Mechanical Stimuli Modulate Lateral Root Organogenesis¹[W][OA]

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Unlike mammals, whose development is limited to a short temporal window, plants produce organs de novo throughout their lifetime in order to adapt their architecture to the prevailing environmental conditions. The production of lateral roots represents one example of such postembryonic organogenesis. An endogenous developmental program likely imposes an ordered arrangement on the position of new lateral roots. However, environmental stimuli such as nutrient levels also affect the patterning of lateral root production. In addition, we have found that mechanical forces can act as one of the triggers that entrain lateral root production to the environment of the Arabidopsis (Arabidopsis thaliana) plant. We observed that physical bending of the root recruited new lateral root formation to the convex side of the resultant bend. Transient bending of 20 s was sufficient to elicit this developmental program. Such bending triggered a Ca²⁺ transient within the pericycle, and blocking this change in Ca²⁺ also blocked recruitment of new lateral root production to the curved region of the root. The initial establishment of the mechanically induced lateral root primordium was independent of an auxin supply from the shoot and was not disrupted by mutants in a suite of auxin transporters and receptor/response elements. These results suggest that Ca²⁺ may be acting to translate the mechanical forces inherent in growth to a developmental response in roots.

Plant development is highly responsive to environmental stimuli. Such plasticity is one way in which plants overcome their inability to move toward areas of high resource availability or away from regions of adverse conditions. Fundamental to this adaptive development is the ability to regulate the timing and placement of postembryonic organ production. In roots, the recruitment of pericycle cells to become founder cells of a new lateral root (LR) primordium represents a key event in establishing the appropriate root system architecture (Casimiro et al., 2003). However, it is unclear why some pericycle cells are directed to initiate a LR developmental program while others are not (Casimiro et al., 2003). Although it is likely that certain cells are determined to founder pericycle cell fate in the basal meristem via an endogenous developmental program (De Smet et al., 2007), LR formation is also strongly influenced by environmental conditions such as nutrient levels (Malamy and Ryan, 2001; Malamy, 2005), suggesting that perception of the state of the surroundings of the root can direct recruitment of pericycle cells to founder cell fate.

Among these environmental factors, stimuli that cause changes in the direction of growth of the primary root can alter LR placement, with LRs emerging from the convex side of the resulting curves rather than in a predetermined distribution (Noll, 1900; Fortin et al., 1989; De Smet et al., 2007; Laskowski et al., 2008; Lucas et al., 2008a, 2008b). This phenomenon occurs whether the curve is the result of mechanical impedance by a barrier (Goss and Russell, 1979) or a change in unidirectional gravitational stimulus (Fortin et al., 1989; Lucas et al., 2008a, 2008b). Such observations led Lucas and colleagues (2008a) to hypothesize that LR initiation and the gravitropic response are controlled by the same signaling components. Considering the close link between auxin redistribution and tropic growth (Ottenschlager et al., 2003) and the key role of auxin in LR development (Dubrovsky et al., 2008), it is tempting to speculate that the auxin movements associated with the gravitropic response could directly play a role in modulating an auxin-dependent system that determines LR positioning. Such a mechanistic link between tropically related auxin movements and LR placement is reinforced by the finding that several mutations simultaneously affect both responses. For instance, the Arabidopsis (Arabidopsis thaliana) mutants auxin resistant4 (axr4 or...
rgr1) and auxin resistant1 (aux1) show a reduced gravitropic response and a decrease in the frequency of LR initiation events (Hobbie and Estelle, 1995; Simmons et al., 1995; Marchant et al., 2002). Indeed, Laskowski and colleagues (2008) have proposed that curve-related LR formation reflects differential dynamics of auxin transport/uptake by cells on each side of the root driven by the differential cell geometry caused by root curvature. Alternatively, Ditengou and colleagues (2008) have argued that such bend-related phenomena act upstream of auxin-dependent processes. However, the precise signaling/response pathways directing LR production to the convex side of curving roots, and whether additional components other than auxin-mediated events are involved in this process, remain to be determined.

We report that the mechanical forces inherent in bending a root can elicit LR placement to the convex side of the bend in the absence of tropic signaling from the root cap. We have observed that a 20-s transient bend is sufficient to trigger this developmental program, suggesting that a mechanically related signal initiates a long-term signaling pathway leading to LR formation. In a bent root, the stretching of cells on the convex side of the curve was observed to elicit a transient increase in cytosolic Ca$^{2+}$, and blocking this Ca$^{2+}$ change with La$^{3+}$ also blocked recruitment of new LRs to the curved region. This bend-related activation of LR formation could potentially act in an auxin-independent manner to specify the position of founder pericycle cell fate.

RESULTS

Curve-Related LR Formation Does Not Require Gravitropic Signaling

We first established that under our growth conditions, LR initiation to the convex side of the curve could be triggered by the stimuli previously reported to elicit curve-related LR formation. We observed that in roots of Arabidopsis (Columbia ecotype), root waving, gravitropic response, and mechanically induced reorientation by contact with a barrier to growth all elicited recruitment of LR formation to the convex side of a curve (Fig. 1, A–C). We observed that gravitropically induced (90° rotation) LR formation was associated with an apical shift in the site of initiation (Fig. 1D). In addition, gravitropic stimulation of as little as 10° led to a significant ($P < 0.05$, t test) induction of LRs to the convex side of the resulting root curvature.

