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Mikaël Lucas, Christophe Godin, Christian Jay-Allemand, Laurent Laplaze

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

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

5 Lucas, M.<sup>1,2</sup>, Godin, C.<sup>2</sup>, Jay-Allemand, C.<sup>3</sup>, and Laplaze L.<sup>1,\*</sup>

6 <sup>1</sup> IRD, UMR DIA-PC (Agro.M/INRA/IRD/UM2), Equipe Rhizogenèse, 911  
7 avenue Agropolis, 34394 Montpellier cedex 5, France

8 <sup>2</sup> INRIA, UMR DAP (Agro.M/Cirad/INRIA/INRA/UMII), Virtual Plants,  
9 TA40/02, Cirad, Avenue Agropolis 34398 Montpellier cedex 5, France

10 <sup>3</sup> Université Montpellier II, UMR DIA-PC (Agro.M/INRA/IRD/UM2), Equipe  
11 Rhizogenèse, Laboratoire de Biochimie et Physiologie Végétale, case 024, 34095  
12 Montpellier cedex 5, France

13

14 Corresponding author: Laurent Laplaze, IRD, UMR DIA-PC, Equipe  
15 Rhizogenèse, 911 avenue Agropolis, 34394 Montpellier cedex 5, France, Tel:  
16 +33 (0)4 67 41 62 02, Fax: +33 (0)4 67 41 62 22, E-mail: [laplaze@mpl.ird.fr](mailto:laplaze@mpl.ird.fr)

17

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19 6 figures

20 6 supplementary figures

21

1 **ABSTRACT**

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

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

15

# 1 INTRODUCTION

2

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

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

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

1 root emergence are regulated independently. The plant hormone auxin plays a  
2 central role in lateral root development. It is the key signal that controls lateral  
3 root initiation (Casimiro *et al.*, 2003; De Smet *et al.*, 2006). Auxin is also  
4 involved in the growth and organisation of lateral root primordia (Benková *et al.*,  
5 2003; Casimiro *et al.*, 2003) and in the emergence of lateral roots from the parent  
6 root (Laskowski *et al.*, 2006).

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

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

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

8

## 9 **MATERIAL AND METHODS**

10

### 11 *Plant Material and Growth*

12

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

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

1 scored using an optical microscope according to Malamy and Benfey (1997).

2 Data were analysed using the Excel statistical package.

3

#### 4 *Microscopy*

5

6 Seedlings were collected and incubated in a solution containing 50 mM sodium

7 phosphate buffer, pH 7.0, 0.5 mM  $K_3Fe(CN)_6$  and  $K_4Fe(CN)_6$ , 0.05% (v/v)

8 Triton X-100, 0.05% (v/v) DMF, 0.02% (v/v) EDTA, and 1 mM 5-bromo-4-

9 chloro-3-indolyl- $\beta$ -glucuronic acid and incubated at 37°C for several hours.

10 Seedlings were then cleared in 70% (v/v) ethanol for 24 hours, before being

11 immersed for 2 hours in 10% (v/v) glycerol 50% (v/v) ethanol; 2h in 30% (v/v)

12 glycerol 30% (v/v) ethanol; 2h in 50% (v/v) glycerol. Seedlings were mounted in

13 50% (v/v) glycerol and visualised using a DMRB microscope (Leica).

14

#### 15 *Design of a mechanistic model of lateral root initiation*

16

17 The mechanistic model of lateral root initiation we introduced (Fig. 3A) was

18 formalized and transcribed in the python programming language as a logical

19 algorithm (Fig S4). Parameter T1 (spontaneous initiation threshold) was

20 estimated directly from the observed data as the mean time between two

21 successive initiations in the control. The two other parameters T2 (induced

22 initiation threshold) and G (cost of gravistimulation) were inferred from

23 observed data, using Python-scripts to explore the parameters-space. Over 1800

24 parameter combinations of T2 and G were tested. The parameter combination

25 corresponding to the best fit of lateral root initiation densities to the observed

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

3

#### 4 *Lateral dissymmetry of soil exploitation along the primary root*

5

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

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

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

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

4 
$$(3) s_1 \propto dl_1 \cdot h_1$$

5 
$$(4) s_2 \propto dl_2 \cdot h_2$$

6 where  $\propto$  stands for proportional and  $h_1$  and  $h_2$  are the respective depth of  
7 exploitation on each side (not to be confounded with root hair length – see Fig.  
8 5C). Under our hypotheses, these surfaces are proportional to the number of root  
9 hairs ( $n$ ) and their harvesting power ( $a$ ). These parameters being the same on  
10 each side of the root, we have:

11 
$$(5) s_1 = s_2 = s \propto n \cdot a$$

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

13 
$$(6) dl_1 \cdot h_1 = dl_2 \cdot h_2$$

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

15 
$$(7) h_1 / h_2 = (\rho + b / 2) / (\rho - b / 2)$$

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

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

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

3

#### 4 *Root hair length analysis*

5

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

12

## 13 **RESULTS**

14

### 15 *Gravistimulation leads to local lateral root initiation*

16

17 Recent studies indicate that lateral root formation is correlated with root waving  
18 in an AUX1-dependent way (De Smet *et al.*, 2007). In order to test whether  
19 gravitropism and lateral root initiation are co-regulated, we tested the effect of  
20 gravistimuli on lateral root initiation. Transgenic *Arabidopsis* plants carrying a  
21 *ProCYCB1::GUS* marker for cell division were grown on vertical plates for 30  
22 hours after germination and then subjected to a gravistimulus (90° rotation) every  
23 12h for 3.5 days. Two different patterns of gravistimulation were used leading to  
24 stair- or crenel-shaped root growth (see Fig. S1). Plants were then left to grow  
25 for an extra 60h before testing for GUS activity. Roots were then cleared and

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

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

20 We next analysed the timing of lateral root initiation following a  
21 gravistimulus. Six batches of *ProCycB1::GUS* plants were grown for 30h after  
22 germination on vertical Petri dishes then subjected to a 90° gravistimulus every  
23 six hours with a one hour delay between each batch. This was done for 24h and  
24 plants were then harvested and stained for GUS activity. This enabled us to  
25 observe gravistimulated zones every hour from 0 to 25h after stimulation. The

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

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

19

### 20 *The rhythm of lateral root initiation is modified by external clues*

21

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

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

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

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

13

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

16

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

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

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

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

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

6 In order to validate the model, we designed a new experiment to evaluate its  
7 predictive power. We selected 6 new gravistimulation patterns (Fig. S5) not  
8 previously tested, with either regular or irregular spacing between  
9 gravistimulations. Based on direct pattern observation, it was not possible to  
10 guess the total number of lateral root initiations that would be produced. Those  
11 patterns were applied on *ProCYCB1::GUS* seedlings for 48h, according to the  
12 previously described protocol of gravistimulation. The total number of lateral  
13 root initiations for the various seedlings groups were scored and compared to the  
14 total number of lateral root initiations predicted by the model (Fig. 3C). This  
15 experiment was repeated twice independently.

