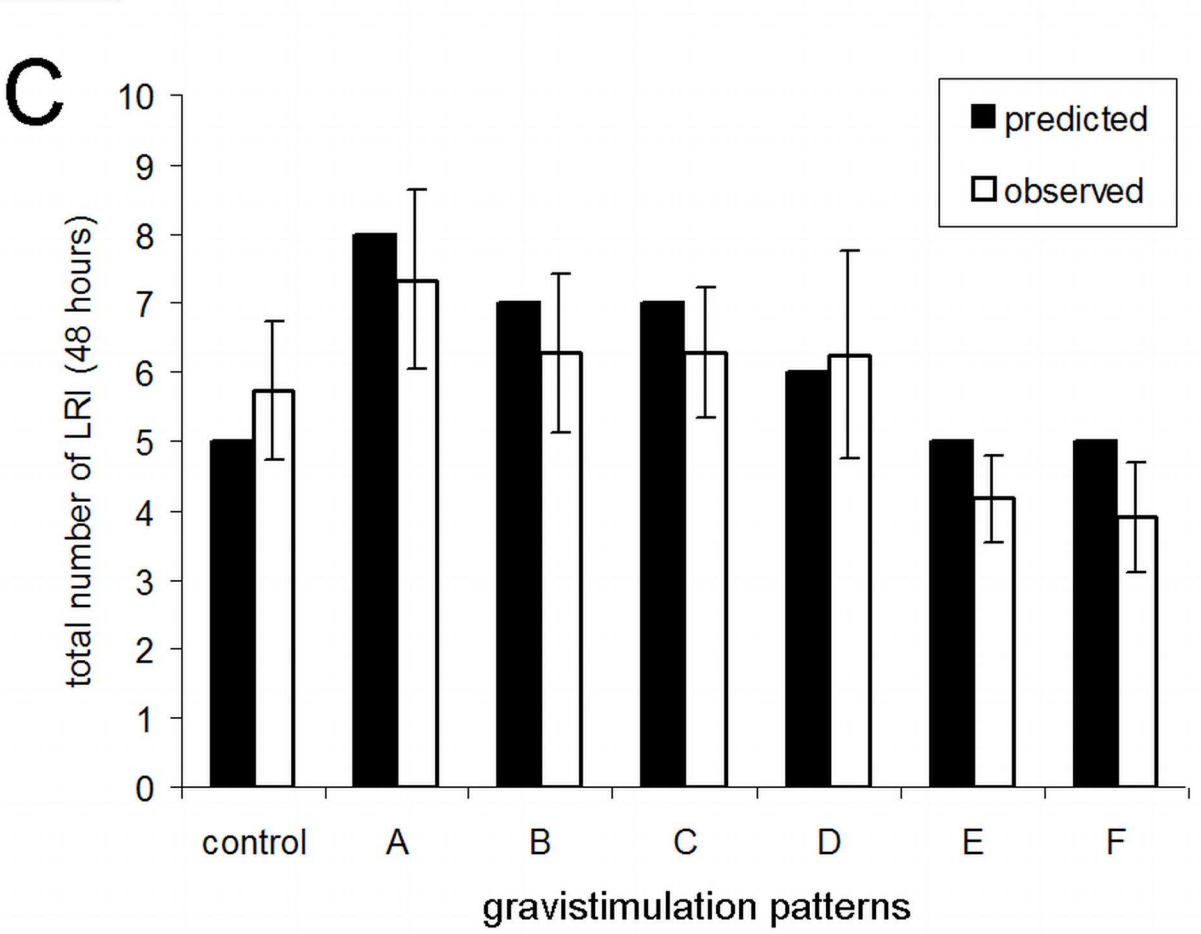

C
% of straight zones with LRI
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
na
3 h
6 h
12 h
24 h
time between rotations

