Cytokinin functions as an asymmetric and anti-gravitropic signal in lateral roots

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Directional organ growth allows the plant root system to strategically cover its surroundings. Intercellular auxin transport is aligned with the gravity vector in the primary root tips, facilitating downward organ bending at the lower root flank. Here we show that cytokinin signaling functions as a lateral root specific anti-gravitropic component, promoting the radial distribution of the root system. We performed a genome-wide association study and reveal that signal peptide processing of Cytokinin Oxidase 2 (CKX2) affects its enzymatic activity and, thereby, determines the degradation of cytokinins in natural Arabidopsis thaliana accessions. Cytokinin signaling interferes with growth at the upper lateral root flank and thereby prevents downward bending. Our interdisciplinary approach proposes that two phytohormonal cues at opposite organ flanks counterbalance each other’s negative impact on growth, suppressing organ growth towards gravity and allow for radial expansion of the root system.

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Root architectural traits define plant performance and yield. The radial spreading of the root system depends on the directional growth of primary and secondary roots. The phytohormone auxin plays a central role in aligning root organ growth towards gravity. In the root tip, columella cells perceive changes in gravity via statolith sedimentation. The relative change in statolith positioning triggers a partial polarization of redundant PIN3, PIN4, and PIN7 auxin efflux carriers towards this side, leading to enhanced auxin transport along the gravity vector. The asymmetric distribution of auxin eventually reduces cellular elongation rates at the lower root flank, which consequently leads to differential growth within the organ and bending towards gravity.

Lateral roots (LRs) substantially differ from main roots, establishing a distinct gravitropic set point angle (GSA). The divergent developmental programs of lateral and main (primary) roots allow the root system to strategically cover the surrounding substrate. In Arabidopsis, LRs emerge from the main root at a 90° angle (stage I LRs) and afterwards display maturation of gravity sensing cells, as well as the de novo formation of an elongation zone. Transcription factors FOUR LIPS and MYB88 define PIN3 expression in columella cells of LRs and its transient expression in columella cells temporally defines asymmetric auxin distribution as well as differential elongation rates in stage II LRs. This developmental stage lasts about 8–9 h and is characterized by asymmetric growth towards gravity at a slower rate than in primary roots. During this phase of development, the primary GSA of LRs is established. The subsequent repression of PIN3 in columella cells of stage III LRs coincides with symmetric elongation, maintaining this primary GSA. Notably, the repression of PIN3 and PIN4 in columella cells of older stage III LRs does not necessarily correlate with additional bending to gravity. This finding illustrates that the primary GSA is developmentally maintained, determining an important root architectural trait. Moreover, a stage III LRs will return to its initial GSA if it is reoriented relative to the gravity vector. Accordingly, the partial suppression of a full gravitropic response in recently emerged LRs is critical for establishing the primary growth direction of LRs, which importantly contributes to the root system architecture.

Despite the apparent importance of directional LR growth for radial exploration of the root system, the underlying suppressive mechanisms are largely unexplored. Using genetic, physiological, computational, biochemical, and cell biological approaches, we reveal that two opposing hormonal cues at the lower and upper lateral root flank counterbalance each other and set directional LR growth.

**Results**

Angular lateral root growth displays substantial natural variation. To examine the natural diversity in radial root growth, we screened 210 sequenced Arabidopsis accessions (Supplementary Fig. 1a, Supplemental Data 1) and quantified their primary GSA of LRs. When grown in vitro on the surface of the growth medium, we observed extensive variation for the mean GSA values, detecting a deviation of about 40° between most extreme natural accessions (Fig. 1a).

Because the in vitro approach allowed only two-dimensional analysis of root growth, we further assessed angular growth of LRs in three dimensional and soil systems. For this purpose, we studied a subset (depicted by red and blue lines in Fig. 1a) of hyper-responsive and hypo-responsive accessions in greater detail (Fig. 1b, c Supplemental Data 2). To allow three-dimensional root expansion in vitro, we grew this subset of accessions in growth medium-filled cylinders (Supplementary Fig. 1b). In addition, we used the GLO-Roots system, which is a luciferase (LUC)-based imaging platform to visualize root systems in a soil-like environment (Supplementary Fig. 1c). Accordingly, we quantified the same subset of accessions with pUBQ:LUC2o, ubiquitously driving LUC expression. In the Col-0 reference accession, about 60% of emerged LRs displayed a GSA between 51° and 70° in all three growth conditions (Fig. 1b–e, Supplementary Data 2). In all three systems, hypo-responsive and hyper-responsive accessions displayed a pronounced shift towards higher (71°–90° and 91°–110°) and lower (31°–50° and 0°–30°) angle categories, respectively (Fig. 1b–e, Supplementary Data 2). This suggests that our two-dimensional, in vitro screen was highly suitable to identify natural accessions with diverging GSA values of their root systems.

GWAS reveals a link between cytokinin and angular growth of LRs. Next, we sought to identify molecular players involved in the LR trait of our interest. To achieve this, we used our quantitative data on primary GSA of LRs and conducted a genome-wide association study (GWAS). We identified several chromosomal regions, displaying associations with our trait (Fig. 2a). A prominent peak at chromosome 2 drew our attention to a thymine (T)/guanine (G) single-nucleotide polymorphism (SNP) located in the CYTOKININ OXIDASE 2 (CKX2) gene (position 8,447,233) (Fig. 2b). Importantly, the minor G allele, showing a frequency of 19.5% in all sequenced and 32.7% in our set of accessions, was associated with increased GSA values, reflecting more perpendicular LR growth to gravity (Fig. 2c). Notably, linkage disequilibrium analysis showed that adjacent SNPs display pronounced non-random association with our SNP of interest (Supplementary Fig. 2), suggesting that the CKX2 gene could be linked to variations in angular growth of LRs.

