Running title:
SHORT-ROOT is a key regulator of root development

Journal research area:
Development and Hormone Action – Associate Editor Richard Amasino

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Article title:
SHORT-ROOT regulates primary, lateral and adventitious root development in
Arabidopsis

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This work was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and Engineering Physics Scientific Research Council (EPSRC) CISB award to the Centre for Plant Integrative Biology (to ML, RS, MH, TP, SM and MJB); Marie-Curie Fellowship funding (to BP); 2009 BBSRC Professorial Research Fellowship award (to MJB); University of Nottingham Interdisciplinary Doctoral Training Centre funding (to SZ and SM); Collaborative Research Center 592; the Excellence Initiative of the German Federal and State Governments (EXC 294); Graduiertenkolleg 1305, Bundesministerium für Forschung und Technik (BMBF); and Deutsches Zentrum für Luft und Raumfahrt and the European Space Agency (to IP and KP).
Abstract:

*SHORT-ROOT (SHR)* is a well characterised regulator of radial patterning and indeterminacy of the *Arabidopsis* primary root. However, its role during the elaboration of root system architecture (RSA) remains unclear. We report that the indeterminate wild-type *Arabidopsis* root system was transformed into a determinate root system in the *short-root (shr)* mutant when growing in soil or agar. The root growth behaviour of the *shr* mutant results from its primary root apical meristem (RAM) failing to initiate cell division following germination. The inability of *shr* to reactivate mitotic activity in the RAM is associated with the progressive reduction in the abundance of auxin efflux carriers, PIN1, PIN2, PIN3, PIN4 and PIN7. The loss of primary root growth in *shr* is compensated by the activation of anchor root primordia whose tissues are radially patterned like wildtype. However, SHR function is not restricted to the primary root but is also required for the initiation and patterning of LRP. In addition, SHR is necessary to maintain the indeterminate growth of lateral and anchor roots. We conclude SHR regulates a wide array of *Arabidopsis* root-related developmental processes.
Introduction:

Higher plants exhibit an amazing diversity of root architectures at both the systems and anatomical levels (Waisel et al., 2002). Many dicotyledonous plants like Arabidopsis have a primary root that repeatedly branches to generate several orders of lateral roots, whereas the root systems of cereal crops such as rice and maize are predominantly composed of adventitious roots (Hochholdinger et al., 2004; Osmont et al., 2007). Despite these anatomical differences, with the exception of the highest orders, roots from all plant species exhibit indeterminate growth behaviour (i.e. able to maintain growth indefinitely; Waisel et al., 2002).

The cellular basis of indeterminate root growth has been best studied in the Arabidopsis primary root. In this experimental system, stem cells (also termed initials) abut specialised organising cells (often denoted as the quiescent centre [QC]) within the root apical meristem (Dolan et al., 1993). When initial cells divide they generate two daughter cells; the one directly abutting the QC remains a stem cell; whilst the remaining daughter cell (also termed a transit amplifying cell) undergoes a finite number of cell divisions before exiting the meristem and elongating (Scheres, 2007). Mutants that disrupt initial cell/QC function often exhibit a determinate root growth phenotype, resulting in the termination of organ growth (Benfey et al., 1993, DiLaurenzio et al., 1996).