![Figure 1](image-url)
(Fig. 1E). This robust “curve-induced” organogenesis provided us with a model to investigate how founder pericycle cells are recruited from the xylem pole pericycle population at bent regions of the root.

In the cases of curve-related LR formation during root waving and upon encountering a barrier to growth seen in Figure 1, A and C, root curvature results from a complex interplay between gravitational signaling and mechanical cues (Massa and Gilroy, 2003; Thompson and Holbrook, 2004). Therefore, to determine whether gravitropic signaling/response was a required component of LR recruitment to a bend, we removed the gravity-perceiving root cap by laser ablation, rendering the roots agravitropic. Importantly, this treatment does not impair root growth (Blancaflor et al., 1998). In order to force the now agravitropic root to make a predictable bend, we then grew the decapped root into a barrier that elicits a mechanically induced curve as the root grows along and around the barrier (Massa and Gilroy, 2003; Monshausen et al., 2009). The normal barrier response of an intact root is shown in Supplemental Movie S1A and Supplemental Figure S1. The response of a decapped root is shown in Supplemental Movie S1B, Supplemental Figure S1, and Figure 2A. In the decapped root, curves formed spontaneously during agravitropic growth or when the root was forced to bend by growing into a barrier. In such cases, the convex sides of these curves were sites of LR development (Fig. 2A). Similarly, mutants altered in gravitropic response, such as pin-formed2 (pin2) and aux1, were not disrupted in bend-induced LR induction (see below). Thus, while the gravitropic response results in LR recruitment to the associated tropically induced bend, gravitropic signaling does not appear to be a required element of bend-associated LR formation.

Mechanical Curvature of the Root Can Elicit LR Formation

Because gravitropic signaling did not appear to be required for curve-related production of LRs, we tested whether the mechanics of the bend itself might be the inductive signal. To ensure the continued distinction between gravitropic and mechanical signaling pathways, we developed a gel-sliding assay that formed two bends in the root without reorienting either the aerial parts of the plant or the root tip (Fig. 2, B and C; Supplemental Fig. S2). Subsequent to such bending, LRs formed on the convex side of the most distal curve in 99% of cases compared with 18% in the equivalent position of unbent controls (Fig. 2, C–E), indicating that mechanical cues are sufficient to trigger LR development.

Mechanical Curvature Can Lead To Multiple, Closely Spaced LRs

It has been proposed that LR spacing is controlled by competition between LR primordia (e.g. for carbon resources or an adequate supply of auxin), preventing LRs from forming next to one another and leading to the observed equal spacing of LRs along the main root axis (O’Brien et al., 2007; Lucas et al., 2008b). Indeed, in vertically growing, nonbent wild-type roots, we found...
that alternately placed LRs were spaced at regular intervals of approximately 1 mm (Fig. 3A). However, upon bending, LRs could be induced to form directly opposite each other across the vasculature (Fig. 3B) or close to one another (Fig. 3, C and D). Such “aberrant” LR positioning occurred with approximately 25% frequency in bends and was never observed in the unbent wild-type control (Fig. 3, A). Aberrant LR positioning was seen in 25% of curves. $n > 100$ roots. Bars = 200 μm.

Curve-Related LR Emergence But Not Initiation Requires an Acropetal Source of Shoot-Derived Auxin

We next asked which signaling pathways are activated by mechanical stimulation to recruit pericycle cells to founder pericycle cell fate. It has been demonstrated that accumulation of auxin in the pericycle is likely sufficient to convert a pericycle cell to a founder pericycle cell (Dubrovsky et al., 2008). Furthermore, Arabidopsis mutants defective in auxin transport or signaling show reduced LR formation (De Smet et al., 2006). In keeping with these findings, we have observed that the GUS reporter driven by the auxin-responsive DR5 promoter is expressed asymmetrically in mechanically induced bends, with the strongest signal localized to the site where LR primordia develop (Fig. 2C).

To determine if auxin indeed plays a role in bend-induced LR formation, we first surgically removed the hypocotyl of Arabidopsis seedlings to deplete the root system of an acropetal source of auxin originating from the shoot (Fig. 4A). This treatment has previously been shown to arrest LR emergence while supporting early primordium formation (Bhalerao et al., 2002). Consistent with recent observations (Ditengou et al., 2008), we found that hypocotyl removal did not disrupt bend-induced LR initiation, although LRs failed to develop past the initial stages of primordium formation (Fig. 4B). This failure of LRs to emerge could be rescued by application of an agar block containing 100 μM of the auxin 1-naphthalene acetic acid (1-NAA) to the cut surface before or after bending of the root (Fig. 4C), in keeping with the known role of shoot-derived auxin in supporting LR emergence.