CKX enzymes are responsible for the irreversible degradation of cytokinins (CKs) via the oxidative cleavage of their side chain. CKXs are the preferred substrate of CKXs and are as expected increased in cks2-1 (Col-0 background) mutant roots (Supplementary Fig. 3a). On the other hand, other types of CKs were downregulated in the cks2-1 mutant background (Supplementary Fig. 3a–e), presumably underlying a compensation mechanism. This measurement reveals that CK metabolism is indeed affected in cks2-1 mutant roots (Supplementary Fig. 3a–e). To test whether active CKs may modulate angular growth of LRs, we initially transferred 7-day-old seedlings of the reference accession Col-0 to medium supplemented with CKs. We observed a strong concentration-dependent increase in GSA values of LRs emerging in presence of active CKs, such as 6-Benzylaminopurin (BAP) (Fig. 2d, Supplementary Data 2), transzeatin (IZ) and isopentenyladenine (iP) (Supplementary Fig. 3f, g, Supplementary Data 2). Conversely, CK receptor double mutant combinations showed a relatively mild, but statistically significant decrease in GSA of LRs (Fig. 2e, Supplementary Data 2), proposing functional redundancy among the cytokinin receptors.

This set of data suggests that cytokinin signaling interferes with downward bending of emerged LRs. Notably, emerging LRs of winter oilseed rape also displayed reduced bending of LRs when treated with BAP (Supplementary Fig. 3h, Supplementary Data 2), suggesting that the effect of CK on directional LR growth is likely to be conserved.

To further assess the importance of CKX2 in GSA establishment, we disrupted CKX activity in the reference accession Col-0. Treatments with the CKX inhibitor INCYDE phenocopied the cks2-1 loss-of-function mutant, both displaying more horizontal LRs when compared to its respective controls (Fig. 2f, g, Supplementary Data 2). On the other hand, CKX2 overexpressing (OX) plants showed accelerated bending of LRs, phenocopying...
the CK receptor mutants (Fig. 2g, Supplementary Data 2). This set of data suggests that CK signaling defines directional lateral root growth by reducing LR bending after emergence.

**Cytokinin response factors define angular growth of LRs.** Our data indicates that CK signaling impacts the angular growth of emerged LRs. Therefore, we assessed whether CK-dependent transcription factors indeed have an impact on LR growth in the reference accession Col-0. It has been previously shown that CK signaling initiates transcriptional changes via the Arabidopsis response regulators (ARRs)\(^{20-22}\) and the cytokinin response factors (CRFs)\(^{23,24}\). According to available LR organ-specific microarray data\(^{25}\), type-B ARRs, such as ARR10 and ARR12, and type-A ARRs, such as ARR3 and ARR4 (Supplementary Fig. 4a, b) were strongly upregulated in mature LRs. However, we did not detect any expression of ARR3, ARR4, ARR10, and ARR12 in young stage II LRs, using respective promoter GUS reporter lines (pARR3::GUS, pARR4::GUS, pARR10::GUS, pARR12::GUS; Supplementary Fig. 4d)). In agreement, angular growth of LRs was not altered in arr3, arr4, arr10, and arr12 single mutants (Supplementary Fig. 4e, Supplementary Data 2). While this approach was not successful in identifying the responsible ARRs in young LRs, we in contrast could confirm expression of CRF2 and CRF3 (25, Supplementary Fig. 4c) in the early stages of LR development (Fig. 3a and Supplementary Fig. 4f).

In agreement with CRF2 and CRF3 expressions in emerged LRs, loss-of-function alleles of crf2 and crf3 displayed enhanced bending of LRs (Fig. 3b, Supplementary Fig. 4h, Supplementary Data 2). Conversely, we found that ubiquitous overexpression of either CRF2 or CRF3 led to more horizontal LRs (Fig. 3b, Supplementary Data 2). When we transferred crf2 and crf3 single
mutants to medium supplemented with CK, we observed partial resistance to CK in both lines (Supplementary Fig. 4i, Supplementary Data 2). This set of data confirms that cytokinin signaling, utilizing transcription factors, such as CRF2 and CRF3, regulates angular growth of LRs.

Cytokinin integrates environmental cues into angular growth of LRs. Our data supports a role for cytokinin signaling in modulating angular growth of LRs. To investigate whether cytokinin modulates angular LR growth in response to environmental cues, we examined whether the primary GSA of Arabidopsis accessions is linked to geographic origins. Intriguingly, accessions with the largest GSA values predominantly originated in Nordic (above 58°N) regions (Fig. 4a). In addition, the above median of LRs in the north of Sweden is most of the year covered with snow (Supplementary Fig. 5a), indicating that these fully-grown leaf rosettes and root systems withstand long-term snow coverage. Snowpack insulation capacity can protect these plants from extreme temperatures, but may also restrict soil–atmosphere gas exchange, eventually leading to hypoxia in the soil27. Additionally, rapid snowmelt in spring can lead to temporary soil flooding, which depletes soil oxygen and may restrict the amount of oxygen reaching the root tissues. Intriguingly, endogenous hypoxia in roots may repress lateral root primordia development28 and hypoxic conditions induce an organ-bending response in the primary root as a possible adaptive avoidance response29. Therefore, we asked whether hypoxia conditions also modulate the bending of LRs. In contrast to the primary root response, we observed that hypoxic stress reduced bending in emerged LRs (Fig. 4c, Supplementary Data 2), demonstrating distinct pathways to regulate root bending in primary and secondary roots. Interestingly, hypoxia stress for 4 h was sufficient to increase GSA of subsequently emerged LRs in Col-0 (Fig. 4c, Supplementary Data 2), mimicking the cxx2-1

**Fig. 2** Genome-wide association study (GWAS) on gravitropic set point angle (GSA). a. Manhattan plot of GWAS results. The dotted horizontal line indicates a significance level of 0.1 after Bonferroni correction for multiple testing. b. Magnification of the peak region on chromosome 2. A highly significant SNP was located at position 8,447,233 in the coding region of CKX2. c. Mean GSA of T and G alleles of CKX2. Horizontal lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the min and max values. Student’s t-test P-value: ***P < 0.001.