16 We found that the total number of lateral root initiations is not governed by  
17 the number of gravistimulations (Fig. 3D). The quantitative model was able to  
18 predict with accuracy the total number of LRI for each pattern, over a large range  
19 of total number of lateral root initiations without loss of accuracy (Fig. 3C,E)  
20 thus showing that the total number of lateral root initiations is actually a function  
21 of the structure of the gravistimulation pattern. Similarly to the inhibitory field  
22 models for the shoot apical meristem (Douady and Couder, 1996; Smith *et al.*,  
23 2006), the proposed model suggests that lateral root initiations are submitted to  
24 inhibition fields (here represented by auxin consumption) that control their  
25 patterning.

1

2 *Common auxin fluxes regulate gravitropism and lateral root*  
3 *initiation*

4

5 Gravitropism and lateral root initiation are both regulated by auxin (Casimiro *et*  
6 *al.*, 2001; Swarup *et al.*, 2005). Gravity is perceived in the central part of the root  
7 cap and gravitropism relies on an AUX1-dependent acropetal auxin flux from the  
8 root apex through the lateral root cap and the elongating root epidermis where it  
9 induces changes in cell elongation (Ottenschläger *et al.*, 2003; Swarup *et al.*,  
10 2005). AUX1 encodes a high-affinity auxin influx carrier (Yang *et al.*, 2006). On  
11 the other hand, very little is known about the auxin fluxes that are responsible for  
12 lateral root initiation in the root pericycle. However, the *aux1* mutant is perturbed  
13 in both lateral root initiation and root gravitropism and recent studies suggest a  
14 common auxin transport pathway for gravitropism and lateral root initiation (De  
15 Smet *et al.*, 2007).

16 Since we found that gravitropism and lateral root initiation are co-regulated  
17 we tested whether both processes were dependent on the same auxin transport  
18 route. We used a transactivation strategy to complement the *aux1* mutant in  
19 different tissues at the root apex as described by De Smet *et al.* (2007) and to test  
20 the effect on lateral root initiation. Plants expressing *UAS:AUX1* under the  
21 control of the GAL4 enhancer trap lines M0013 (root cap) or J0951 (root cap and  
22 expanding root epidermis) in an *aux1-22* mutant background (Swarup *et al.*,  
23 2005) were grown for 10 days on vertical plates. They were then harvested and  
24 scored for gravitropism and lateral root primordia density. Our results on lateral  
25 root initiation (Fig. 4A) were similar to those obtained by De Smet *et al.* (2007)

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

4 We next tested whether auxin needs to be perceived in the tissues through  
5 which it flows for lateral root initiation. We transactivated a dominant negative  
6 version of the AXR3 protein (*axr3-1*) that was previously shown to inhibit auxin  
7 response in different root tissues (Swarup *et al.*, 2005) and tested the effects on  
8 gravitropism and lateral root initiation. F1 plants were grown for 10 days on  
9 vertical plates before analysis. Our results on gravitropism were similar to those  
10 of Swarup *et al.* (2005). When *axr3-1* was transactivated in the root cap, using  
11 ET line M0013, it had no effect on gravitropism or lateral root initiation (Fig.  
12 4B). When *axr3-1* was transactivated in both the root cap and the root epidermis  
13 using enhancer trap line J0951, it abolished the gravitropic response of the root  
14 but did not perturb lateral root initiation (Fig. 4B). Thus our results suggest that  
15 in contrast to gravitropism, auxin does not need to be perceived in the root  
16 epidermis in order to direct lateral root formation.

17

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

20

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

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

20

## 21 **DISCUSSION**

22

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

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

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

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

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

7 Finally, our experimental data suggest a mechanism for co-regulation of  
8 gravitropism and lateral root initiation (Fig. 6). Auxin, the key signal that  
9 controls both processes, is produced in leaf primordia and transported to the root  
10 via the vascular basipetal flow (Friml *et al.*, 2006). Root meristems and lateral  
11 root primordia can also produce auxin (Ljung *et al.*, 2005). An auxin maximum  
12 is generated in the root columella (Sabatini *et al.*, 1999) and auxin is  
13 redistributed in the meristem from the columella in a PIN3-dependent way. Upon  
14 gravistimulation, PIN3 is retargeted to the lower face of columella cells thus  
15 creating an asymmetric auxin distribution (Friml *et al.*, 2002). Auxin is  
16 transported from the root tip through the lateral root cap and in the elongating  
17 root epidermis in an AUX1/PIN2-dependent way thus generating an acropetal  
18 auxin flux (Swarup *et al.*, 2005). Auxin perception in the epidermis is then  
19 responsible for root gravitropism by changing the relative elongation of  
20 epidermal cells (Swarup *et al.*, 2005). Our transactivation experiments together  
21 with previous results (De Smet *et al.*, 2007) indicate that the same acropetal flux  
22 is responsible for lateral root initiation further up the root. This is consistent with  
23 previous data indicating that acropetal auxin transport from the root tip is  
24 responsible for lateral root initiation (Casimiro *et al.*, 2001; Bhalerao *et al.*,  
25 2002). Moreover, our *axr3* transactivation data suggest that while gravitropism

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

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

22

23 **SUPPLEMENTARY DATA**

24

1 **Fig. S1.** Gravistimulation protocols. Seedlings were grown on vertical plates and  
2 gravistimulated by a periodic (period T) 90° rotation of the growth plates. Two  
3 different rotation protocols were used to generate either crenel-shaped or stair-  
4 shaped roots. Roots subjected to these protocols were grown under stimulation  
5 for 3.5 days and with no stimulation for an additional 2.5 days before harvesting.  
6

7 **Fig. S2.** Localisation of lateral root initiation in a gravistimulated root. A  
8 Pro<sub>CYCB1</sub>::GUS seedling was subjected to crenel gravistimulation at 12-h  
9 intervals. Lateral root initiations were localised and their development scored.  
10

11 **Fig. S3.** Influence of varying gravistimulation on lateral root initiation density  
12 (crenel-shaped roots). (A) Vertically grown Pro<sub>CYCB1</sub>::GUS seedlings were left to  
13 grow (control; n=20) or were subjected to gravistimulation at intervals of 1 h (n  
14 = 24), 3 h (n = 20), 6 h (n = 21), 12 h (n = 21) or 24 h (n= 24) over a period of  
15 3.5 days (1), then left to grow for 2.5 days without stimulation (2). Bars = 1 cm.  
16 (B) Occurrence of LRI in root turns. (C) Occurrence of lateral root initiation  
17 between root turns. Due to the particular configuration of roots subjected to  
18 gravistimulation at 1-h and 3-h intervals (respectively presenting no visible turns  
19 and only turns), some values were not determined (na = not applicable). (D)  
20 Effect of gravistimulation on root growth. Length of the gravistimulated root  
21 segments (first 5 days of growth) and non-gravistimulated root segments (last 2.5  
22 days of growth) were also determined. (E) Lateral root initiation densities were  
23 determined in gravistimulated and non-gravistimulated root segments. Different  
24 letters indicate significantly different results as tested by a Student T-test ( $P <$   
25 0.01).