**The Reduced LR Formation Phenotype in aux1 and axr4 Mutants Is Suppressed by Bending**

To further test if mechanical stimulation specifies founder pericycle cell fate via an auxin-dependent pathway, we investigated bend-induced LR formation in a range of mutants in root-expressed auxin transporters. Lesions in the PIN and ABCB proteins responsible for auxin efflux from root cells do not alter normal LR production (Biliou et al., 2005; Wu et al., 2007) and had no significant effect on bend-induced

![Figure 3](image-url)  
**Figure 3.** Curve-related LRs can form in positions not seen in unbent controls. A, Unbent wild-type control showing regular alternating LR formation (arrowheads) at approximately 1 mm spacing. This image is representative of more than 100 separate roots. B to D, Mechanical bending of the root leads to multiple LR production sites (asterisks) in patterns not seen in unbent controls, with LRs forming across the vasculature (B), multiple LRs forming throughout the curve (C), or multiple LRs emerging in very close proximity (D). Aberrant LR positioning was seen in 25% of curves. $n > 100$ roots. Bars = 200 μm.

![Figure 4](image-url)  
**Figure 4.** Effects of shoot removal on bend-induced LR formation. A, Schematic representation of surgical removal of the hypocotyl to deplete the root of an acropetal source of shoot-derived auxin and replacement of the auxin source with an agar block. B, Removal of the hypocotyl prevented bend-induced LR emergence but still supported bend-induced primordium formation. C, An agar block containing 100 μM 1-NAA was added to the cut surface before or after bending, as indicated, and LR emergence was scored. Note that removal of the hypocotyl blocks LR emergence but not primordium formation in the curve. Replacing the acropetal source of auxin with a 1-NAA-containing agar block restored LR emergence. The proportion of roots showing LR production in response to adding the agar block before or after bending was not significantly different ($P > 0.05, t$-test). Results show means ± s; $n \geq 15$ in four separate experiments.
LR formation (Fig. 5A; Supplemental Fig. S3A). On the other hand, a number of mutants related to auxin transport, which normally show a reduced frequency of LR production, were rescued to wild-type frequencies of LR production to the curve by the bend induction process. For example, even though mutants in the AUX1 auxin permease responsible for auxin influx into the cell normally exhibit an approximately 50% reduction in LR density (Supplemental Fig. S3B), they show a wild-type frequency of LR induction by bending (Fig. 5A; Supplemental Figs. S3 and S4). The production of LR to bends in aux1 induced by the growth response to a barrier (Fig. 5B; Supplemental Fig. S4) shows that such responses are elicited by the normal forces inherent in growth and are not an artifact of the experimental manipulations related to mechanically bending the root. In keeping with this model of suppression of the aux1 phenotype, bending also compensated the reduced LR phenotype of axr4 (Fig. 5A). AXR4 is thought to play a major role in correctly localizing AUX1 (Dharmasiri et al., 2006).

Reduced LR Formation in the tir1 But Not the axr1 Mutant Is Suppressed by Bending

To investigate whether mechanical induction of LR formation requires other components of an auxin signaling pathway, we monitored LR formation in response to bending in a range of mutants in root-expressed auxin receptor/response elements. TRANSPORT INHIBITOR RESPONSE1 (TIR1) is a family member of the well-characterized group of AFB auxin receptors (Tan et al., 2007), but of these, only tir1 shows severely reduced LR density under normal growth conditions (Ruegger et al., 1998; Dharmasiri et al., 2005; Supplemental Fig. S3B). However, upon mechanical bending, 90% of tir1-1 roots produced LRs to the convex side of the curved region (Fig. 5A), demonstrating that TIR1-dependent signaling is not required for bend induction of LR formation. Similarly, tir1-1 mutants were observed to make LRs in the curves elicited upon encountering and growing past a barrier to growth (Fig. 5C).

Figure 5. Effects of mutations in auxin transport/signaling on bend-induced LR formation. A, Plants of each genotype were subjected to bending, and the number of LR primordia plus emerged LRs was scored on the convex side of the bend (gray bars). Data expressed separately as emerged LRs or unemerged primordia are shown in Supplemental Figure S3. Results represent means ± se; n > 15 from more than five separate experiments. Letters represent results significantly different from bent wild-type (WT) controls (P < 0.05, t test). White bars indicate the effect each mutation has on normal LR frequency (calculated as in Supplemental Fig. S3B). Asterisks denote genotypes where bend-induced LRs are at a frequency significantly higher than the values predicted from their normal frequency (P < 0.05, t test). B, aux1-7 shows a wild-type-like frequency of LRs formed at the bend that was induced by growing into a barrier. Col WT, Columbia wild type. C, Roots of the tir1-1 mutant show LR formation in bends induced by growing into a barrier (1) and in curves elicited by the gravitropic response as the root grows past the end of the barrier (2). The results shown are representative of more than 50 separate plants. Bar = 1 mm.
Of all the auxin-related mutants tested (Fig. 5A), none were seen to selectively suppress bend-induced LR formation. Thus, for example, although we observed that bend-induced LR formation was suppressed in the axr1 background, this lower frequency was equivalent to the reduction seen in LR production during normal straight growth, suggesting that a lesion in AXR1 neither selectively promotes nor inhibits bend induction of LRs.