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**Fig. 3** a. Bar chart showing the percentage of LRs with different GSA in different treatments. b. Box plot showing the distribution of GSA in untreated and 6-Benzylaminopurin (BAP)-treated Col-0 seedlings. c. Box plot showing the distribution of GSA in untreated and INCYDE-treated Col-0 seedlings. d-g. Representative images and GSA distributions of untreated and 6-Benzylaminopurin (BAP)-treated Col-0 wild type, Col-0 wild type, ahk2 ahk3, ahk2 ahk4 and ahk3 ahk4, untreated and INCYDE-treated Col-0 wild type, Col-0 wild type, cxx2-1 and CKX2OX** seedlings. Kolmogorov–Smirnov test P-values: *P < 0.05, **P < 0.01, ***P < 0.001 (compared to DMSO solvent or Col-0 wild type control). Mean ± SEM, n = 5 plates (16 seedlings with 65–160 LRs per plate). Scale bars, 2 mm.

d-g. Experiments were repeated at least three times.
loss of function phenotype. Furthermore, hypoxic stress did not further increase GSA in the *ckx2-1* mutant (Fig. 4c, Supplementary Data 2), proposing that CK metabolism could mediate hypoxia-dependent repression of LR bending. Furthermore, the LR of *ahk2 ahk4* CK receptor mutants were insensitive to the hypoxia-induced repression of LR bending (Fig. 4d, Supplementary Data 2). Accordingly, we conclude that CK signaling integrates environmental signals, such as hypoxia, into GSA establishment of emerged LRs.

**Single base-pair variation in CKX2 impacts on its in planta activity.** Our data proposes that variation in CKX2 is linked to the control of radial root system expansion in natural *Arabidopsis* accessions. The previously mentioned G allele of CKX2 is associated with higher GSA values (Fig. 2c), which phenocopies the loss of CKX2 function or increase in CK levels in the reference accession *Col-0* (Fig. 2d, Supplementary Data 2). Accordingly, we conclude that CK signaling integrates environmental signals, such as hypoxia, into GSA establishment of emerged LRs.

![Figure 3](https://doi.org/10.1038/s41467-019-11483-4) **Fig. 3** Characterization of cytokinin response factors (CRFs) in lateral roots. **a** Representative images of pCRF2::GFP/GUS and pCRF3::GFP/GUS in stage I–III LRs. Scale bar, 25 µm. **b** Representative images and GSA distribution of *Col-0* wild type, *crf* mutants, and CRFOX lines. Kolmogorov-Smirnov test *P*-values: ***P < 0.001 (compared to DMSO or Col-0). Mean ± SEM, *n* = 5 plates (16 seedlings with 50–120 LRs per plate). Scale bars, 2 mm. **a, b** Experiments were repeated at least three times.

The SP processing is an important determinant of the mature protein and, hence, we examined the enzymatic CKX2 activity in the presence and absence of the signal peptide. We expressed full length SPCKX2 and SPCKX2M as well as the SP-lacking counterparts -SPCKX2 and -SPCKX2M in *Escherichia coli* and measured their ability to oxidize CKs. Both SP-lacking forms -SPCKX2 and -SPCKX2M showed a 10-fold higher activity compared to the SP containing versions (Fig. 5d). This in vitro data suggests that SP processing is required to ensure full enzymatic activity of CKX2.

Next, to assess whether the T to G mutation also affects CKX2 activity in planta, we expressed full length *pCKX2::CKX2* and *pCKX2::CKX2M* encoding versions in the *ckx2-1* mutant background. As expected, the wild-type (*Col-0*) CKX2 was able to fully complement the *ckx2-1* mutant phenotype (Fig. 5e, Supplementary Fig. 5c, Supplementary Data 2). In contrast,
Thus, we conclude that variation in SP processing of CKX2 contributes to the natural variation of CK-dependent angular LR growth in *Arabidopsis*.

**CKX2 does not detectably interfere with auxin signaling in emerged LRs.** Next, we investigated the cellular mechanism by which CKX2 activity modulates the primary GSA of LRs. We first inspected the spatial expression of CKX2 to identify cells in which CKX2 may directly regulate angular growth in LRs. The CKX2::mTurquoise was weakly expressed in the tip of stage I LRs but showed increased expression in stage II and III LRs (Fig. 6a). We confirmed that endogenous CKX2 transcripts are strongly up-regulated in stage II and III LRs by examining expression in excised LR tissue using qPCR (Fig. 6b). Notably, the CKX2::mTurquoise was not readily detectable in the primary root tip (Fig. 6b and Supplementary Fig. 6a), proposing that CKX2 might preferentially act in secondary root organs. In agreement, gravitropic response of cxx2-1 mutant main roots were largely not distinguishable from wild type roots (Supplementary Fig. 6b).

We next aimed to investigate how deviations in CKX2-dependent modulation of CK in LRs may modulate their directional growth. CKs signaling impairs PIN-dependent auxin transport in main roots as well as in LR primordia. We therefore assessed whether CKX2 activity regulates auxin transport in emerged LRs. Because PIN3 is the main regulator of asymmetric auxin redistribution in columella cells of emerged LRs, we initially assessed whether the cxx2-1 mutant shows defective abundance or localization of functional pPIN3::PIN3-GFP in columella cells. At the time of GSA establishment (stage II LRs), PIN3-GFP abundance and asymmetry were not detectably altered in cxx2-1 mutants when compared to wild type (Fig. 6c and Supplementary Fig. 6c). Next, we used the auxin responsive promoter DR5 fused to GFP and assessed whether auxin signaling is affected in cxx2-1 mutant LRs. In accordance with proper PIN3 localization, DR5 signal intensity in columella cells, and asymmetric signal in the flanks was similar in cxx2-1 mutant and wild type stage II LRs (Fig. 6d and Supplementary Fig. 6d).

Overall, this set of data illustrates that auxin responses in gravitropic LR are not detectably altered by CKX2, suggesting that CKX2 modulates angular growth by an alternative, CK-dependent mechanism in emerged LRs.

**Emerged LR display asymmetric cytokinin signaling.** Our data indicates that CK regulates angular LR growth. To further assess the mechanism by which CK modulates GSA establishment in developing LRs, we visualized the spatial distribution of CK signaling, using the two-component signaling sensor (TCSn) transcriptionally fused to GFP (TCSn::GFP). We observed increased CK signaling on the upper side of stage II LRs, coinciding with gravitropic bending (Fig. 6e). This asymmetry declined in stage III LRs, which maintain the previously established GSA (Fig. 6e and Supplementary Fig. 6e). In agreement with the anticipated reduction in CK degradation, the magnitude of asymmetric CK signaling was increased in cxx2-1 mutant LRs (Fig. 6f and Supplementary Fig. 6f). Conversely, asymmetric CK signaling was reduced in the CK receptor double mutant ahk2 ahk4 (Fig. 6g and Supplementary Fig. 6f). These data propose that the increased magnitude of asymmetry in CK signaling across the root tip correlates with reduced LR bending towards gravity.