Genetic studies have identified several genes that regulate indeterminate root growth in Arabidopsis. One of these genes encodes the SHORT-ROOT (SHR) protein that has been shown to be essential for root apical meristem (RAM) function (Benfey et al., 1993, Scheres et al., 1995). In mutant plants lacking SHR, primary roots are dramatically shortened (Benfey et al., 1993, Scheres et al., 1995). Another related protein, SCARECROW (SCR), has a similar role alongside SHR (Benfey et al., 1993, DiLaurenzio et al., 1996). SHR and SCR genes encode closely related transcription factors belonging to the GRAS gene family (DiLaurenzio et al., 1996; Helaruitta et al., 2000). SHR has been demonstrated to directly regulate the expression of genes including SCR (Levesque et al., 2006) and a number of cell cycle components including the D-type cyclin, CYCD6;1 (Sozzani et al, 2010).
The spatiotemporal roles of SHR, SCR and various others genes such as PLETHORA1 (PLT1) and 2 (PLT2) in establishing and maintaining the RAM have been extensively studied. In the model proposed by Blilou et al. (2005), redistribution of polar localised auxin efflux carriers of the PIN-FORMED (PIN) family results in accumulation of auxin at the prospective meristem stem cell niche; this maximum is maintained throughout post-embryonic elaboration of the root (Aida et al., 2002, Friml et al., 2003, Sabatini et al., 1999, Blilou et al. 2005). The distal auxin maximum then determines the expression pattern of the transcription factors, PLETHORA1 (PLT1) and PLT2, which are required for quiescent centre (QC) identity and stem cell specification from embryogenesis onwards (Aida et al., 2004). The distal auxin maximum maintained by PIN proteins restricts PLT expression to the basal pole of the pro-embryo; this facilitates the activation of the root primordium. In turn, PLT positively regulate PIN expression (PIN3, PIN4 and PIN7) (Blilou et al., 2005), which reinforces the flux of auxin into the RAM, thereby maintaining the postembryonic position of the distal stem cell niche. The radial expression domains of the transcription factors SHR and SCR participate in the positioning of the stem cell niche. The SHR gene is transcribed in the stele, but the mobile SHR protein migrates outwards one cell layer to the adjacent QC, cortex-endodermis initial (CEI) cell and endodermis layer (Helariutta et al., 2000, Nakajima et al., 2001) where it activates expression of SCR (Nakajima et al., 2001). The endodermal/QC domain of SHR/SCR protein expression overlaps with the distal PLT expression domain, with the highest level of both components defining the stem cell niche.

Despite of the importance of SHR for regulating RAM function and indeterminate primary root growth, no studies have addressed its impact on root system architecture (RSA) to date. The progression of a plant root system from a single meristem-containing primary root, formed during embryogenesis, to the extensively elaborated RSA of a mature plant involves numerous exogenous abiotic and biotic factors plus endogenous genetic factors. One of the most important factors determining total RSA is the post-embryonic appearance of lateral root primordia (LRP) and ultimately lateral roots (LR) that branch off from the primary root (Nibau, 2008). Given the fact that lateral root development is considered to be a broad recapitulation of equivalent processes in the primary root, we investigated whether SHR plays a role in LRP development, and consequently on global RSA. We report that SHR is indeed a key regulator, controlling primary, lateral and anchor root development at the macroscopic scale, and patterning of lateral (but not anchor) roots at the microscopic scale.
Results:

The short-root mutation causes root system architecture to become determinate

The short-root mutants were initially identified based on their severely reduced primary root growth and radial patterning defects (Benfey et al., 1993, Scheres et al., 1995). Nevertheless, short-root mutant seedlings are able to grow and complete their lifecycle, somehow compensating for the dramatic reduction of their primary root length.

We initially investigated the morphological effect the short-root (shr) mutation had on the development of the root system in soil. X-ray Computed Tomography (CT) was employed to investigate the 3D root architecture of three weeks-old wild-type and shr seedlings grown in a sandy loam soil (Figure 1A-C, Supplemental Movies SM1 & SM2). This technique relies on discriminating differences in attenuation density between the soil matrix and live roots. Difficulties can arise when discriminating differences in attenuation density between water filled pore space and live roots. Nevertheless, the CT scan and subsequent 3D reconstruction allowed us to retrieve > 50% of the root tissues of the scanned plants. This amounted to a total root volume of 0.3 mm³ for shr and 1 mm³ for the wild-type (root volume was automatically estimated by the 3D reconstruction software – see material and methods for additional details). Roots were relatively straight, with a mean tortuosity of 1.05 both in shr and wild-type (tortuosity being the ratio of the root length over the shortest distance between the two extremities of the measured root). However, shr did not exhibit a strong 3D branching phenotype when grown in the sandy loam soil compared to the wild-type.