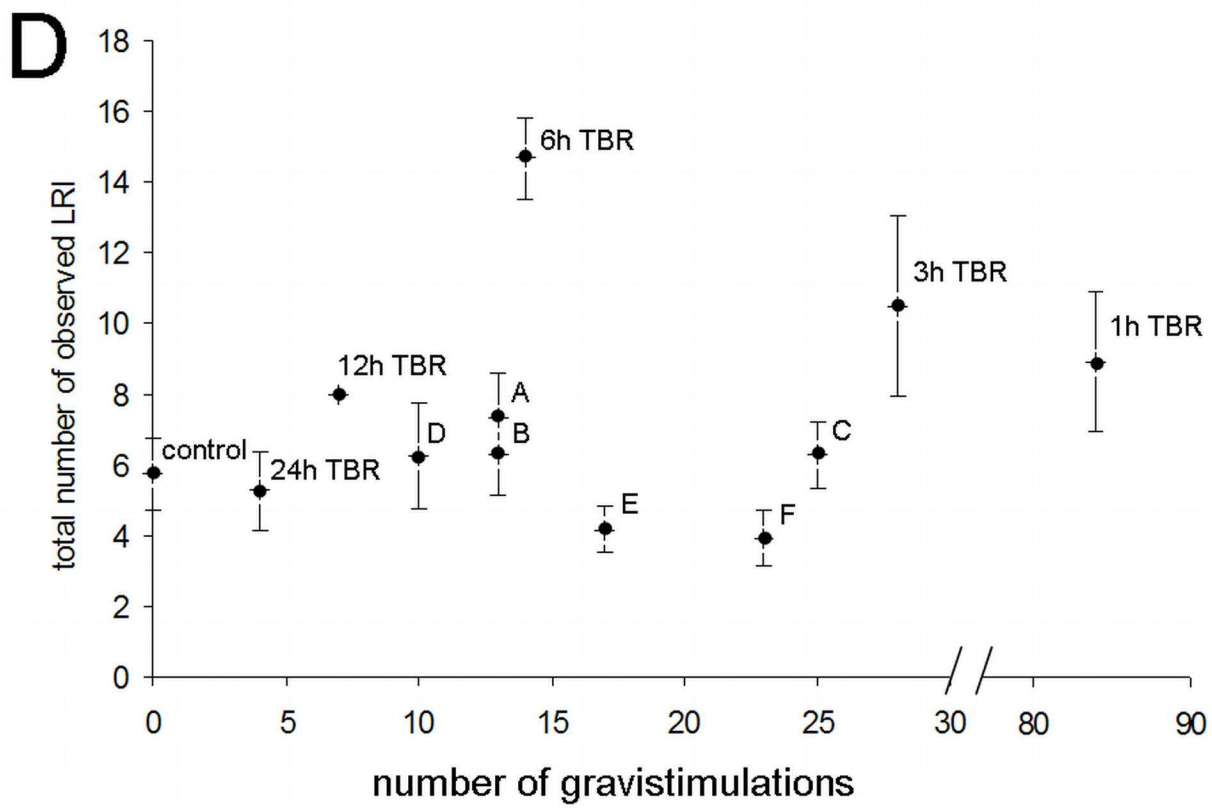

D
root length (cm)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
(a)
(b)
(b)
Control
1h
3h
6h
12h
24h
whole root
gravistimulated segment
non-gravistimulated segment