To determine whether asymmetric CK signaling regulates bending specifically in LRs, we examined the distribution of CK signaling in primary roots responding to gravity. Importantly, we did not observe asymmetric CK signaling in unstimulated or gravity-stimulated primary roots (Supplementary Fig. 6g–h).

the mutated CKX2<sup>M</sup> version was not able to reverse the reduced LR bending of cxx2-1 mutants (Fig. 5e and Supplementary Fig. 5c). Overall, our data suggests that the T to G mutation found in natural accessions renders CKX2 to be largely non-functional in planta by disrupting its secretion and/or SP processing.

**Fig. 4** Cytokinin signaling integrates environmental signals into angular lateral root growth. **a** Comparison of the mean GSA distribution and its geographical (latitude) distribution of the phenotyped accessions. T and G allele of CKX2 are depicted in blue and green, respectively. **b** Relative geographical distribution of the T and G allele of CKX2 in all sequenced Swedish *Arabidopsis* accessions. The distribution of accessions is visualized by R package "rworldmap". **c** Representative images and GSA distributions of Col-O wild-type and cxx2-1 or Col-O wild type and ahk2 ahk4 with and without hypoxia treatment for 4 h. Scale bars, 2 mm. Kolmogorov-Smirnov test *P*-values: ** ***P* < 0.001 (compared to DMSO solvent or Col-O wild type control). Mean ± SEM, *n* = 4 plates (10 seedlings with 60–120 LRs per plate). Experiments were repeated at least three times.
were repeated at least three times. We used the non-parametric Kolmogorov–Smirnov test to determine the statistical significance of the differences in the growth rates between the upper and lower epidermal layers of emerged LRs. The Student’s t-test P-value for the comparison of upper and lower LRs was calculated using the measure of GSA (Fig. 7f, Supplementary Fig. 7a, b).

Accordingly, we conclude that TCSn-based asymmetric CK signaling is specific to LRs and thus contributes to the distinct establishment of primary GSA in LRs. Previous work proposed a hypothetical gravitropic offset component at the upper root flank of LRs that functions as an anti-gravitropic component to LRs and thus contributes to the distinct establishment of primary GSA in LRs. Previous work (Supplementary Fig. 8a, b). Next, we experimentally assessed whether the loss of CKX2 or CK application interferes with cell elongation in stage II LRs. Wild type seedlings showed asymmetric elongation (longer cell length at the upper compared to the lower flank) in stage II LRs (Supplementary Fig. 8a, b, Supplementary Fig. 8a, b). In agreement with reduced LR bending, the cks2-1 loss-of-function mutant, as well as wild type plants treated with BAP showed abolished asymmetry in cellular elongation (Supplementary Fig. 8a, b). Our previous work revealed that differential elongation is a major factor controlling LR bending. However, the loss of CKX2 reduces cellular elongation at the upper root flank in average only by 10% (Fig. 7f, Supplementary Fig. 7a, b).

CKX2 activity determines cellular elongation in emerged LRs. Light sheet-based live cell imaging showed that cells on the upper and lower flanks of emerged LRs show differential elongation for about 8–9 h. To test if this difference can account for the primary GSA establishment, we used these quantitative growth parameters to construct a dynamic computational model of LR bending (Fig. 7a–d and Supplementary Fig. 7a–f). This model incorporates cellular mechanics to simulate cell elongation using stretchable strings as a manifestation of the cell wall elasticity and internal turgor pressure in the cell (see the “Methods” section). The anisotropic growth is simulated by extending the resting length of the string to account for three-fold differences in the growth rates between upper and lower flanks. The resulting model predicts that the incorporation of measured elongation rates on the upper LR flank is able to realistically recapitulate LR bending angle of wild type plants, reaching an angle of about 62–63° within 8–9 h (Fig. 7b, Supplementary Fig. 7a, b).

Cytokinin alters cell division rates and defines angular growth of LRs. In primary roots, CK reduces not only cellular...
elaboration, but also cell proliferation by distinct mechanisms. Moreover, our computational model predicts that the rate of LR bending could be restricted by the number of cells (Supplementary Fig. 7a, b). Thus, we tested if CK might also affect the meristem of LRs and used cell division marker pCycB1:1::GUS in wild type and cdx2-1 mutant background. BAP and INCYDE treatment reduced the abundance of pCycB1:1::GUS at the upper flank of stage II LRs (Supplementary Fig. 8c, d). In agreement, cdx2-1 mutants displayed a more pronounced and statistically significant asymmetry in meristematic cell numbers when compared to wild type stage II LRs, revealing slightly less and more cell divisions at the upper and lower LR flanks, respectively (Fig. 7f). This set of data suggests that CK signaling not only restricts cellular elongation, but also induces a slight asymmetry in cell proliferation in emerged LRs.