Transient Bending Is Sufficient to Induce LR Formation

In order to define when the signal for bend-induced founder pericycle cell specification was generated, we next characterized the inductive window required for LR formation. By providing a transient bending stimulus (Supplemental Fig. S5), we determined how long a root needed to be bent in order to trigger production of a LR in the curve. Figure 6 shows that a stimulus as short as 20 s yields a significant ($P < 0.05$, t test) increase in the number of LRs on the convex side of the former bent regions. This observation indicates that the mechanical stresses of the bend likely rapidly generate a signal, which then triggers subsequent signal transduction events to elicit founder pericycle fate specification even when the bend has disappeared.

Curvature Is Accompanied by Ca$^{2+}$ Changes in the Pericycle

Mechanical stimulation is closely linked to the rapid generation of Ca$^{2+}$ signals (for review, see Monshausen et al., 2008b). Indeed, bending is known to elicit Ca$^{2+}$ changes in roots (Monshausen et al., 2009), providing a strong candidate for a bend-related signal linked to LR formation, although whether bend stimulation triggers Ca$^{2+}$ changes to the pericycle destined to become a founder cell was previously undefined. Therefore, we monitored the cytosolic Ca$^{2+}$ concentration using confocal imaging of Arabidopsis expressing the fluorescence resonance energy transfer (FRET)-based Ca$^{2+}$ sensor YC3.6 (Nagai et al., 2004; Monshausen et al., 2008a, 2009). Bending the root elicited a rapid biphasic Ca$^{2+}$ increase in epidermal, cortical, endodermal, and pericycle cells on the convex side of the curve (Fig. 7, A–C; Supplemental Movie S2). Equivalent changes were never seen in unbent regions of the root (Supplemental Fig. S8). Although we were unable to specifically identify the xylem pole pericycle cell files known to be the sites of founder cell fate specification in this analysis, in all bending experiments the pericycle on the convex side of the resulting curve showed similar Ca$^{2+}$ increases, implying that both xylem pole and nonxylem pole pericycle were capable of generating changes in Ca$^{2+}$ levels upon being stretched.

To inhibit such bend-elicited Ca$^{2+}$ transients, we treated roots with the Ca$^{2+}$ channel blocker La$^{3+}$. Because prolonged exposure to La$^{3+}$ had adverse effects on root growth and development, we preincubated roots with 1 mM La$^{3+}$ for only 5 min and then provided a short bending stimulus of 5 min, which is well within the inductive window required to elicit LR formation (Fig. 6) and allowed LRs to subsequently develop in La$^{3+}$-free medium. This treatment abolished detectable bend-induced Ca$^{2+}$ transients (Fig. 7, B and D; Supplemental Movie S3). Identical treatment of the root also abolished bend-induced LR production to the former convex region of the curve (Fig. 7E). Importantly, LR formation elsewhere (e.g. to the concave side of the formerly bent region) was unaffected, indicating that the La$^{3+}$ treatment was selectively inhibiting the bend-induced promotion of LR formation but not nonspecifically altering the production of all LRs. These observations are consistent with a possible role for the cytoplasmic Ca$^{2+}$ changes caused by the stretching of cells during bending as a signal leading to founder pericycle cell fate specification. Treatments at less than 1 mM La$^{3+}$ or with up to 1 mM of the L-type Ca$^{2+}$ channel blockers verapamil and nifedipine were ineffective at inhibiting mechanically induced Ca$^{2+}$ changes or LR formation (data not shown), suggesting either a failure of their penetration into the root or lack of targets for their action.

DISCUSSION

The regulated proliferation of LRs allows the root system to more effectively explore and exploit the soil. For example, the modulation of LR production by nutrient levels has been well documented (Malamy and Ryan, 2001) and reflects the need for the plant to tailor its root system architecture to the prevailing local nutrient status of the soil. We report here that the geometry of the root system itself also has an important role in determining the precise placement of LRs.

Figure 6. Effects of transient bending of the root on LR formation. Roots were bent for the indicated time periods and then returned to straight growth, and LR formation was assayed over the following 72 h. Asterisks indicate LR formation at levels significantly higher than the control ($P < 0.05$, t test). Results represent means ± s.e.; n = 20 in four separate experiments.

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Thus, in response to gravitropic stimulation, waving, and bending upon contacting a physical barrier to growth (Noll, 1900; Fortin et al., 1989; De Smet et al., 2007; O’Brien et al., 2007; Laskowski et al., 2008; Lucas et al., 2008a, 2008b), LRs emerge at the convex side of the resultant bend. For roots growing in the physically complex environment of the soil, such mechanically induced LR patterning may play an important role in the generation of root system architecture, enhancing both the volume of soil explored and the anchorage produced by the root system as a whole. Figure 8 shows the development of an Arabidopsis root system experiencing repeated redirection of root growth due to barrier contact. At each reorientation of the root, a curve-related LR is initiated, leading to a significant impact on the overall root system architecture.