1

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

4

5 **Fig. S5.** Gravistimulation patterns used for the evaluation of our model. Six  
6 previously non-tested gravistimulation patterns were applied to seedlings over a  
7 48h period starting 30 hours after germination. Gravistimulation are indicated by  
8 black dots. The total number of gravistimulation for each pattern varies between  
9 10 and 25. After the last gravistimulus, seedlings were left to grow undisturbed  
10 for 24h before harvest and observation.

11

12 **Fig. S6.** Curve radius, depth of exploration and root hair length. (A) Curve of  
13 the function  $h_1 / h_2 = (\rho + b / 2) / (\rho - b / 2)$ . (see figure 4 for additional details on  
14 the parameters) (B) Curve radius estimated for a portion of a gravistimulated root  
15 ( $90^\circ$  re-orientation).  $b$  and  $\rho$  are the thickness and the curve radius of the chosen  
16 root portion respectively. (C) Curve radius estimated for various root turns of a  
17 waving root. (D) Ratio of exploration depths ( $h_1 / h_2$ ) for various values of ( $\rho$ ).  
18 (E) Direct visualisation of root hair on both sides of a root turn. (F) Root hair  
19 length was measured on both sides of root turns ( $n=20$ ) and straight roots.  
20 Different letters indicate significantly different results as tested by a Student T-  
21 test ( $P < 0.01$ ).

22

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24

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3 Plants project). M.L. is the recipient of a PhD grant from the French *Ministère de*  
4 *l'Enseignement Supérieur, de la Recherche et de la Technologie.*

5

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23

24 **FIGURE LEGENDS**

25

1 **Fig. 1.** Influence of gravistimulation on lateral root initiation (LRI). (A)  
2 Segmentation of the root between gravistimulated (turn) and non-gravistimulated  
3 (straight) zones used for determination of the position of LRI. (B) Percentage of  
4 lateral root initiations in the gravistimulated and non-gravistimulated zone of  
5 crenel-shaped (n = 23) and stair-shaped (n = 24) roots gravistimulated at 12-hour  
6 intervals (see supplementary figure 1 for additional details on the  
7 gravistimulation protocol). (C) Kinetic of lateral root initiation after  
8 gravistimulation. Six batches of roots (n = 40) were gravistimulated every 6  
9 hours over a 24-hour period before harvest and GUS staining, with a one-hour  
10 shift between each batch. This led to the formation of root turns aged from 0 to  
11 25 hours, which were scored for lateral root initiation. (D) Kinetic of LRP  
12 development after gravistimulation. Arrows indicate the period of time over  
13 which the different developmental stages were observed.

14

15 **Fig. 2.** Influence of the gravistimulation interval on lateral root initiation  
16 density. (A) Vertically grown *ProCYCB1::GUS* seedlings were left to grow (control;  
17 n=20) or were subjected to gravistimulation at intervals of 1 h (n = 24), 3 h (n =  
18 20), 6 h (n = 21), 12 h (n = 21) or 24 h (n= 24) over a period of 3.5 days (1), then  
19 left to grow for 2.5 days without stimulation (2). Bars = 1 cm. (B) Occurrence of  
20 lateral root initiation in root turns. (C) Occurrence of lateral root initiation  
21 between root turns. Due to the particular configuration of roots subjected to  
22 gravistimulation at intervals of 1 h and 3 h (respectively presenting no visible  
23 turns and only turns), some values were not determined (na = not applicable). (D)  
24 Effect of gravistimulation on root growth. The length of the gravistimulated root  
25 segments (first 5 days of growth) and non-gravistimulated root segments (last 2.5