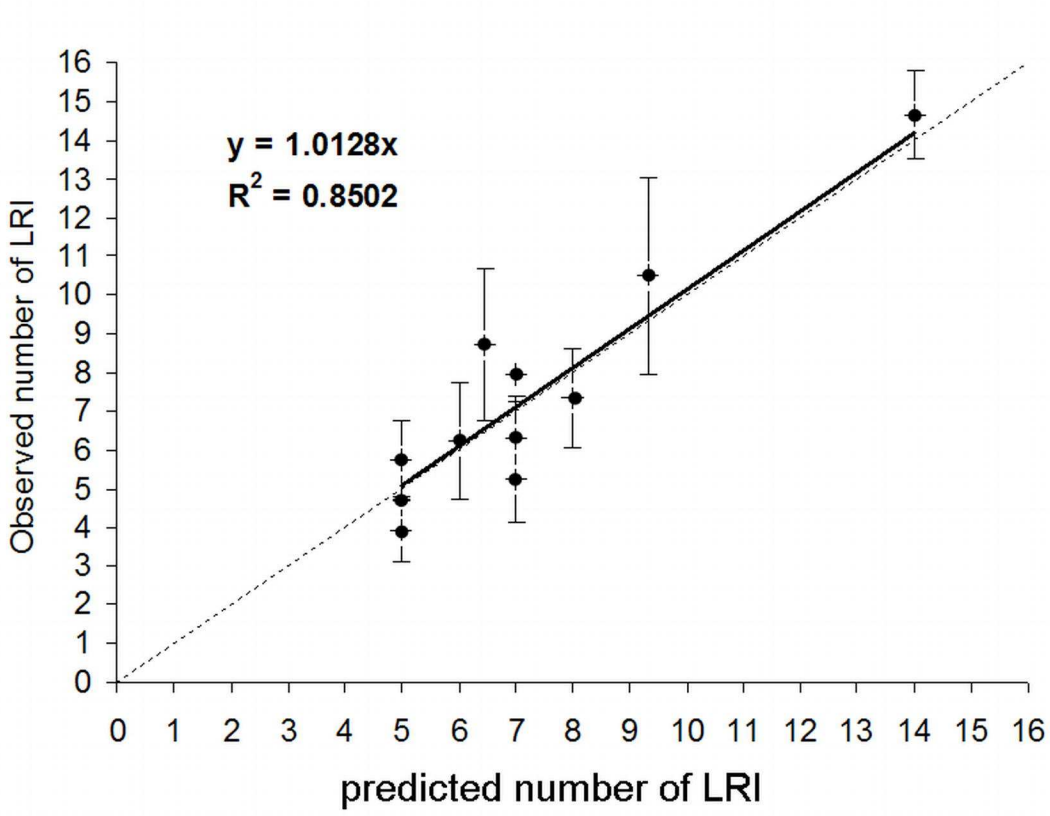

E
LRI density (event/cm)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
(a)
(a)
(b)
(c)
(a)
(d)
(a)
(d)
Control
1h
3h
6h
12h
24h
gravistimulated segment
non-gravistimulated segment

