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

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Received 19 February 2007; Revised 28 June 2007; Accepted 2 July 2007

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 it is shown that lateral root initiation and root gravitropism, two processes that are regulated by auxin, are co-regulated in Arabidopsis. A mathematical model was generated 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 optimizing soil exploration as supported by a simple quantitative analysis.

Key words: 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-characterized 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 organization 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, efforts were focused on auxin fluxes in the root apex. Auxin fluxes have already been studied in the apical root meristem (Bilou et al., 2005), but little is known about the fluxes that are responsible for lateral root initiation. Interestingly, data suggest a link between root waving, which depends on gravitropism/thigmotropism and lateral root initiation (Fortin et al., 1989; De Smet et al., 2007). 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 it is shown that a gravistimulus quickly leads to lateral root initiation at the site of reorientation of root growth. Gravistimulation was used to analyse the pattern of lateral root initiation. The 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. The data were used to generate a mathematical model that can predict the effects of gravistimulations on lateral root initiation density. Moreover, it was observed that the auxin flux responsible for lateral root initiation goes through the same route as the auxin responsible for gravitropism, thus explaining the co-regulation of these two processes. Finally, mathematical modelling suggests that the co-regulation of root bending and branching optimizes soil exploration by the root system.

Materials and methods

Plant material and growth

Wild-type (Col-0) seeds were obtained from the NASC. Pro_CYP81F1::GUS (Col-0 background) seeds were provided by Dr P Doerner (University of Edinburgh, UK). J0951, M0013, and UAS-axr3 lines in wild-type (Col-0) background and J0951, M0013, and 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 Fig. S1 at JXB online. 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/idesc.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 K3Fe(CN)6, and K4Fe(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 h, before being immersed for 2 h in 10% (v/v) glycerol, 50% (v/v) ethanol; 2 h in 30% (v/v), glycerol 30% (v/v) ethanol; 2 h in 50% (v/v) glycerol. Seedlings were mounted in 50% (v/v) glycerol and visualized using a DMRB microscope (Leica).

Design of a mechanistic model of lateral root initiation

The mechanistic model of lateral root initiation (Fig. 3A) was formalized and transcribed in the Python programming language as a logical algorithm (see Fig. S4 at JXB online). Parameter T1 (spontaneous initiation threshold) was estimated directly from the data observed 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. The effect of such dissymmetry was quantified in terms of the availability of local resources using simple mathematical modelling. As lateral root initiation in Arabidopsis thaliana takes place in a plane defined by the two protoxylem strands, this analysis was made in a two-dimensional space. In addition, a number of simplification hypotheses was made. 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). It was considered that each root hair harvests a fixed pool of resources (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 two-dimensional space, here the corresponding exploited surfaces, s1 on the inner side and s2 on the outer side are considered (Fig. 5C). Also it is assumed that each root turn corresponds to a portion of a circle of radius r. Considering an infinitesimal portion of root turn defined by the angle dα, soil exploitation takes place over dl1 (inner side) and dl2 (outer side; Fig. 5B). If b represents the thickness of the root (assumed to be constant in the zone concerned), then

\[ dl_1 = (r - b/2)dα \]  

\[ dl_2 = (r + b/2)dα \]

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

\[ s_1 = \pi dl_1 b_1 \]  

\[ s_2 = \pi dl_2 b_2 \]

where a stands for proportional and h1 and h2 are the respective depth of exploitation on each side (not to be confused with root hair length).
length; see Fig. 5C). Under the present hypotheses, these surfaces are proportional to the number of root hairs \( n \) and their harvesting power \( a \). As these parameters are the same on each side of the root

\[
s_1 = s_2 = s = na
\]  

(5)

From (3), (4), and (5)

\[
dl_1 h_1 = dl_2 h_2
\]  

(6)

and from (1), (2), and (6)

\[
h_1/h_2 = (\rho + b/2)/(\rho - b/2)
\]  

(7)

This equation gives the ratio between the depth of exploration on each side as a function of \( \rho \) (see Fig. S6A at JXB online). If \( \rho \) tends toward infinity, i.e., the root becomes completely straight (infinite impossibility (roots turn with an inner side of negative length was possible to estimate various values for \( \rho \)).