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

5

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

25

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

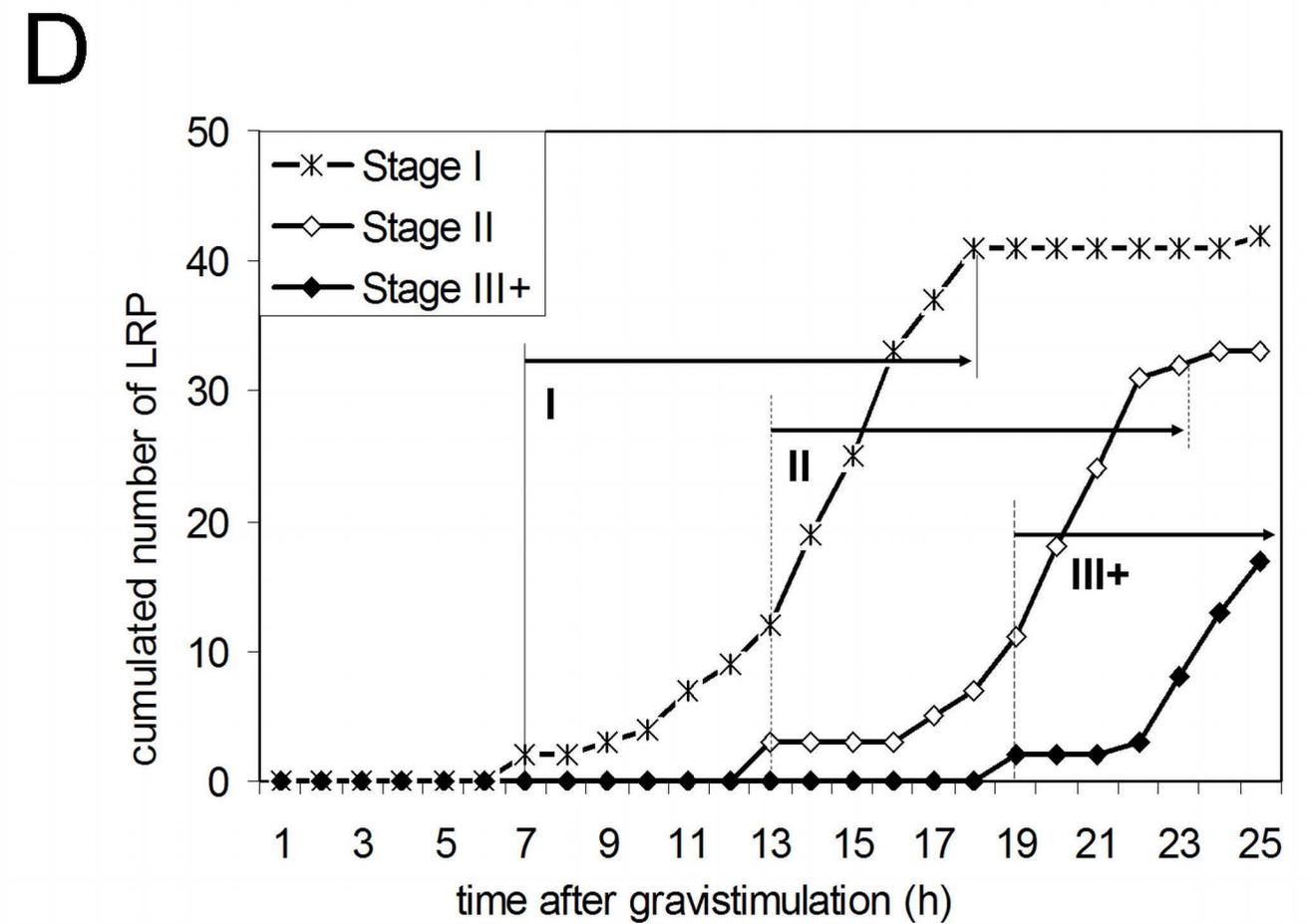
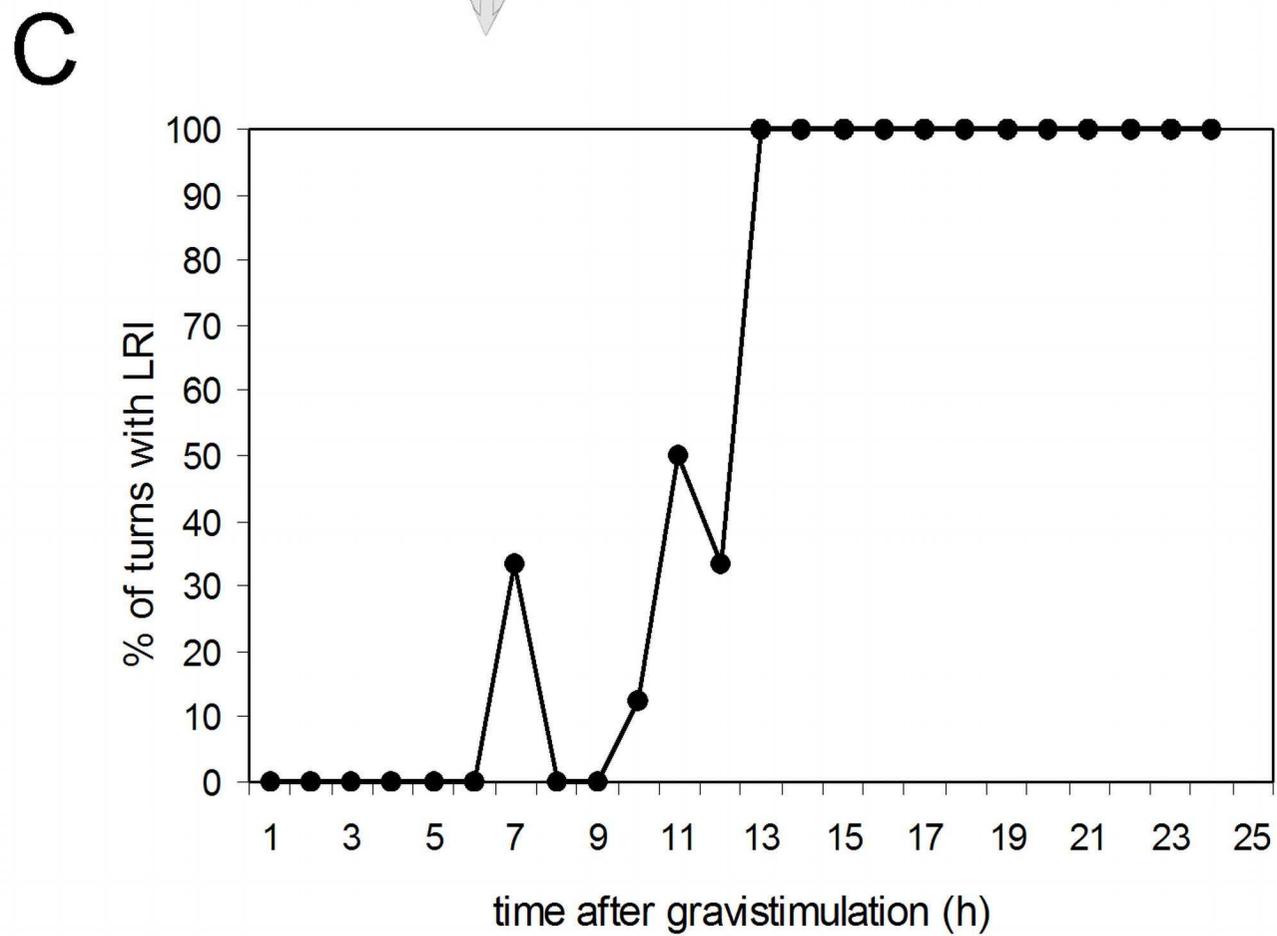
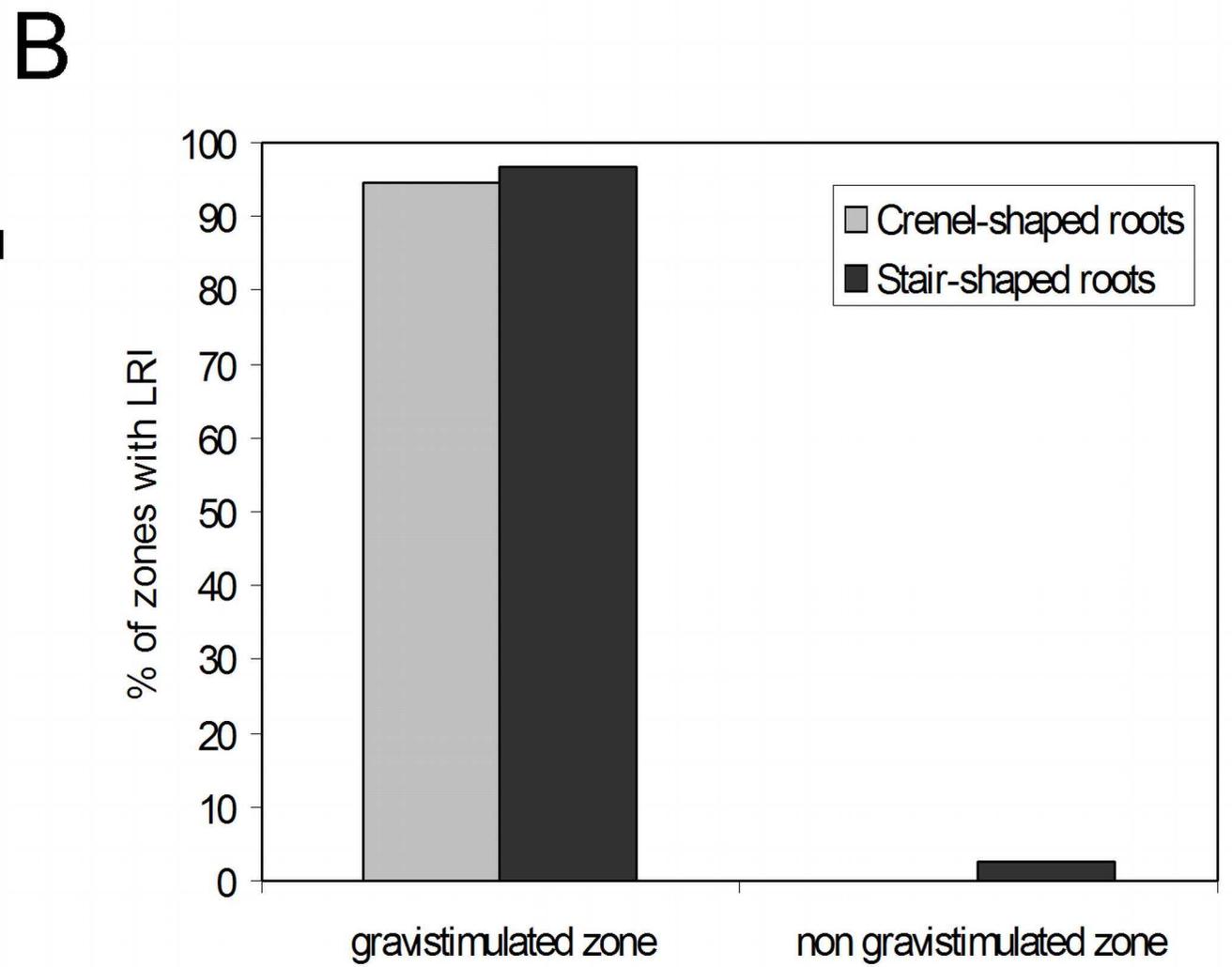
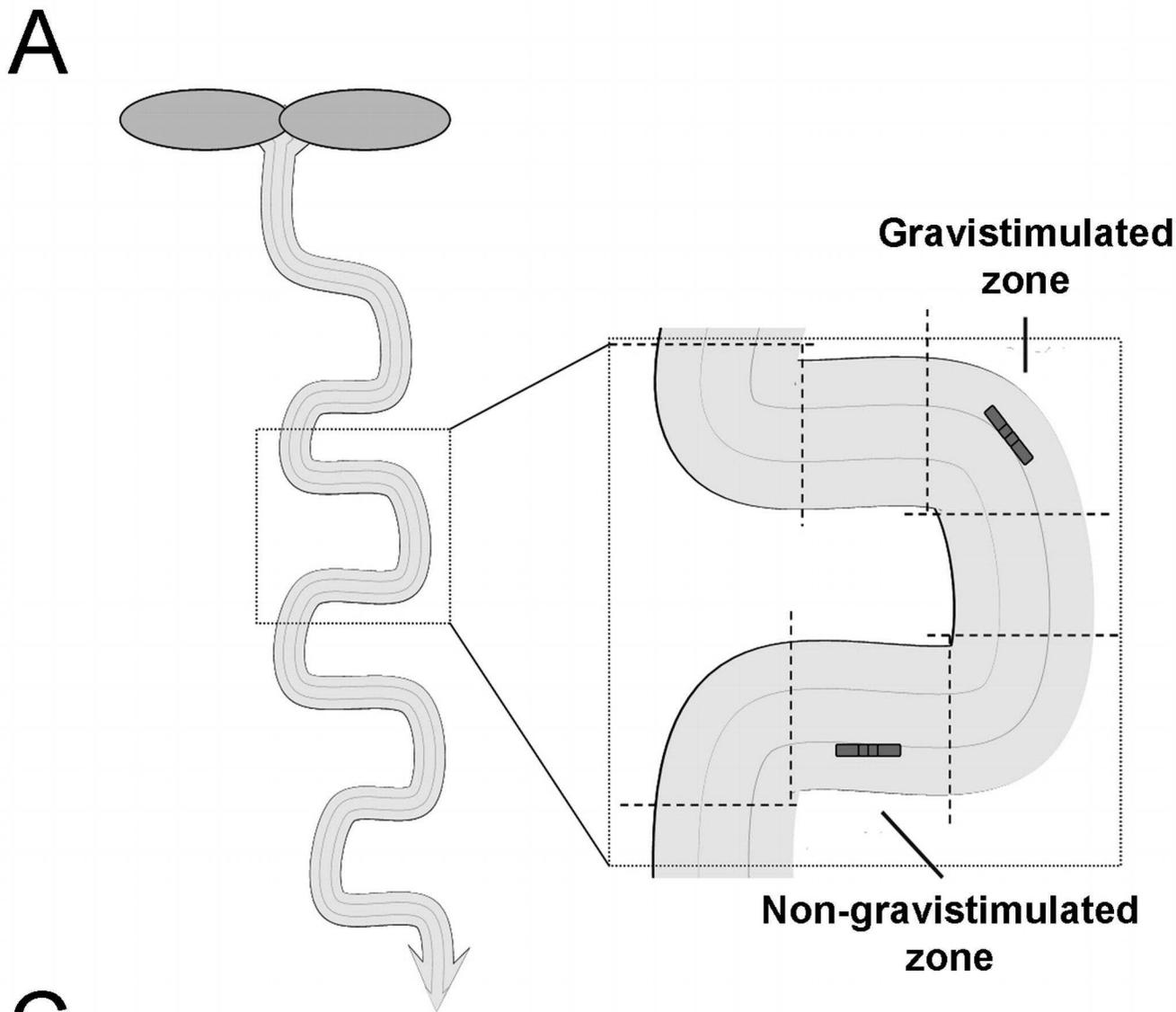
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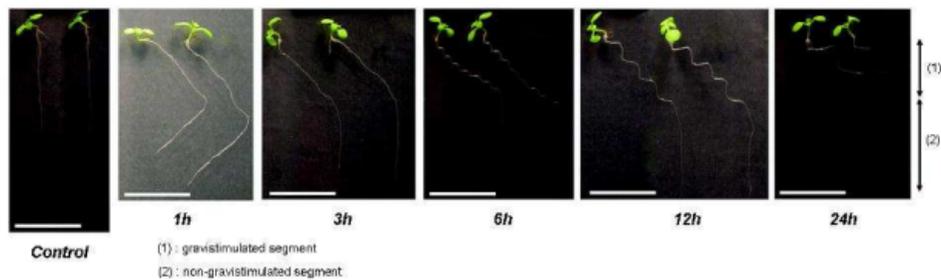
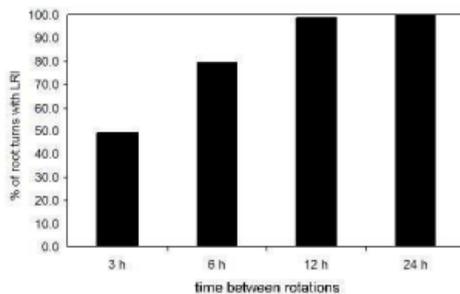