Our results support the idea that gravitropically induced bending can promote LR production to the convex side of a bend with very small displacements from the vertical (Fig. 1). We observed that gravitropic reorientation can also lead to LR formation closer to the tip than seen in vertically growing roots (Fig. 1). This observation implies that the gravitropic response is not simply inducing LR formation from cells at a stage predetermined by the endogenous LR patterning program (De Smet et al., 2007) but is capable of recruiting pericycle cells to founder cell fate earlier in their developmental progression through the root. Lucas and colleagues (2008b) have shown that gravitropic stimulation can promote LR emergence, leading to a model where the tropically induced bend somehow lowers the threshold levels of auxin required to

Figure 7. Effects of bending of the root on Ca\(^{2+}\) levels. A, Cytoplasmic Ca\(^{2+}\) levels were imaged using the YC3.6 Ca\(^{2+}\) sensor driven by the CaMV 35S promoter and calculated from the ratio of the sensor’s Ca\(^{2+}\)-dependent FRET signal/cyan fluorescent protein (CFP) signal. Ca\(^{2+}\) levels have been pseudocolor coded according to the scale at right. Note the increase in Ca\(^{2+}\) in cells under tension. B, Quantitative analysis of Ca\(^{2+}\)-dependent ratio values from YC3.6 was made in the regions indicated in Supplemental Figure S7. C and D, Roots were pretreated for 5 min with or without 1 mM LaCl\(_3\) and subjected to bending, and Ca\(^{2+}\) levels were analyzed as in A and B. Note that bending triggers Ca\(^{2+}\) increases in cells under tension. La\(^{3+}\) treatment blocks these Ca\(^{2+}\) changes elicited by bending the root. Results are representative of 15 separate experiments. E, La\(^{3+}\) treatment selectively blocks LR formation in bends of the root. Roots were pretreated for 5 min with or without 1 mM LaCl\(_3\) and subjected to bending for 5 min and then returned to straight vertical growth in La\(^{3+}\)-free medium. LR formation was scored after 72 h of further growth. Note that La\(^{3+}\) treatment selectively blocks LR recruitment to the convex side of the curve in the root but does not block all LR formation. Results show means ± se; n = 15 from four separate experiments.
transient bending of 20 s was sufficient to induce LR formation (Fig. 6). In these “bend-back” experiments, the root was returned to its straight growth position after bending, with no obvious asymmetry in the geometry on either side of the root (Supplemental Fig. S5). This observation suggests that, in these cases, the mechanical/bending stimulus rapidly triggers a signal transduction cascade linked to founder pericycle cell fate determination rather than operating through alterations in cell geometry. However, whether LR induction through tropically induced curvature and mechanically induced bends operates through the same signaling pathways remains to be determined. Mechanical induction of LR formation seems to involve the elevation of cytosolic Ca\(^{2+}\) levels (see below), but to date, analogous increases in Ca\(^{2+}\) have not been reported during the development of tropic curvature of the root, suggesting the possibility of different mechanisms.

It is also important to note that although mechanical bending via gel sliding does elicit LR production, it is possible that this stimulus does not mimic one normally experienced by the plant, so the LR formation under these conditions would lack a true physiological context. However, the initial phase of the growth response of a root encountering a barrier to downward growth reflects mechanical sliding sideways of the root tip as it encounters the obstacle (Massa and Gilroy, 2003; Monshausen et al., 2009). The bends in the root elicited by the gel-sliding approach also occur in the same region of the root as this growth-related mechanical response. Taken together, these observations suggest that manual bending of the root is likely mimicking a stimulus that the root encounters during normal growth and development.

Although the decapitation experiments shown in Figure 4 show that auxin is clearly intimately involved in the emergence of LRs induced by bending and auxin is strongly linked to the induction of LR formation (De Smet et al., 2007; Dubrovsky et al., 2008), we observed that bending could suppress the reduction in the frequency of LR formation seen in several auxin transport/signaling mutants. Thus, mutants in the AUX1 permease responsible for auxin uptake nor-
mallly exhibit an approximately 50% reduction in LR density. However, LRs formed in nearly 100% of the mechanically induced curves (Fig. 5). This observation is consistent with the reported LR production to the outside of bends in aux1 roots undergoing coiling growth (De Smet et al., 2007; Ditengou et al., 2008) and our observation of LR production by aux1 roots that bend upon encountering barriers to growth (Fig. 5; Supplemental Fig. S4). Differences in the bending protocol used may explain why Ditengou et al. (2008) observed only 45% of aux1-7 plants showing LR formation in manually imposed bends in the root. Consistent with this curvature-induced suppression of

promote LR formation. This promotion of emergence by bends would fit well with the high levels of emerged LRs seen in the mechanically induced bends in our experiments. However, consistent with the report of Ditengou et al. (2008), we have determined that the root cap, which contains the gravity-sensing columella cells (Kiss, 2000), was not required for this response (Fig. 2). Similarly, in the gel-sliding approach we developed (Fig. 2B), bends were produced with no reorientation (i.e. no gravistimulation) of the gravity-sensing regions of either the root tip or the shoot system but still led to curve-related LR formation (Fig. 2, C–E). Taken together, these observations indicate that gravitropic signaling is not a required component of this bend-related LR inductive response.