Notably, CDKB1;1 and other cell cycle promoting genes are down-regulated in the cef1,3,5,6 quadruple mutant. Hence, we assumed that CRF-dependent control of the cell cycle may contribute to the CK-mediated establishment of GSA in emerged LRs. To reduce cell cycle progression, we used the dominant negative (DN) allele of CDKB1;1 and the cdkb1;1 cdkb1;2 double mutant (Fig. 7g, Supplementary Data 2), as well as the cell cycle inhibitor Roscovitine (Supplementary Fig. 8f, Supplementary Data 2). Both genetic and pharmacological interference with the cell cycle interfered with the LR bending (Fig. 7g, Supplementary Data 2, Supplementary Fig. 8e), phenocopying cdx2-1 mutants. Intriguingly, CDKB1;1DN as well as cdkb1;1 cdkb1;2 showed a meristematic asymmetry, displaying a higher number of meristematic cells in the lower flank as compared to the upper flank (Fig. 7h). This is somewhat reminiscent to the cdx2-1 mutant phenotype and we...
Fig. 7 Cytokinin affects cell elongation and number in lateral roots. a Sketch shows a simplified geometry of a lateral root (LR). LR model consists of tip and elongation zones. The cell elongation rate (visualized as red spot inside the cell) linearly increases from lower flank towards the upper flank of the LR (up to three-fold) based on estimates derived from previous work. Bottom panel, color coding bar for cell elongation rates. b Time-lapse model simulations (screenshots) which lead to the in vivo observed LR bending (63°) after ~8 h. c Left panel corresponds to b. Right panel, 10% decrease in elongation rate only on the upper root flank (white arrow heads). Each simulation represents LR status after 9 h of dynamic elongation. d Time evolution of set-point angle corresponding to different scenarios in c. e Representative image and quantification of first two elongated cells of lateral roots in stage II. f Quantification of the cell number in the upper and lower meristem of Col-0 and cxx2-1. g Representative images and GSA distributions of Col-0 wild-type, CDKB1;1 DN (dominant negative) and cdkb1;1 cdkb1;2. Kolmogorov–Smirnov test P-values: ***P < 0.001 (compared to Col-0). Mean ± SEM, n = 5 plates (16 seedlings with 100–180 LRs per plate). Scale bars, 2 mm. h Quantification of the cell number in the upper and lower meristem of Col-0, CDKB1;1 DN and cdkb1;1 cdkb1;2. e, f, h One-way ANOVA P-values: *P < 0.05, **P < 0.01, ***P < 0.001. Horizontal lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the min and max values, n = 10–20 individual LRs. Scale bar, 10 μm. e–h Experiments were repeated at least three times.
assume that the CK-dependent, asymmetric interference with the cell cycle is amplified in these cell cycle mutants when compared to wild type. In agreement with such a scenario, the gravity response kinetics of primary roots, which lack asymmetric TCSn-based CK signaling, were not affected by the CDKB1;1DN as well as cdkb1;1 cdkb1;2 mutations (Supplementary Fig. 8f, g). This suggests that not only cellular elongation but also the negative impact of CK on the cell cycle in stage II LRs is a particular determinant of directional LR growth.

Overall, this set of data suggests that CK modulates both differential cell elongation and cell proliferation to interfere with growth at the upper flank of LR, ultimately regulating angular LR growth and radial expansion of the root system.

**Discussion**

Because root systems are hidden beneath the soil, the study and directed improvement of root architectural traits in crop breeding programs have been delayed. There is growing interest to alleviate the harmful effects of drought stress by modulating the primary GSA of LRs. Despite the apparent importance of the root system depth, the molecular mechanisms regulating the direction of LR growth are poorly understood. Thus, understanding the molecular mechanisms establishing the primary angular growth in LRs could guide future engineering of plants to suit certain habitats. Anticipating that natural variation could provide valuable insights on how to sustainably engineer root systems, we focused on the primary growth direction of LR in natural *Arabidopsis* accessions.

We reveal that the primary growth direction of LR varies substantially within a population of natural *Arabidopsis* accessions. Primary LR angles of hypo-responsive or hyper-responsive accessions followed a similar trend regardless of whether they were grown in soil, two-dimensional or three-dimensional in vitro systems. We thus conclude that this approach is suitable to assess the genetic control of angular LR growth. Using a GWAS approach, we show that angular growth of LR is controlled by CKX2-dependent metabolism of the phytohormone CK. CKX2 contains a SP to enter the secretory pathway, which could be crucial for its impact on CK perception. However, the precise site of CK receptor activity (plasma membrane and/or endoplasmic reticulum) is still under debate. We conclude that variation in an amino acid substitution after the predicted cleavage site impacts on SP processing of CKX2, which consequently obstructs CKX2 activity in planta. Our data suggest that the lack of SP processing abolishes the secretion and enzymatic activity of CKX2, thereby contributing to CK-dependent GSA trait variation in natural *Arabidopsis* accessions.

Nordic accessions preferentially express an inactive CKX2 variant, which prompted us to investigate whether environmental cues further define the root system in a CK-dependent manner. We revealed that hypoxic conditions induce more horizontal LR growth through CK signaling. The increased frequency of an inactive CKX2 allele in Nordic accessions suggests that the allele may have been selected for in these populations, promoting more horizontal root growth. It is an intriguing possibility that more horizontal, near surface roots may rectify gas exchange under hypoxia conditions, potentially alleviating the harmful effects of hypoxic stress in these Nordic, snow covered habitats. This assumption, however, awaits further experimental validation.

Our analysis suggests that primary and secondary roots have distinct responses to CK. While CK signaling abolishes PIN-dependent transport in main roots, we show that CKX2-dependent interference with endogenous CK levels does not affect PIN3 and auxin signaling in emerged LRs. Moreover, CK signaling is asymmetric in emerged lateral, but not primary roots, proposing a unique role of CK in regulating asymmetric growth responses in LRs. Also abscisic acid signaling displays distinct activities in main and lateral root organs, presumably allowing distinct organ growth rates in response to environmental stresses. Thus, we propose that hormone signaling might be generally co-opted in primary and secondary roots to facilitate diverged growth responses to the environment.

The increase and decrease of CK levels slightly accelerate the rate of gravitropic bending in primary root, but the developmental importance of this effect remains uncertain. In contrast, we show here that CK signaling plays a developmental role in establishing the primary GSA of LRs. Moreover, an increase and decrease of CK signaling correlate with reduced and enhanced down-ward bending of LRs, respectively. Mechanistically, we showed that CK signaling interferes with cellular elongation and proliferation in emerged LR to reduce LR organ bending towards gravity. These stage II LRs undergo de novo formation of the elongation zone. During this short developmental time window, the CK-dependent reduction in cell proliferation could have hence caused an immediate influence on the number of elongating cells. We detected only a mild asymmetry in cell numbers at the LR organ flanks of cka2 mutants, proposing that these LRs display only few cells less at the upper compared to the lower root flanks. Such a small impact could nevertheless compromise angular LR growth, because our computational model predicted that an asymmetric reduction in cell number at the upper root flank (Supplementary Fig. 7c–f) or gradually along the LR organ (Supplementary Fig. 9a–c) would induce some constraints, additionally limiting organ bending. However, this aspect awaits experimental validation, requiring detailed live cell imaging and possibly mechanical constraint measurements in LRs.