Equation (7) is not valid for \( \rho \) inferior to \( b/2 \) as this is a biological impossibility (roots turn with an inner side of negative length \( dl_1 \)).

Using pictures of gravistimulated root turn and waving roots, it was possible to estimate various values for \( \rho \) (see Fig. S6B, C at JXB online). These values correspond to a ratio \( h_1/h_2 \) varying between 1.4 and 3 (see Fig. S6D at JXB online). 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 described previously (Laplae et al., 2005). Plates were then subjected to three 90° gravistimulations at 12 h time intervals, starting 30 h after germination. Pictures of the plants were obtained using an MZFLIII (Leica) dissecting microscope equipped with a digital camera. Root hair length was measured with the UTHSCSA ImageTool package.

**Results**

**Gravistimulation leads to 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, the effect of gravistimuli on lateral root initiation was tested. Transgenic Arabidopsis plants carrying a Pro\( \text{CYCB1} \cdot \text{GUS} \) marker for cell division were grown on vertical plates for 30 h after germination and then subjected to a gravistimulus (90° rotation) every 12 h for 3.5 d. Two different patterns of gravistimulation were used leading to stair- or crenel-shaped root growth (see Fig. S1 at JXB online). Plants were then left to grow for an extra 60 h 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.

It was observed that lateral root initiation occurred in >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, it was observed that it took 4 h in the present growth conditions for all root apices to reorient their growth direction after a 90° gravistimulus (data not shown) in agreement with previous studies (Swarup et al., 2005). In the present experiment, there was about 4 h of gravistimulated growth followed by about 8 h of non-gravistimulated root growth. If lateral root initiation occurs randomly or regularly, about two-thirds of the lateral root primordia would be expected to occur in the non-gravistimulated zone. Therefore it is concluded that lateral root initiation is induced in response to gravitropic root bending.

Next the timing of lateral root initiation following a gravistimulus was analysed. Six batches of Pro\( \text{CYCB1} \cdot \text{GUS} \) plants were grown for 30 h after germination on vertical Petri dishes then subjected to a 90° gravistimulus every 6 h with a 1 h delay between each batch. This was done for 24 h and plants were then harvested and stained for GUS activity. This enabled gravistimulated zones to be observed every hour from 0 h to 25 h after stimulation. The occurrence and stage of development of lateral root primordia in root turned were noted (Malamy and Benfey, 1997). The first occurrence of stage I lateral root primordia was found 7 h after gravistimulation (Fig. 1C). All the gravistimulated zones showed lateral root initiation 13 h after gravistimulation (Fig. 1C). Stages II and III of lateral root development occurred 6 h and 12 h after lateral root initiation, respectively (Fig. 1D). The present data therefore indicate that lateral root initiation occurs rapidly after gravistimulation.

It was observed that lateral root primordia always appeared on the external side of the bend (100%, \( n = 2677 \) lateral root primordia observed; see Fig. S2 at JXB online). 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. By contrast, the crenel-shaped roots had two initiations on one side followed by two initiations on the other side (see Fig. S2 at JXB online). In this case, it was 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

It was shown that lateral root initiation can be initiated by gravistimuli applied every 12 h. 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 h. In order to test whether lateral root initiation was strictly controlled by an internal rhythm, the experimental design described previously was used and gravistimuli were applied every 1, 3, 6, 12, or 24 h (Fig. 2A; see Fig. S3A at JXB online). Similar results were obtained for stair- and crenel-shaped roots (Fig. 2; and see Fig. S3 at JXB online, respectively). For periods of 6, 12, and 24 h between gravistimuli, lateral root initiation was found in >90% of root turns (gravistimulated zones; Fig. 2B). This value was reduced to about 50% for roots gravistimulated every 3 h (Fig. 2B). For roots stimulated every hour, the roots did not have enough time to reorientate their growth and it was therefore not possible to measure the percentage of turns showing lateral root initiation. Lateral root initiation occurred between turns only in roots subjected to gravistimulation at 12 h (<10%) or 24 h intervals (>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.