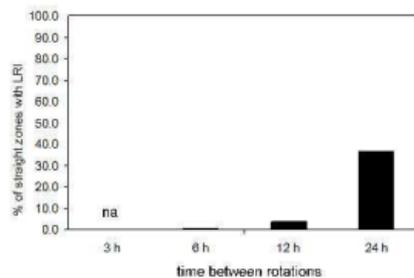
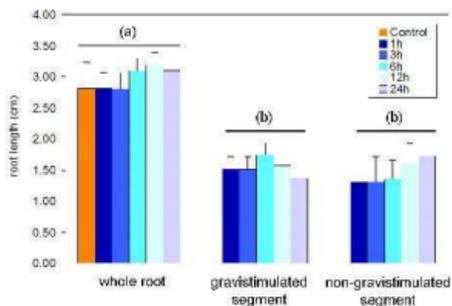
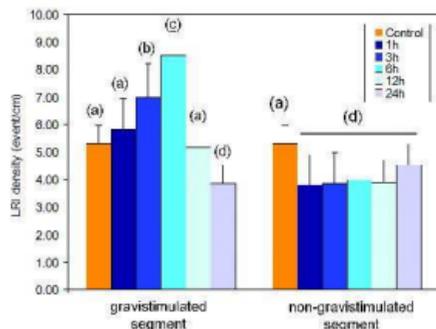
6 **Fig. 5.** Influence of root bending on resource exploitation. (A) Exploitation of  
7 soil resources by a bent root (grey zone). (B) Infinitesimal portion of root turn.  
8 Parameters are ( $n$ ), number of root hairs; ( $\rho$ ), curve radius of selected zone; ( $b$ ),  
9 thickness of the root; ( $d\alpha$ ), angle made by selected zone; ( $dl_1$ ) and ( $dl_2$ ), length of  
10 curved zone on each side of the root turn. (C) Area of soil exploited. Parameters  
11 are ( $s_1$ ) and ( $s_2$ ), area of soil exploited each side of the root turn; ( $a$ ), absorption  
12 strength of a single root hair ; ( $h_1$ ) and ( $h_2$ ), depth of soil exploited on each side  
13 of the root turn. (D) Transversal profile of soil exploitation at a root turn. (E)  
14 Corresponding depletion of resources.