We illustrate that CKX2 contributes to the rate of asymmetric CK signaling, but CKX2 expression did not show a pronounced asymmetry. Similarly, the CK response factors CRF2 and CRF3 are not asymmetrically expressed in emerged LR. We, hence, conclude that additional so far unknown factors play decisive roles in establishing asymmetric CK signaling across the LR tip. Our work proposes that an auxin transport mechanism promotes the asymmetry of CK signaling. Accordingly, auxin could generate an anti-gravitropic signal to interfere with its own gravitropic impact in LRs. Unlike auxin, the mechanisms of intercellular CK transport are poorly characterized. One intriguing possibility is however that the asymmetric auxin signal could favor CK relocation towards the upper side of LRs, inducing differential CK signaling and growth repression on this side. However, it is also possible that differential CK signaling occurs at the level of signal integration and might be independent of differential distribution of CK. Future work will examine these possibilities to uncover the mechanism by which CKX2 is linked to differential CK activity across a stage II LR.

At the transition to stage III, PIN3 expression in columella cells transiently decreases. This temporal absence of PIN proteins in gravity sensing columella cells correlates with the onset of non-differential auxin redistribution and symmetric (non-gravitropic) LR organ growth. In agreement, also the asymmetry in CK signaling declines at the onset of stage III LR, proposing that gravity-induced, differential growth at upper and lower organ flanks is generally switched off at this stage. Stage-specific repression of PIN3 and PIN4 expressions in columella cells of stage III LRs does not induce alterations in GSA of LR, suggesting that the gravitropic machinery is similarly inactive in
Methods

Plant material and growth conditions. Seeds of Arabidopsis thaliana accessions were kindly provided by Magnus Nordborg and Wolfgang Busch. The following lines and constructs have been described previously: akh2-7 (akh2), akh3-7 (akh3)39, crel-12 (akh4)40, akh2 akh3, akh3 akh439, arr3, arr4, arr10-5, arr12-1 / PARR3::GUS, PARR4::GUS, PARR10::GUS, PARR12::GUS41, CDKB1;1 DN42, cdkb1;1 cdkb2;143, 35S::CKX244, cks2-145, cysf-2, cysf-1, cysf-3, cysf-2, RPS5a::CFR2, 35S::CRF514, pCRF2::GFP/GUS, pCRF3::GFP/GUS45, pCRF3::GFP/GUS56, pCYS1:: GUS7, pDR5::GFP57, pPIN3::PIN3-GFP48, TCSn::GFP32, TCSn::GFP cre1-12, pARR3::GUS, pARR4::GUS, pARR10::GUS, pARR12::GUS41, CDKB1;1 DN42, cdkb1;1 cdkb2;143, 35S::CKX244, cks2-145, cysf-2, cysf-1, cysf-3, cysf-2, RPS5a::CFR2, 35S::CRF514, pCRF2::GFP/GUS, pCRF3::GFP/GUS45, pCYS1::GUS7, pDR5::GFP57, pPIN3::PIN3-GFP48, TCSn::GFP32, TCSn::GFP akh2 akh449, pUBQ10::LUC2o15. Seeds of Brassica napus (L.) were kindly provided by Saatzucht Donau. Seeds were surface sterilized, stratified at 4 °C for 2 days in the dark. Seeds were grown vertically on half Murashige and Skoog medium (1/2 MS salts (Duchefa), pH 5.9, 1% sucrose, and 0.8% agar). Plants were grown under long-day conditions (16 h light, 8 h dark) and transferred into a box with water and allowed to absorb water overnight from the bottom side. Seeds containing the pUBQ10::LUC2o transgene35 were stratified for 2 days at 4 °C in distilled water and three seeds were sown in the center of each rhizotron. Each rhizotron was equipped with a unique barcode. All rhizotrons were sprayed down with water and sealed with a transparent lid and packing tape. Plants were grown at 22/18 °C (day/night) under long-day conditions (16 h light, 8 h dark) using LED lights (Vayola, C-Series, N12 spectrum) with a light intensity of about 130 µmol m⁻² s⁻¹. After 2 days, the transparent lid was unscaled, rhizotrons were watered with 100 µl of a transferring pipettes of water, and the lid left loose for an additional day. After removing the lid, rhizotrons were watered twice per day with two transferring pipettes of water each time until 9 days after sowing.

Plant imaging in rhizotrons. 20 days after sowing, the automated rhizotron handling system (designed by Modular Science, San Francisco) added 50 ml of 300 mM NaCl (Sigma) as electron acceptor and different concentrations of N6-(2-isopentenyl)adenine (Sigma) as substrate. 20 days after sowing, the automated rhizotron handling system (designed by Modular Science, San Francisco) added 50 ml of 300 mM NaCl (Sigma) as electron acceptor and different concentrations of N6-(2-isopentenyl)adenine (Sigma) as substrate. To identify the genetic basis of the IV transition of LRs.

Hypoxia treatment. All following treatments were performed in air-tight glass desicicators in which seedlings grown on vertical agar plates were carefully placed with the lids removed. Seedlings were exposed to a hypoxia treatment by flushing the desicicators with humidified air. Flow rates were controlled by mass flow controllers (MAS-VIEW, Bronkhorst). At the end of the hypoxia treatment, plates were removed from the desicicators, closed, and transferred back to the climate chamber. The plates remained in the climate chambers under control growth conditions for 5 days after the treatment after which they were scanned using an EPSON Scanner V300.

DNA constructs. The promoter region and full-length CKX24 or coding DNA sequence (CDS) were amplified by PCR (Supplementary Data 3) from genomic DNA or cDNA using Q5 high-fidelity DNA polymerase (NEB) and cloned either alone or under of the 35S promoter together with GFP and mScarlet-i into pPLV03 or pGEX5x3 using Gibson Assembly Master Mix (NEB). Subsequently, this plasmid were used for (3) in vitro mutagenesis (Supplementary Data 3) to obtain CKX2M. The resulting constructs were transferred into Col-0 and cks2-2 plants using the floral dipping method or for transient transformation in tobacco plants.