Then the effect of the gravistimuli on the density of lateral root initiation was determined. First, it was 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). Then it was observed that gravistimulation changed lateral root density with an optimum for gravistimulation at 6 h intervals (Fig. 2E). Taken together the present 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, it is also shown that, in the present experimental conditions, two subsequent lateral root initiations cannot occur at intervals which are too short. Indeed, when the interval between two successive gravistimulations was ≤3 h, the percentage of turns with lateral root initiation dropped and lateral root primordia density returned to the non-stimulated level. Moreover, the present 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 lateral root primordia density...
cannot be reduced below a minimal level that is close to non-stimulation conditions. It is concluded from the present 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

As these first results on gravistimulation showed a global consistent rationale an attempt was made to capture it quantitatively through the design of a simple mechanistic model. This model was based on an auxin budget system (Fig. 3A) and its aim was to explain the effects of gravistimulations on lateral root initiation. When a root
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 $T_1$ a lateral root 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 $T_2$. (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 parameter 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 ($n=20$) for additional details on the treatments, see Fig. S4 at JXB online. The control is a non-gravistimulated seedlings lot grown in the same conditions as the gravistimulated seedlings ($n=20$). (D) There is no relationship 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 closely match the predicted value.
growth unperturbed it initiates new lateral root primordia regularly. This phenomenon was modelled 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). The threshold value $T1$ was estimated to be equivalent to 12 h of auxin production/accumulation in the present conditions, as initiation density in the control corresponds to a 12 h 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 concentrates 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. Thus, in the present model, the spontaneous lateral root initiation threshold $T1$ and the lower threshold $T2$ corresponding to gravistimulation-induced initiation can be distinguished. In addition, each gravistimulation induces 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).

A computer algorithm implementing the above mechanistic model controlling lateral root initiation in time was designed as described in Fig. 3A. This model takes as an input parameter $T1$, estimated from the data observed ($T1$=12 h, 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, an extensive exploration of the parameter space was conducted and the number of lateral root initiations of the returned initiation patterns compared with the observed number of lateral root initiations of gravistimulated roots. The values of $T2$ and $G$ giving the best fit were $T2 \sim 0.4T1$ and $G \sim 0.05T1$. 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, a new experiment was designed to evaluate its predictive power. Six new gravistimulation patterns (see Fig. S5 at JXB online) not previously tested, with either regular or irregular spacing between gravistimulations, were selected. 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 Procycb1::GUS seedlings for 48 h, according to the previously described protocol of gravistimulation. The total number of lateral root initiations for the various seedling groups was scored and compared with the total number of lateral root initiations predicted by the model (Fig. 3C). This experiment was repeated twice independently.

It was 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 lateral root initiations for each pattern, over a large range of total numbers 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 it was found that gravitropism and lateral root initiation are co-regulated, a test was carried out to find out if both processes were dependent on the same auxin transport route. A transactivation strategy was used 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 d on vertical plates. They were then harvested and scored for gravitropism and lateral root primordia density. The present results on lateral root initiation (Fig. 4A) are similar to those obtained by De Smet et al. (2007) on lateral root density.
Therefore it is concluded 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.