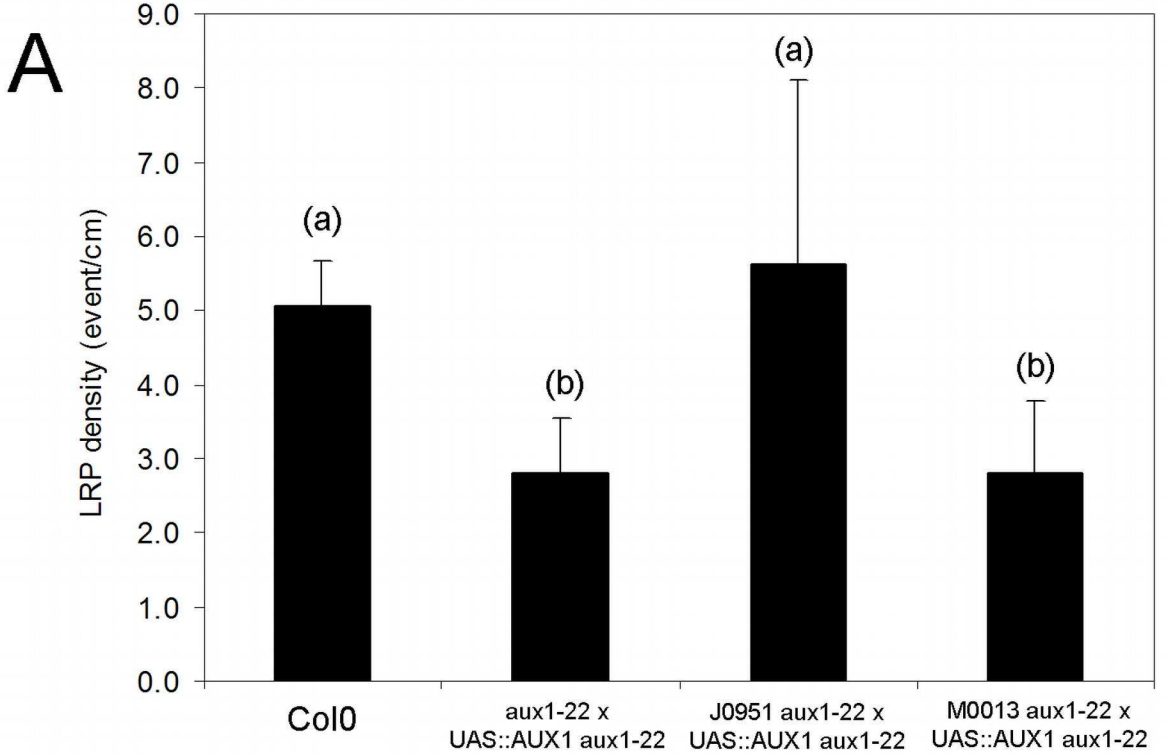

A
return to initial state
T1
T2
P
flush
spontaneous initiation
LRI
gravistimulation
(1)
sufficient auxin for LRI
gravitropism without initiation
G
induced initiation
flush
auxin consumption
(2)
insufficient auxin for LRI

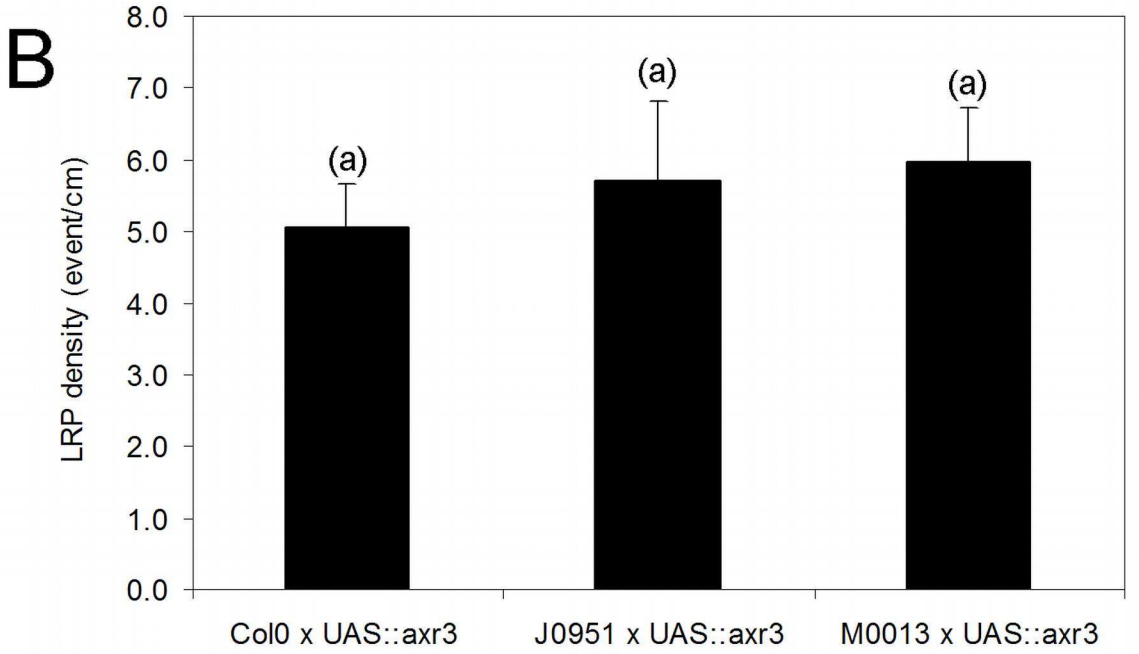

B
Best fit parameters :
T1 = 12 h
T2 = 0.4 T1
G = 0.05 T1
model - best fit
observed
total number of LRI (3.5 days)
1h
3h
6h
12h
24h
time between rotations


C
predicted
observed
total number of LRI (48 hours)
control
A
B
C
D
E
F
gravistimulation patterns