Activity measurement of recombinant proteins. Recombinant proteins were expressed as GST fusion proteins and in Escherichia coli BL21 codon plus strain. Proteins were purified using the Sepharose beads affinity method (Glutathione Sepharose 4B; GE Healthcare).

The activity was measured using a modified end-point method previously described31. In brief, the samples were incubated in a reaction mixture (total volume of 600 µl) that consisted of 200 mM McIlvaine buffer (100 mM citric acid and 200 mM Na2HPO4) pH 7.4, 500 µM 2,6-dichlorophenol indophenol (DCPIP; Sigma) as electron acceptor and different concentrations of N6-(2-isopentenyl)adenine (IP; Sigma) as substrate. The volume of the enzyme sample used for the assay was adjusted based on the enzyme activity. The incubation time at 37 °C was 1 h. The enzymatic reaction was stopped after incubation by adding 300 µl of 40% trichloroacetic acid (TCA), then 200 µl 2% 4-aminophenol (Sigma) (in 6% TCA) was added and the sample was centrifuged at 20,000 × g for 5 min to remove protein precipitate. 200 µl supernatant was used to measure the absorption spectrum from 352 to 500 nm to determine the concentration of produced Schiff base with ε352 = 15.2 M⁻¹ cm⁻¹ using a plate reader.

Microscopy. Confocal microscopy was performed using a Leica SP5 (Leica). Fluorescence signals for GFP (excitation 488 nm, emission peak 509 nm), mScarlet-i (excitation 561 nm, emission peak 607 nm), mTurquoise (excitation 434 nm, emission peak 474 nm) and propidium iodide (PI) staining (20 µg ml⁻¹) (excitation 569 nm, emission peak 593 nm) were detected with a ×40 or ×63 (water immersion) objective. The fluorescence signal intensity (mean gray value) of the presented
markers was quantified using the maximum projections obtained from a Z-stack series that were taken and analyzed using the Leica software LAS AF 3.1. The region of interest (ROI) was defined for each individual seedling.

To determine meristematic cell numbers and cell size in stage II LRs, 8-day-old seedlings (n = 10–15) were stained with PI and microscopy was performed using confocal microscope (see above). The first two epidermal cells (adjacent to the main root) were considered for cell size measurements in stage II LR. Epidermal cell numbers were counted between the quiescent center and the first elongating cell (twice as long as wide) at the upper and lower flank of stage II LRs. Graphpad Prism software was used to evaluate the statistical significance of the differences observed between control and genotype/treatments (one-way ANOVA).

Gravitropic set-point angle measurements. Plates with 14-day-old seedlings were scanned and the initial gravitropic set-point angle (gSA) of individual LR was measured with reference to the gravity vector* using Image J software. All available LR from each seedling were measured. Individual GSA values were then sorted into eight categories: 0–30°, 30–50°, 51–70°, 71–90°, 91–110°, 111–180°. Percentages of incidence were calculated for each category and graphs of GSA distribution were generated. The test of Kolmogorov–Smirnov (KS-test) was used online (http://www.physics.csbsju.edu/stats/KS-test.n_plot_form.html) to statistically evaluate the GSA data sets generated from mutants and treated seedlings in comparison to wild type and untreated controls, respectively.

Histochemoical GUS staining. The GUS histochemical staining of acetone-fixed 7-day-old seedlings containing pCYC815:GUS fusion constructs followed a previously described method52, using 5-Bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-Gal, Carl Roth) as substrate. In brief: seedlings were fixed in 90% aceton for 30 min. After washing with 0.1 M Na-phosphate buffer (pH 7) seedlings were incubated for 2 h at 37 °C in the GUS staining solution (2 mM X-Glc (dissolved in DMSO), 0.1% Triton X-100, 10 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM ferricyanide, 120,000 U/ml Na-phosphate buffer pH 7). Examination of stained seedlings and image acquisition were performed with a light microscope (Zeiss Observer D1) equipped with a DFC 300 FX camera (Zeiss). The intensity of the staining was quantified as described ref. 53 in a region of interest (ROI), which was kept constant. Graphpad Prism software was used to evaluate the statistical significance of the differences observed between control and treated groups (One-way ANOVA).

Transient transformation and western analysis. The Agrobacterium tumefaciens strain GV3101 was transformed with the respective construct and grown for 2 days at 28 °C in 5 ml Luria-Bertani (LB). The preculture was used to inoculate 25 ml LB and incubated at 28 °C in 5 ml Luria-Bertani (LB). The preculture was used to inoculate 25 ml LB and incubated at 28 °C for 18 h. The bacterial suspension was harvested and resuspended in 5 ml of 5% sucrose and in 0.1% Tween 20, with freshly added proteinase inhibitor cocktail (Roche). Protein concentration was assessed using the Bradford method. Membranes were probed with a 1:5000 dilution of GFP antibody (Ab2390, abcam) or 1:20,000 of tubulin antibody (T6074-200UL, Sigma). Goat IRDye 800CW anti-mouse (926-32210, LI-COR) or goat IRDye 800 CW anti-rabbit (926-32211, LI-COR) was used as secondary antibody. The signals were detected and quantified using the Odyssey Imagine System (LI-COR).

RNA extraction, cDNA synthesis and quantitative PCR. RNA extraction was done as described previously54, in brief: a pool of 10 LR or root tips were collected in 30 µl of 100% RNAAlater (Thermo Fisher) and 500 µl of TRIzol (Sigma) was added followed by brief vortexing (2x for 2 s each) and incubating at 60 °C for 30 min. 100 µl of chloroform was added, and then, samples were vortexed briefly (2x for 2 s each) and incubated at room temperature for 3 min. After centrifugation at 12,000 × g for 15 min at 4 °C, the aqueous phase was transferred to a new tube. To precipitate the RNA, an equal volume of isopropanol and 1.5 µl of GlycolBlue (Thermo Fisher) were added followed by a ~20 °C incubation for 15–18 hours and centrifugation at 12,000 × g for 60 min at 4 °C. After removal of the supernatant, the pellet was washed by adding 500 µl of 75% ethanol, vortexing briefly and then centrifuged at >20,000 × g for 15 min at 4 °C. The 75% ethanol wash step was repeated 1×. As much ethanol as possible was removed followed by the drying of the pellet by letting the Eppendorf tube sit on ice with lid open for 10 min. Precipitated RNA was then resuspended with 5–12 µl of nuclease-free water, stored at −80 °C. cDNA synthesis was performed using SuperScript II (Thermo Fisher) and qPCR using 2× Taqman for SYBR Assay—no ROX (Eurogentec) following the manufacturer’s instructions on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Expression values were normalized to the expression of ubiquitin 5 (UBQ5) and translation initiation factor EIF4A.