One possible explanation for the finding that mechanical bending without gravitropic stimulation leads to LR formation is that the new cellular geometry imposed by the bend, with cells being slightly stretched on the convex side and compressed on the concave side, is leading to this developmental response. This geometrical mechanism was proposed by Laskowski et al. (2008), whose modeling of auxin transport suggests that the slightly larger cells on the stretched, convex side of the bend act as more efficient auxin sinks. Over time, these cells would accumulate enough auxin to trigger LR formation. Such a mechanism would fit well with the LR induction events that occur in response to the extended curves generated by a range of different mechanical/tropic mechanisms (Noll, 1900; Fortin et al., 1989; De Smet et al., 2007; Laskowski et al., 2008; Lucas et al., 2008a). However, in the case of mechanical bending, we observed that a
the aux1 LR phenotype, we observed that bending also suppressed the 50% reduction in LR frequency seen in aux4 roots (Fig. 5A). AXR4 is thought to be required to correctly traffic AUX1 to the plasma membrane (Dharmasiri et al., 2006), providing a possible mechanistic link between the similar effects of bending on LR formation in both mutants. However, the observation that the aux1-7/aux4-2 double mutant more severely reduces LR formation than predicted from aux1- or aux4-related effects alone has been used to argue for other roles for AXR4 than simply positioning AUX1 in this process (Hobbie and Estelle, 1995). This idea is supported by our finding that the reduction in LR formation in the aux1-7/aux4-2 mutant could not be compensated by the bend induction process (Fig. 5).

Of the AFB family of auxin receptors, only mutants in TIR1 are reported to affect normal LR formation, showing an approximately 50% reduction in LR frequency (Ruegger et al., 1998; Dharmasiri et al., 2005; Supplemental Fig. S3). Bend induction also suppressed this phenotype, yielding approximately 90% of the curves with LR to the convex side (Fig. 5). Consistent with this compensation of the aux1, aux4, and tir1 LR phenotypes by bending, Ditengou et al. (2008) have reported partial suppression of the reduced LR phenotype of mutants in a SOLITARY ROOT mutant (slr-1, 10% increase in production of LR) and arf7/19 (50% increase in production of LR) by root curvature. SOLITARY ROOT and ARF7/19 are components directly involved in the auxin-dependent transcriptional response machinery. Taken together, our results and those of Ditengou et al. (2008) are consistent with a model where bending elicits a signaling pathway that (1) acts to increase the sensitivity of the system to ambient auxin levels, possibly recruiting redundant auxin response elements, (2) operates downstream of TIR1, or (3) operates in parallel to the normal TIR1-dependent pathway (Fig. 9).

The precise nature of this possible signaling pathway activated by bending remains to be defined. However, we observed bending to cause a biphasic Ca2+ increase in the epidermal, cortical, endodermal, and pericycle cells of the root (Fig. 7). Ca2+ signals and subsequent transduction events are well known to be elicited in plants and other organisms in response to mechanical stimulation (Monshausen et al., 2008b, and refs. therein). The results shown in Figure 7 suggest that it is tension in either the cell wall and/or the plasma membrane that is triggering this Ca2+ change, as only cells on the convex/stretched side of the root showed a clear Ca2+ response to curvature (Monshausen et al., 2009). Although stretch-activated ion conductances have been well characterized in plants through electrophysiology, to date, a candidate stretch-activated mechanoreceptor defined to the molecular level has remained elusive (Monshausen and Gilroy, 2009). Our observation that the bend-induced elevation in Ca2+ can be abolished by pretreatment with La3+ tentatively implicates such a Ca2+-permeable channel in this process. However, it is important to note that Ca2+ increase in the epidermal, cortical, endodermal, and pericycle cells of the root (Fig. 7). Ca2+ signals and subsequent transduction events are well known to be elicited in plants and other organisms in response to mechanical stimulation (Monshausen et al., 2008b, and refs. therein). The results shown in Figure 7 suggest that it is tension in either the cell wall and/or the plasma membrane that is triggering this Ca2+ change, as only cells on the convex/stretched side of the root showed a clear Ca2+ response to curvature (Monshausen et al., 2009). Although stretch-activated ion conductances have been well characterized in plants through electrophysiology, to date, a candidate stretch-activated mechanoreceptor defined to the molecular level has remained elusive (Monshausen and Gilroy, 2009). Our observation that the bend-induced elevation in Ca2+ can be abolished by pretreatment with La3+ tentatively implicates such a Ca2+-permeable channel in this process. However, it is important to note that Ca2+ increase in the epidermal, cortical, endodermal, However, we observed bending to cause a biphasic way activated by bending remains to be defined. However, the observation that the aux1-7/aux4-2 double mutant more severely reduces LR formation than predicted from aux1- or aux4-related effects alone has been used to argue for other roles for AXR4 than simply positioning AUX1 in this process (Hobbie and Estelle, 1995). This idea is supported by our finding that the reduction in LR formation in the aux1-7/aux4-2 mutant could not be compensated by the bend induction process (Fig. 5).