D
total number of observed LRI
control
24h TBR
12h TBR
D
6h TBR
A
B
E
F
C
3h TBR
1h TBR
number of gravistimulations

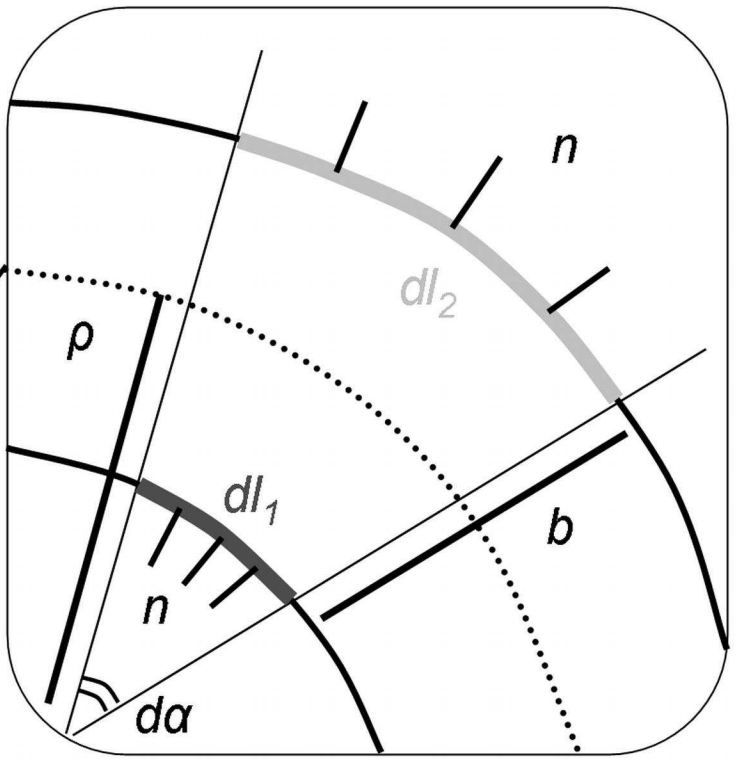

E
y = 1.0128x
R2 = 0.8502
Observed number of LRI
predicted number of LRI


A
LRP density (event/cm)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
(a)
(b)
(a)
(b)
Col0
aux1-22 x UAS::AUX1 aux1-22
J0951 aux1-22 x UAS::AUX1 aux1-22
M0013 aux1-22 x UAS::AUX1 aux1-22

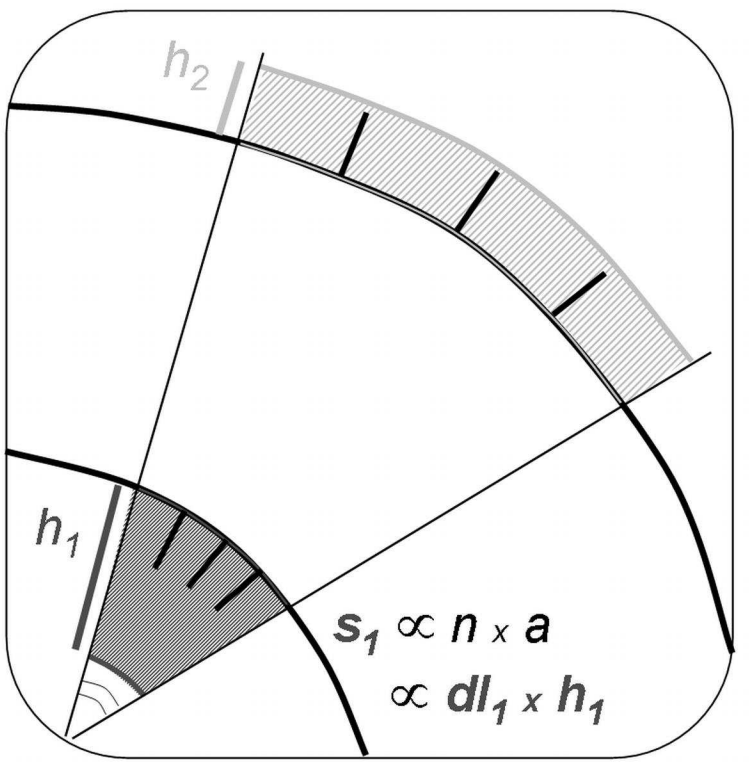

B
LRP density (event/cm)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
(a)
(a)
(a)
Col0 x UAS::axr3
J0951 x UAS::axr3
M0013 x UAS::axr3