Next a test was carried out to find out if auxin needs to be perceived in the tissues through which it flows for lateral root initiation. A dominant negative version of the AXR3 protein (axr3-1) that was shown previously to inhibit auxin response in different root tissues (Swarup et al., 2005) was transactivated and the effects on gravitropism and lateral root initiation tested. F1 plants were grown for 10 d on vertical plates before analysis. The present 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 the present results suggest that, by 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 optimize soil exploration?**

The present results indicated that lateral root initiation and gravitropism/thigmotropism are, at least in part, co-regulated. Next it was wondered if co-regulation could have some selective advantage. Simple geometrical considerations were used to evaluate the potential effect of co-regulation on resource exploitation (Fig. 5). The volume of soil explored by a root (for details, see Materials and methods) was estimated using three simplifying assumptions: (i) the volume of soil exploited by a given root segment is proportional to the number of root hairs; (ii) resources (water and nutrients) diffuse in the soil according to their concentration gradient; and (iii) 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 ($h_1$, Fig. 5C) than on the external side ($h_2$). 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 optimize soil exploitation (Fig. 5E).

In the present model it was considered that root hair length was identical on both sides 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, it was found that root hairs were significantly longer on the inside and shorter on the outside of a bend than control root hairs (see Fig. S6E, F at *JXB* online). This will therefore increase the depletion effect that was observed in the present model on the inside of the bend.

**Discussion**

The present 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 optimizing soil exploration.

The present 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, it was possible to change root architecture simply by applying gravistimulations at different intervals. The present data also point out internal characteristics of the regulatory system such as the minimum/maximum time between two successive initiations. These results were

Fig. 5. Influence of root bending on resource exploitation. (A) Exploitation of soil resources by a bent root (inner grey zone). (B) Infinitesimal portion of root turn. Parameters are: \( n \), number of root hairs; \( \rho \), curve radius of selected zone; \( b \), thickness of the root; \( d \), 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 \( s_2 \), 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.
used to create a mathematical model that can explain and predict the effects of gravistimulations on lateral root initiation density. The present model suggests that, by creating an asymmetric distribution of auxin in the apex using gravistimulations, it is possible 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. The present 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 organization of the cytoskeleton that might prepare the first cell division, i.e. lateral root initiation.

Finally, the present 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). The present transactivation

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**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.
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, the present 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 localization 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 relocalization, 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 stages 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. Currently an in silico model based on this and previous studies (Blilou et al., 2005; Swarup et al., 2005) is being built to try to understand how the redistribution of auxin in the root apex controls root branching.

Supplementary data

Supplementary data (Figs S1–S6) can be found at JXB online.

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 d and with no stimulation for an additional 2.5 d before harvesting.

Fig. S2. Localization of lateral root initiation in a gravistimulated root. A ProCYCB1::GUS seedling was subjected to crenel gravistimulation at 12 h intervals. Lateral root initiations were localized and their development scored.

Fig. S3. Influence of varying gravistimulation on lateral root initiation density (crenel-shaped roots). (A) Vertically grown ProCYCB1::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 d (1), then left to grow for 2.5 d without stimulation (2). Scale bars=1 cm. (B) Occurrence of lateral root initiation (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 d of growth) and non-gravistimulated root segments (last 2.5 d of growth) were also determined. (E) Lateral root initiation (LRI) densities were determined in gravistimulated and non-gravistimulated root segments. Different letters indicate significantly different results as tested by Student’s 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 the model. Six previously non-tested gravistimulation patterns were applied to seedlings over a 48 h period starting 30 h after germination. Gravistimulations are indicated by black dots. The total number of gravistimulations for each pattern varies between 10 and 25. After the last gravistimulus, seedlings were left to grow undisturbed for 24 h before harvest and observation.

Fig. S6. Curve radius, depth of exploration, and root hair length. (A) Curve of the function h1/h2=(p+b/2)/(p–b/2) (see Fig. 4 for additional details on the parameters). (B) Curve radius estimated for a portion of a gravistimulated root (90° re-orientation). b and p 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 (h1/h2) for various values of (p). (E) Direct visualization of root hairs 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 Student’s t test (P<0.01).

Acknowledgements

We thank Dr P Doumas and Dr D Bogusz (Equipe Rhizogenèse) for critical reading of the manuscript. This work was supported by IRD and INRIA (Virtual Plants project). ML is the recipient of a PhD grant from the French Ministère de l’Enseignement Supérieur de la Recherche et de la Technologie.

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