La3+ has been reported to affect processes other than Ca2+ channel activity in plants (e.g. inhibiting anion channel action; Lewis and Spalding, 1998). Unfortunately, the other putative Ca2+ channel blockers we tested (verapamil and nifedipine) were found to be ineffective at blocking mechanically induced Ca2+ changes at levels that sustained root growth and development. However, our imaging of the effect of La3+ on bend-induced Ca2+ changes in the pericycle indicates that irrespective of other possible actions, this treatment did inhibit the mechanically induced Ca2+ increase. Taken with the observation that this treatment also selectively inhibited recruitment of LRs to the convex side of the bend but did not inhibit LR formation per se, and of the ubiquitous nature of Ca2+ as a signaling element in touch responses (Monshausen et al., 2008b), these findings are consistent with a possible role for a mechanically induced Ca2+ increase in the signaling pathway triggering LR initiation.

We also observed that the bend stimulus, whether applied manually by gel sliding or through the endogenous forces of growth upon encountering a barrier, can elicit LR formation in positions not seen in vertically growing, unbent roots (Fig. 3). Thus, bending caused LRs to form opposite each other across the vasculature and side by side in the curve. It is possible that the bending stimulus can trigger multiple primordium specification events, although in the case of the root shown in Figure 4B this would mean the extremely novel idea of bends inducing LR to both the
convex and concave sides of the curve. Alternatively, the endogenous developmental program for LR initiation (De Smet et al., 2007) may have already specified a LR in one position onto which the bend-induced program of LR induction was superimposed. Both visual inspection and DR5 reporter analysis did not reveal clear preexisting primordia in the region of the root where we made the curves at the time bending was performed, suggesting that any prepatterning of LRs would have to have been at the earliest stages of founder cell specification. However, neither of these possibilities for how bends can induce aberrant LR positioning (multiple, closely spaced bend-induced founder events or a bend-induced founder event being superimposed on a preexisting primordium) can be completely explained by current models of LR spacing based on inhibitory fields of auxin depletion (Lucas et al., 2008a, 2008b) or resource competition but instead point to a complex, plastic regulation of LR patterning.

MATERIALS AND METHODS

Plant Material

Arabidopsis (Arabidopsis thaliana) seeds of the Columbia and Landsberg erecta ecotypes were used in experiments involving wild-type plants. Seeds of tir1-1 and agr1/pin2 were kindly supplied by Dr. Elisabeth Blancfoll (The Noble Foundation) and tir1-1 by Dr. Mark Estelle (University of California, San Diego). All other seed stock was obtained from the Arabidopsis Biological Resource Center at Ohio State University. With the exception of agri1pin2 (Landsberg erecta), the mutants and transgenic plants used were all in the Columbia background.

Unless otherwise stated, all chemicals were obtained from Sigma. Seeds were surface sterilized and grown in half-strength Epstein’s medium, 0.5% (w/v) Suc, and 1.5% (w/v) phytagel as described previously (Wymer et al., 1997). Growth was under continuous fluorescent light (90 mmol m⁻² s⁻¹) and at room temperature (22°C).

Shoot Removal and Auxin Treatment

Agar blocks (approximately 1 mm³) containing a final concentration of 100 μM 1-NA was made by adding 1-NA (GIBCO) to cooling phytagel. Nutrient medium from an ethanol stock of 100 mM. Four days after germination, shoots were surgically removed just below the hypocotyl-root junction. Roots were bent 12 h after shoot removal to induce LR formation. Subsequent LR initiation in unbent samples was 75% of nondecapitated controls (decapitated LR density = 0.014 ± 0.004 lateral per mm primary root versus 0.05 ± 0.015 lateral per mm primary root in controls). 1-NA-containing agar blocks were applied to deshooted seedlings either directly after severance or immediately after root bending as noted.

Application of LaCl₃

By carefully removing the gel covering the first 1 mm of the root tip, a 1- to 2-mm-wide well was created to allow the addition of liquid to that section of the root. LaCl₃ at the indicated concentration was then added to the well. To remove the LaCl₃ prior to growth analysis, the root tip was rinsed with water. After subsequent root bending, the well was filled with nutrient medium containing 1% (w/v) low-melting-point agarose (Sigma type IV; added at approximately 30°C) and LR development was scored after 72 h.

Histochemical GUS Staining

GUS staining was performed by incubating seedlings overnight at room temperature in a solution containing 4 μM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (GOLD Biotech), 2.5 mM K₃Fe(CN)₆, 2.5 mM K₄Fe(CN)₆, H₂O₂, and 0.5% (v/v) Triton X-100 in 100 mM sodium phosphate buffer containing 39% (w/v) NaH₂PO₄ and 61% (w/v) Na₂HPO₄ at pH 7.

Microscopy

Laser ablation of the root cap was performed using the Camellion T-saphire multiphoton laser of the Zeiss LSM 510 NLO system tuned to 720 nm and running at 50% transmission power, which was sufficient to ablate cells. A region of interest was defined at the level of columella tier 1 in the root cap using the LSM 510 photobleaching software, and the multiphoton laser was scanned in this region for as a photobleaching protocol while slowly focusing through the root cap, thereby severing the cap from the root body.