A

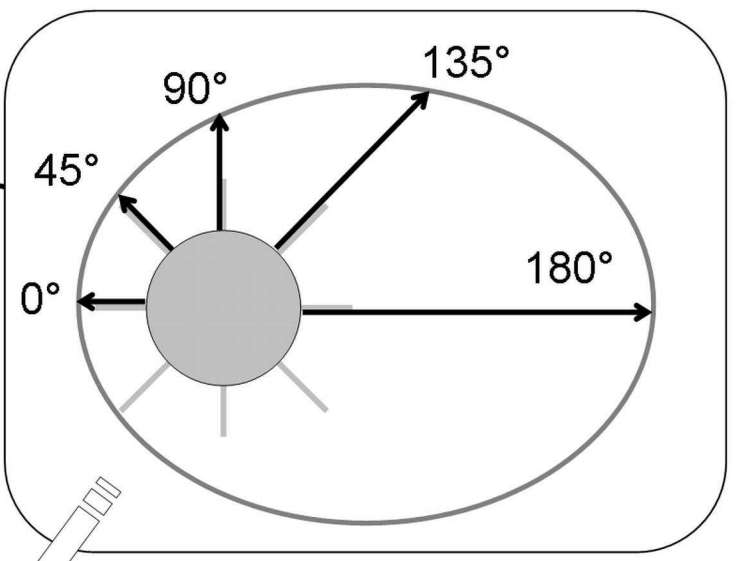
B


n
dl2
ρ
dl1
b
n
dα

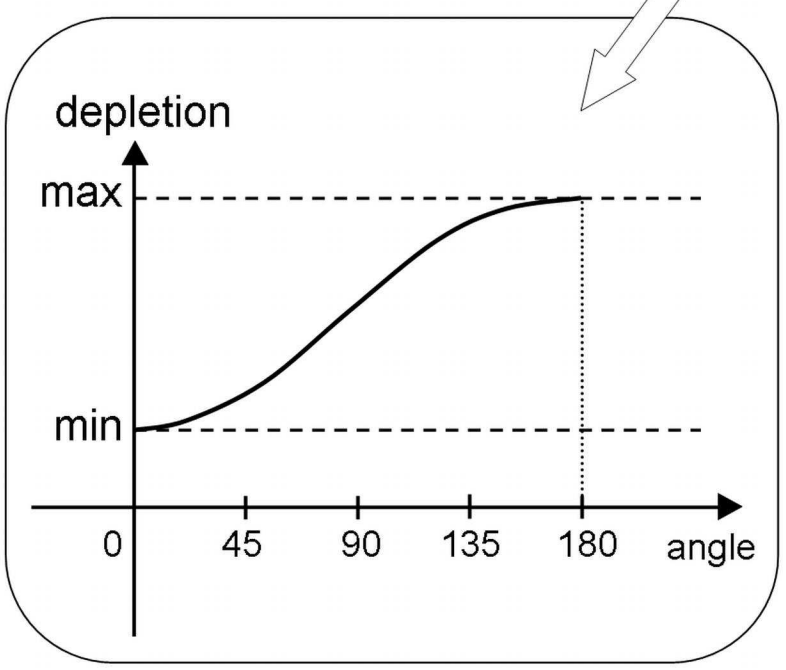
C

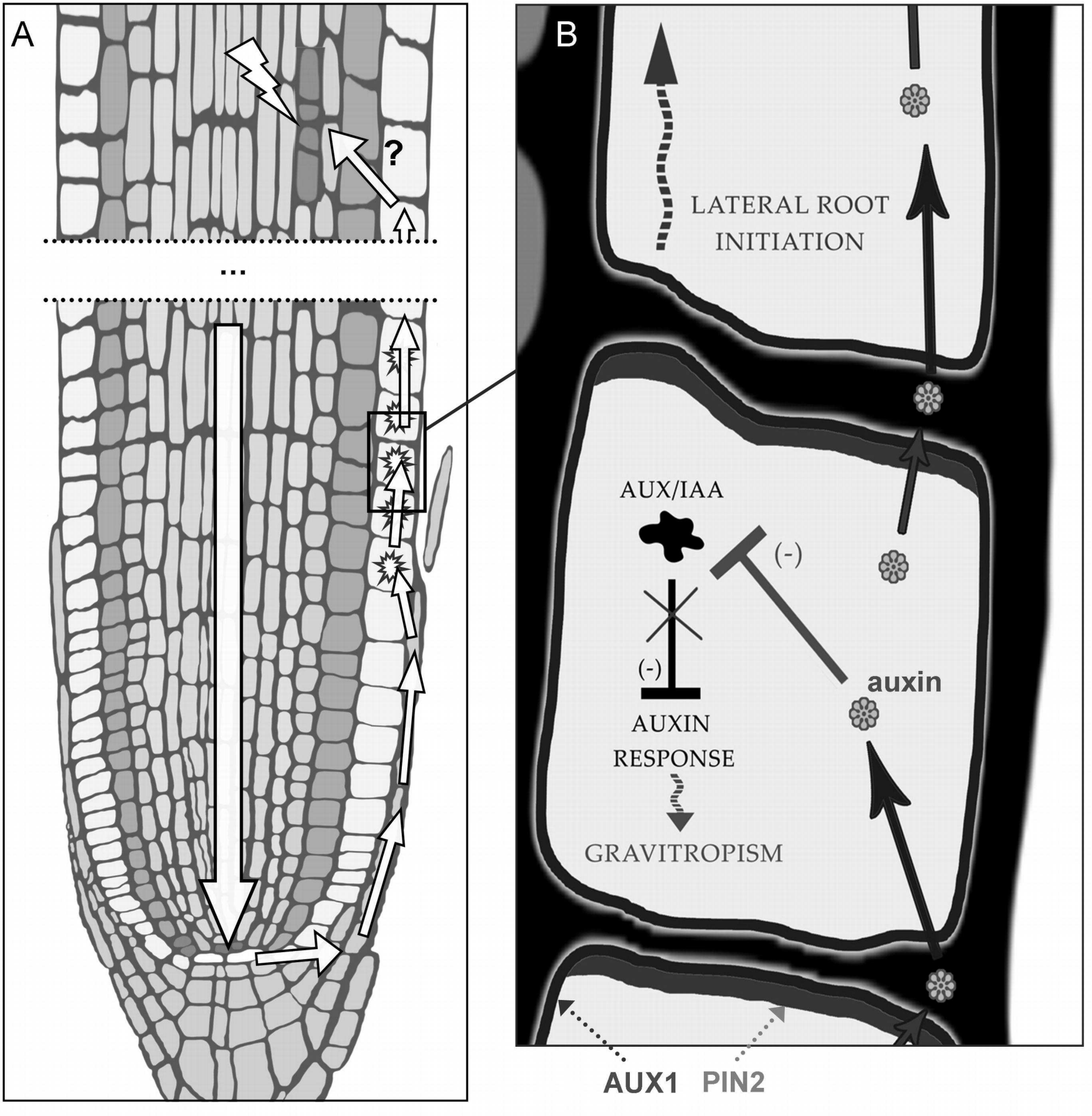

h2
h1
s1 ∝ n x a
∝ dl1 x h1

D


90°
135°
45°
180°
0°

E


depletion
max
min
0
45
90
135
180
angle


A
?
...
B
LATERAL ROOT
INITIATION
AUX/IAA
(-)
(-)
auxin
AUXIN
RESPONSE
GRAVITROPISM
AUX1
PIN2