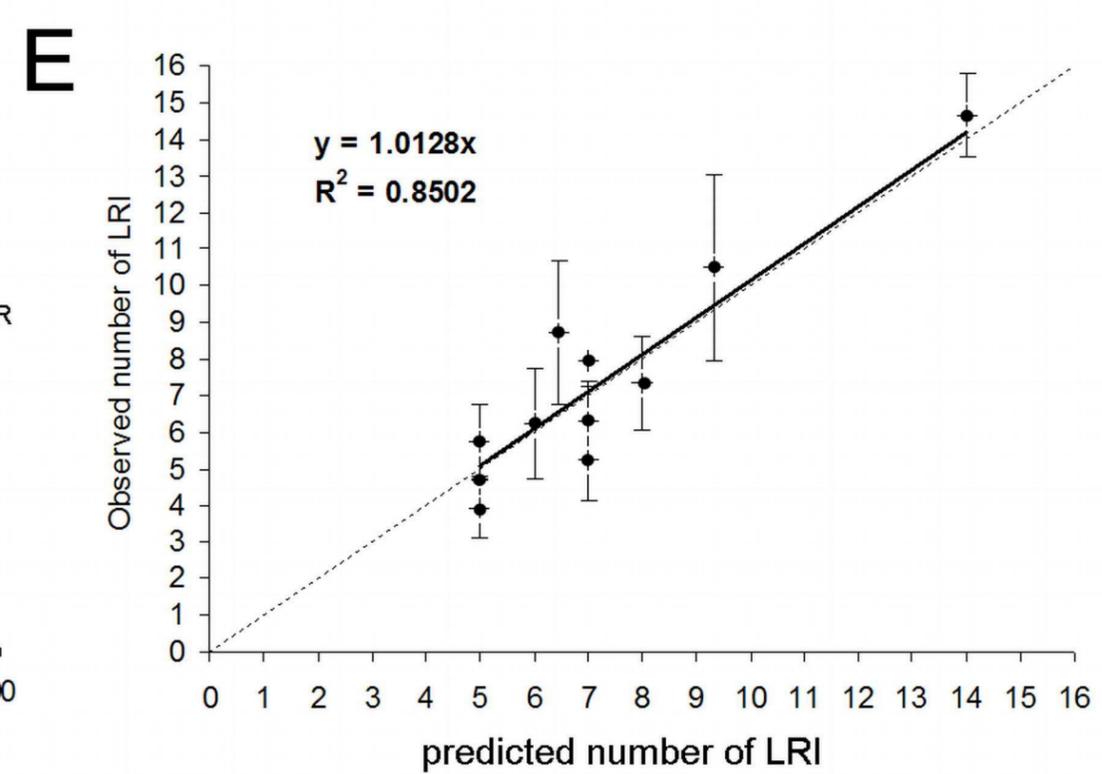
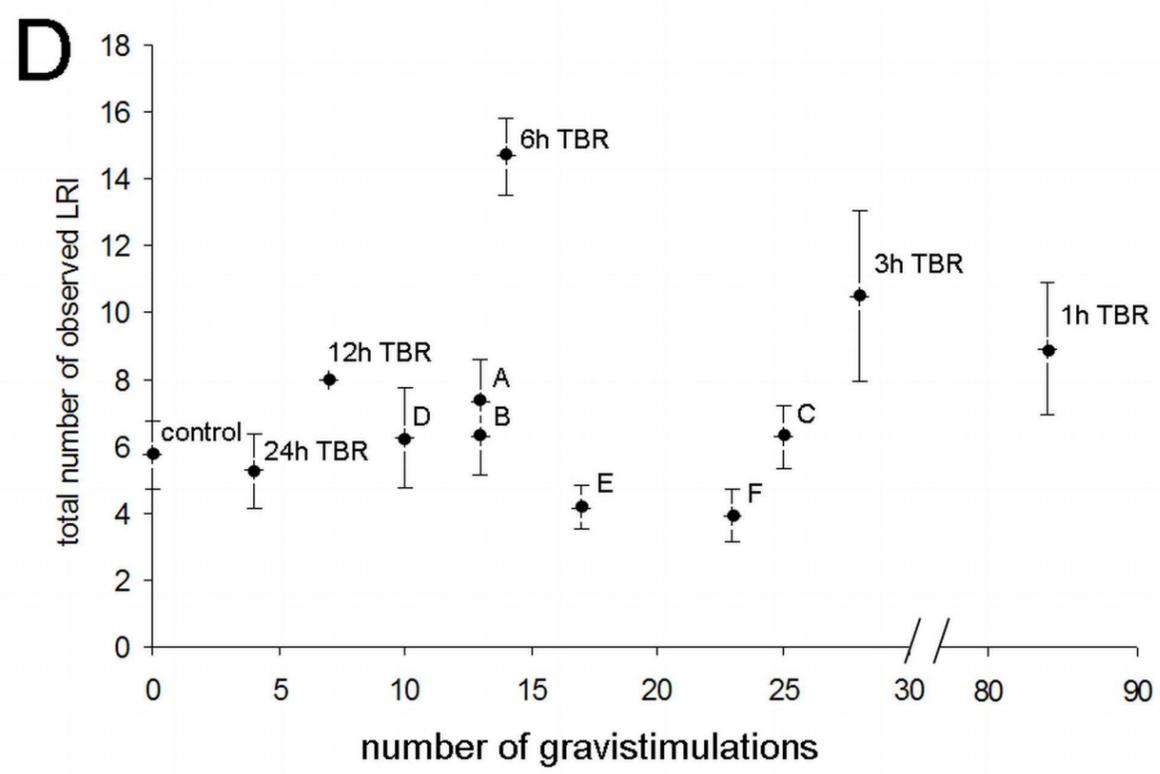
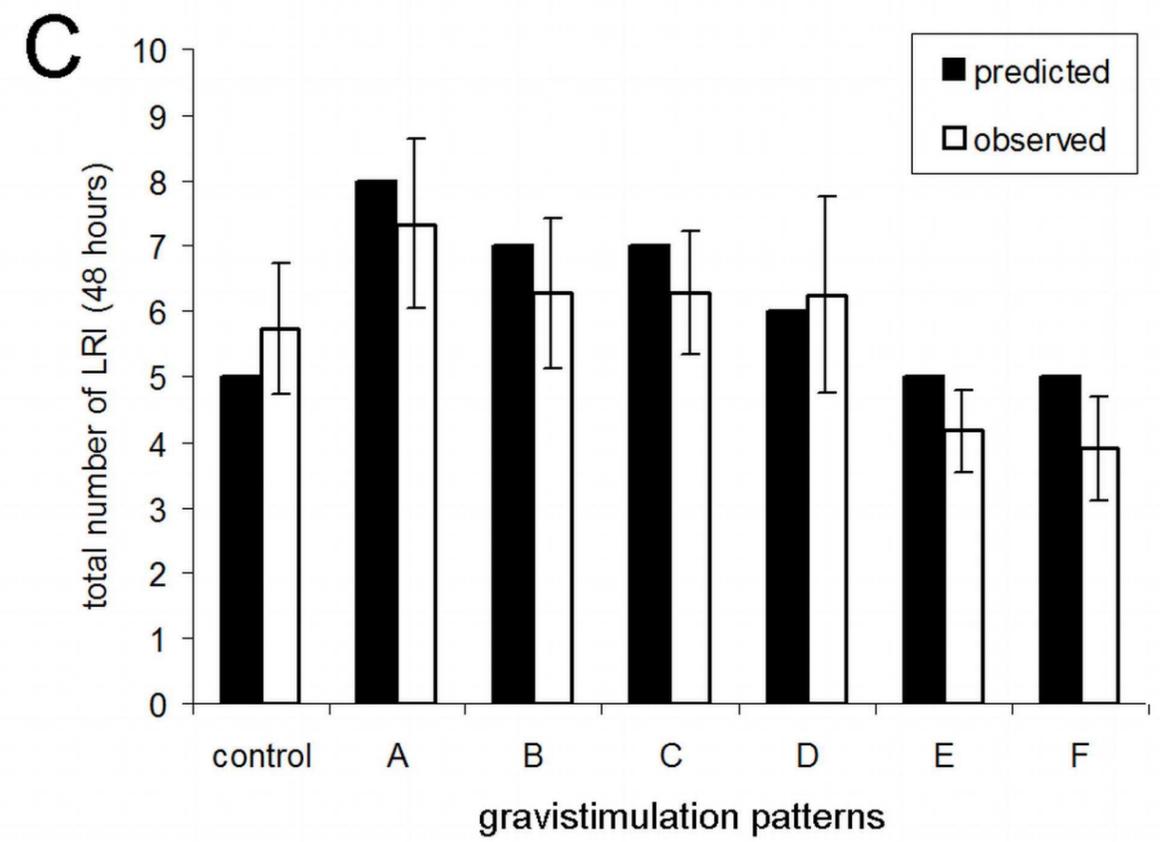
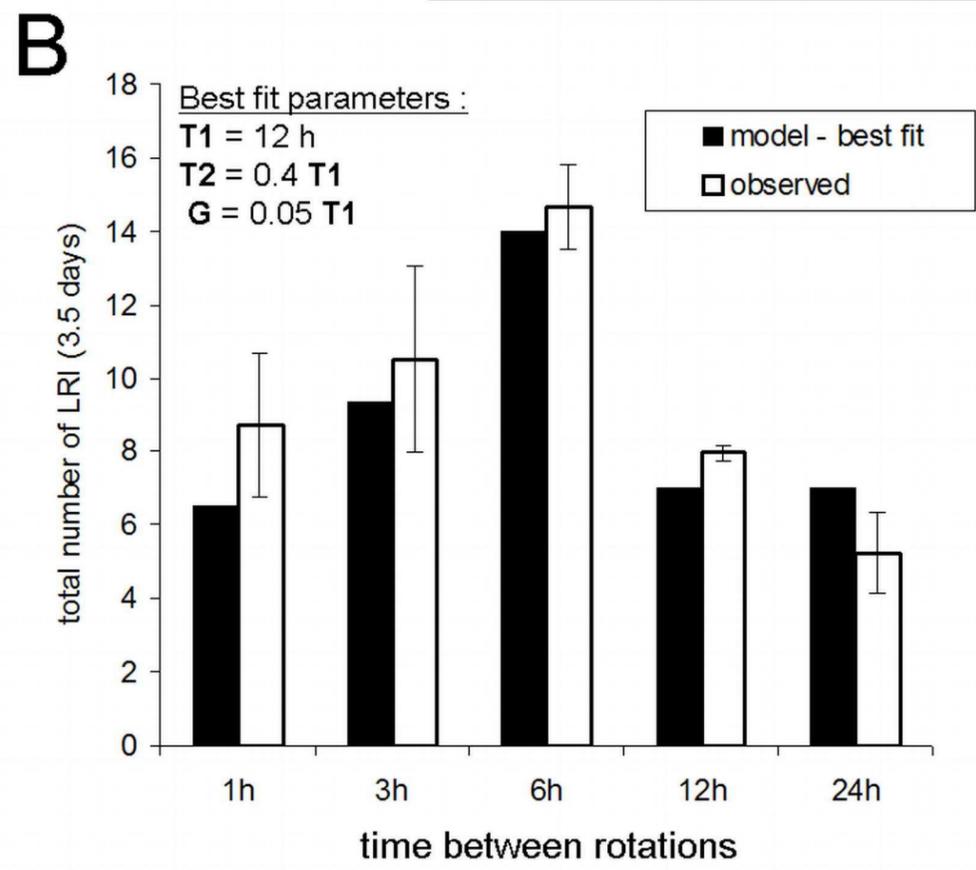
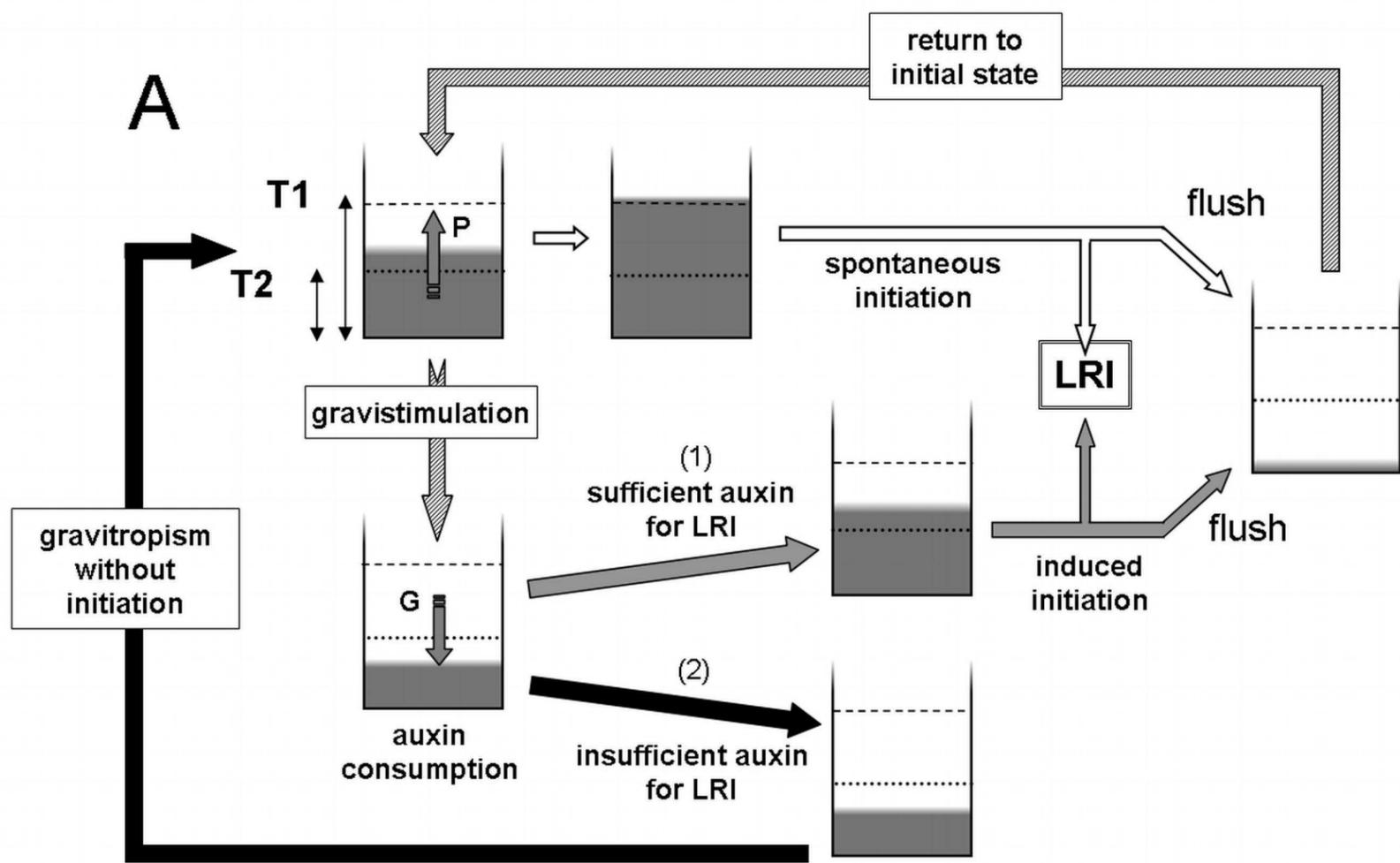
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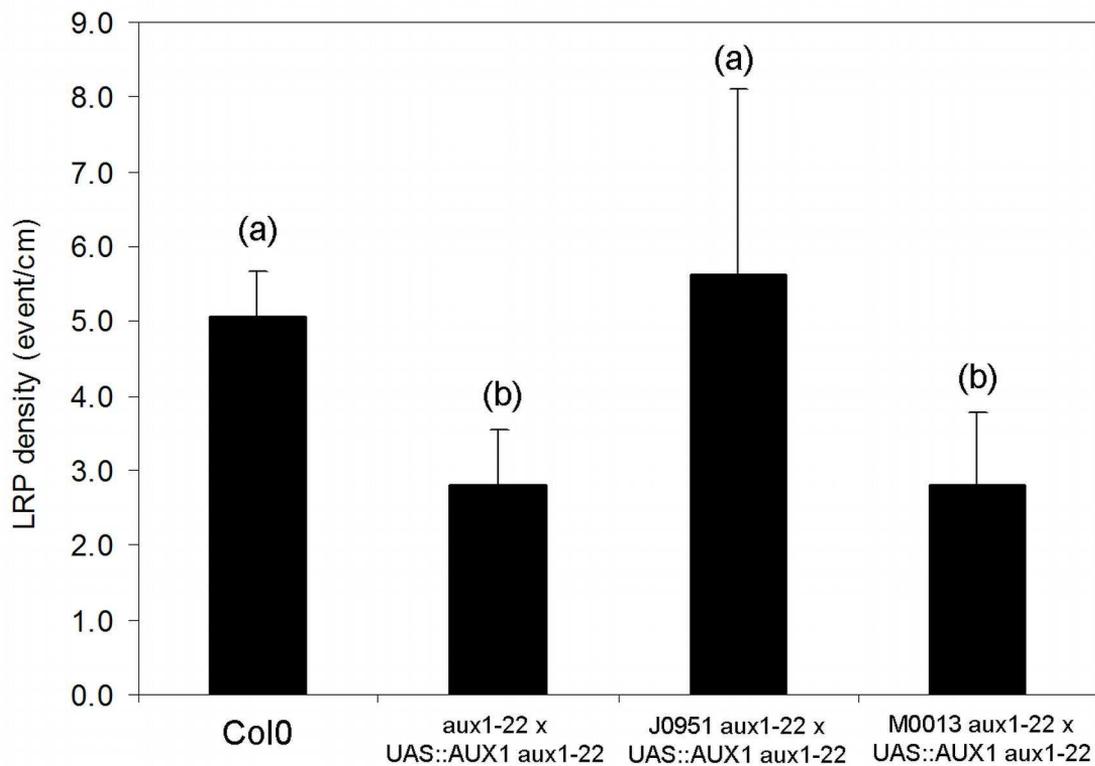
16 **Fig. 6.** Model of gravitropism and lateral root initiation signalling pathways.  
17 Auxin fluxes responsible for gravitropism (A) pass through the lateral root cap  
18 and are perceived in the epidermal cells via the AUX/IAA molecular pathway,  
19 eliciting auxin response (B). Auxin fluxes responsible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

23



**A****B****C****D****E**



**A****B**