Ca²⁺ Imaging

To measure cytosolic Ca²⁺ levels, Arabidopsis seedlings expressing the FRET-based Ca²⁺ sensor yellow cameleon YC3.6 (Nagai et al., 2004) driven by the CaMV 35S promoter were transferred to purpose-built cuvettes and mounted as described previously (Monschau et al., 2008a). After several hours of recovery from transfer to the cuvettes, roots were ratio imaged with the Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss) using a 20× water-immersion objective (1.2 numerical aperture; C-Apochromat) and an argon laser. Excitation was set at 458 nm using the primary 458-nm dichroic mirror, and the Meta detector of the LSM 510 was used to capture cyan fluorescent protein (473–505 nm) and FRET-dependent cpVenus (526–537 nm) emissions. Bright-field images were acquired simultaneously using the transmission detector of the microscope. For time-lapse analysis, images were collected every 2 s, with each individual image scan lasting 1.97 s. As other circularly permuted yellow fluorescent proteins to the venus yellow fluorescent protein in YC3.6 have been reported to be responsive to reactive oxygen species (Wang et al., 2008) and extracellular reactive oxygen species are produced during mechanical responses in plants (Mori and Schroeder, 2004), we tested for possible interference in Ca²⁺ measurements using recombinant protein in vitro. 6×His-tagged YC3.6 was purified on HisPur resin (Pierce) according to McCubbin et al. (2004) and imaged on the Zeiss LSM 510 as described above. No detectable sensitivity to up to 1 mM hydrogen peroxide was detected in this in vitro test, suggesting that Ca²⁺ measurements made using the cytosolically targeted YC3.6 are not contaminated by an effect of permeation of hydrogen peroxide generated through dismutation of extracellularly produced superoxide. Similarly, cytosolic pH changes accompany mechanoresponses in plants (Monschau et al., 2009). However, YC3.6 is insensitive to pH changes in the range 6.5 to 9.2 (Nagai et al., 2004), suggesting that the Ca²⁺ changes observed are also not an artifact of these pH swings.

Root Barrier Response, Waving, and Bending Assays

The growth response of roots encountering barriers to growth was measured as described previously (Massa and Gilroy, 2003). Briefly, roots were grown on coverslips coated with a 1-mm-thick layer of phytagel medium as described above. A Number 1 coverslip was then inserted into the phytagel medium 1 to 2 mm ahead of the root, perpendicular to the direction of root growth. The root was then placed vertically and allowed to grow down and onto the surface of the coverslip.

Root-waving assays were performed on hard agar plates inclined to 45° according to Thompson and Holbrook (2004).

To manually bend roots, one of two methods was employed. The first method caused neither the root apex nor the shoot to undergo reorientation and is shown schematically in Figure 2B. This nonorienting bending process was achieved by cutting (without severing the root) the agar medium in a box shape around the first 1 to 2 mm of the root tip. Then, a section of agar directly adjacent to this box was removed, and the box containing the root tip was gently slid into the open space over the course of 30 s. This created two bends in the root, the most distal of which (approximately 1 mm from the root tip) was used to score LR initiation events. In order to generate the large number of measurements required for the mutant analysis described in Figure 6, this approach was modified such that the box of agar that was slid sideways contained only the first 1 mm of the root apex. When this box was pushed into the adjacent vacated space, a single 90° bend was created with the root tip oriented sideways. The petri dish was then reoriented such that the root apex was realigned perpendicular to the gravity vector. For the bend-back experiments, the agar medium was completely removed from the apical 1 mm of the root.
root tip, and an adjacent box-shaped piece of agar approximately 2 to 3 mm square was cut and used to bend and restore the root tip to its original resting position.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Laser ablation of the root cap causes loss of the normal barrier-tracking response of the root.

Supplemental Figure S2. Images of root undergoing bending without accompanying gravitimization of the shoot or root apex.

Supplemental Figure S3. Auxin transport, signaling, or response mutations affect LR induction.

Supplemental Figure S4. Bending-induced formation of LRs in aux1-7 upon encountering a barrier.

Supplemental Figure S5. Bending and returning the root to vertical does not induce long-term deformation of the root.

Supplemental Figure S6. Non-color-coded images from Figures 2 and 8.

Supplemental Figure S7. Regions of interest for quantification of Ca2+ changes in convex and concave regions of epidermis and pericycle used for Figure 7.

Supplemental Figure S8. Unbent roots do not show the changes in Ca2+ seen in roots upon bending.

Supplemental Movie S1. A, Normal response of an Arabidopsis root as it encounters a barrier to growth; B, a decapped root shows gravitropic behavior upon encountering a barrier to growth.

Supplemental Movie S2. Cytoplasmic Ca2+ increases in cells undergoing stretch upon bending the Arabidopsis root.

Supplemental Movie S3. Cytoplasmic Ca2+ increases in cells undergoing stretch upon bending the Arabidopsis root are blocked by pretreatment with 1 mM La3+ for 5 min.

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LITERATURE CITED