Cytokinin measurements. Quantification of cytokinin metabolites was performed according to the method described by Svacina et al., including modifications described in ref. 55. Briefly, root samples (20 mg FW) were extracted in 1 ml of modified Bieleski buffer57 together with a cocktail of stable isotope-labeled internal standards used as a reference (0.25 pmol of CK bases, ribosides, N-glucosides, and 0.5 pmol of CK O-glucosides, nucleotides per sample added). The extracts were purified using the Oasis MCX column (30 mg/1 ml, Waters) and cytokinin levels were determined using the LC–MS/MS system consisting of an ACQUITY UPLC System and a Xevo TQ-S triple quadrupole mass spectrometer (Waters). Results are presented as the average of five biological replicates ± standard deviation in pmol/g FW. Statistical examinations were made between Col-0 wild type and cks2-1 roots using one-way ANOVA analysis.

Assessment of gravitropic main root growth. We used infrared-based time lapse of gravitropic main roots (90° tilted plates) in the dark. Growth rate normalization was performed as described in ref. 58.

Maps. The maps were created in R using the package “swordmap”59.

Description of the computer model of LR. For the sake of simplicity, our model is composed of rectangular grid in which each box represents a single cell—a basic space discretization unit in the model. Cell walls are modeled as a linear elastic spring (connecting two adjacent vertices) that can expand and contract in order to minimize forces acting on each spring. The magnitude of force exerted by this spring is $k_s \cdot (\mathbf{l}_{\text{vert}} - \mathbf{p}_u - \mathbf{p}_l)$ and is positive for spring compression. $k_s$ characterizes the stiffness of the spring and was set to 0.9 in all simulations. This force is in the direction of the spring from $p_u$ to $p_l$. $p_u$ is the position of vertex $u$, and $p_l$ is the position of neighboring vertex $v$. The total force exerted on vertex $u$ located at position $p_u$ by all such springs can be written as

$$F_u^{\text{linear}} = \sum_{v \in \mathcal{N}_u} k_s \cdot (\mathbf{l}_{\text{vert}} - \mathbf{p}_u - \mathbf{p}_l) - \mathbf{p}_u - \mathbf{p}_l$$

where $\mathcal{N}_u$ is the set of vertices adjacent to vertex $u$. The norm symbol indicates the Euclidean distance between the points.

In addition to the forces acting on a vertex due to springs, a force due to the turgor pressure inside the cell ($p_u = 0.05$) acts in the direction normal to each wall ($n_u$):

$$F_u^{\text{pressure}} = p_u \cdot n_u \cdot \mathbf{r}$$

This cell turgor pressure helps to maintain cell shape protecting from artificial deformation60.

Combining the individual force components, the total force acting on a vertex $u$ is the sum of forces acting on each cell wall and internal pressure inside the cells. According to the second Newton’s Law of dynamics we calculated the velocity ($\mathbf{v}_u$) and position ($\mathbf{p}_u$) of vertex $u$ over time for point mass $m_u = 1$ with the following formulas:

$$\frac{d\mathbf{v}_u}{dt} = \frac{F_u^{\text{total}} - \mathbf{M}_u \cdot \mathbf{a}_u}{m_u}$$

where $\mathbf{r} = 0.2$ is a damping constant.

Other details of a model setup and methodology as well as its successful applicability for resolving the organ bending simulations can be found in ref. 60. The LR root model is spatially divided into two zones (root tip and cell elongation zones) along z-axis based on the threshold parameter that defines distance between individual cell center and right-most cell centers. The right-most boundary vertices were fixed in x-direction to mimic connection to the main root axis (outmost right) such that growth occurs only from the main root axis as observed experimentally. We use thresholds that controls either the length of whole elongation zone or simply the number of elongating cells only on the upper root flank or progressively from the top to the bottom to mimic the input of an asymmetric meristem in our simulations (Supplementary Figs. 7 and 9). Along y-axis axes elongate at different rates based on the linear interpolation between the minimal measured elongation rate at the bottom part of the LR (5 µm h−1) to the maximal elongation rate at the top flank of the LR (15 µm h−1). Furthermore, we found that linear interpolation gave the best fit to the experimental observations in terms of >2-fold change in cell size between upper and lower LR root flanks. To simulate reduced elongation, we consequently reduced the maximal rate of cell elongation on upper root flank by 10%. Cell elongation is simulated by expanding the rest length of linear springs at each growth time step (0.003). The growth time step occurs after mass-reduced system reaches the transient equilibrium such that the slower growth and faster mechanics steps follow consecutive iterations60.

Maps. The geometry of the model was created using a version of the VV simulator52,62 embedded in the modeling software 1-studio52 (http://algorithmicbotany.org/). Cell mechanics and growth steps was solved using the forward Euler method. All simulations were terminated after 9 h of growth to match the experimental observations.
Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data generated or analyzed during this study are included in this article and its supplementary information files. Seeds and plasmids are available from the corresponding author upon request. The GWAS data set has been deposited at https://gwas.gmi.oeaw.ac.at/#/analysis/12722/overview.

Code availability
All information for building the model is indicated in the manuscript. Further information is available from the corresponding author upon request.

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Author contributions

S.W. performed most experiments. M.R.R. initiated the project, M.S., E.S. and K.D. performed confocal microscopy. H.L., T.L.R. and J.R.D. contributed GLO-Roots data. I.P. and O.N. conducted quantification of endogenous cytokinins. S.M. and R.S. performed hypoxia experiments K.W. designed and described the dynamic computer model simulation. J.K.-V. devised and coordinated the project. S.W. and J.K.-V. wrote the manuscript. All authors saw and commented on the manuscript.

Additional information

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