To investigate further the capability of shr to develop a branched root architecture, we then compared 4 weeks-old compost grown wild-type and shr seedlings (Figure 1D). Seedlings grown in compost exhibited a much bigger root system compared to soil-grown plant. The dimorphism between shr and the wild-type was also amplified compared to soil-grown plants. Whereas wild-type plants had an indeterminate tap root system, the shr mutant exhibited a fibrous determinate root system. The shr seedling had numerous short roots of equivalent lengths, each one thicker than individual wild-type roots. Microscopic observation of the shr roots revealed a low degree of branching towards the apex of roots, with no discernable
primordia or young laterals along the bottom-half of roots (data not shown). Instead new roots appeared to originate and branch near the seedling root-shoot junction. Similar alterations of RSA can be similarly observed in agar-grown shr seedlings, which exhibit reduced growth and a characteristic tripod architecture after one week of growth (Figure 1E).

The short-root mutant fails to reinitiate cell division in the primary root following germination

We next investigated how the shr mutation could lead to this dramatic change in overall RSA. To understand the basis for the determinacy of the root apical meristem in shr, we studied the mitotic activity of the mutant pre- and post-germination using the cyclin1:1:GUS marker (Figure 2).

As the Arabidopsis embryo develops almost every cell is continuously dividing as revealed by the mitotic marker cyclinB1:1:GUS (Figure 2A). No major differences in mitotic marker expression were observed between shr and the wild-type during embryogenesis. After seedling germination cell division must be reinitiated in the root apical meristem (Masubelele et al., 2005). SHORT-ROOT and SCARECROW gene products are required to maintain the activity of this population of root stem cells (Sabatini et al., 2003). However, we observed that in the shr mutant background expression of the mitotic marker was largely absent in cells close to the root apex post-germination (Figure 2B). Hence, SHR appears to be required for the reactivation of the Arabidopsis root apical meristem following germination. Despite this root division defect, shr seedlings germinated normally (Figure S1). However, root length is severely reduced compared to wildtype or scr mutant (Figure S2).

As a result of the failure to reactivate mitotic activity in the primary root, the root apical meristem became progressively smaller (Figure 2C). This reduction in root apical meristem size was monitored using several markers including the LAX3 pro:GUS reporter (Swarup et al., 2008) which is expressed in stele cells entering the elongation zone. We observed that the LAX3 pro:GUS marker moves progressively closer to the root tip in older short-root seedlings (Figure 2C). We conclude that as the shr root grows, cells exit the arrested meristem for the elongation zone until the meristem is largely depleted of cells. SHR is required to maintain indeterminate growth (and therefore appears to function as an indeterminacy factor) in the post-embryonic primary root meristem.
The short-root mutant is perturbed in auxin abundance and biosynthesis

The short-root mutation disrupts the maintenance of RAM activity, a process which is closely associated with the mitotic signal auxin. To investigate whether there was a reduction in the accumulation and/or synthesis of auxin in mutant root tissues, we analysed IAA concentration and biosynthesis rate in the root apex of wild-type and shr seedlings during the first 10 days of germination using mass spectrometry (as described in Edlund et al., 1995; Ljung et al., 2005). Surprisingly, IAA concentration was significantly elevated in shr root tips compared to wild-type in 3 and 6 DAG seedlings (pooled 1mm root tips) (Figure 3A). However, no significant difference in IAA concentration between wild-type and shr was observed in root tips from 10 DAG seedlings.

In parallel, the IAA synthesis rate in root tips from wild-type and shr seedlings was also analysed. 5-day-old excised seedling roots were incubated in liquid medium containing 30% deuterated water (²H₂O). Excised roots were incubated instead of whole seedlings in order to separate IAA synthesised de novo in the root system from IAA synthesised in the aerial part of the seedling and then transported to the root. After 24 h of incubation, 1mm root tips were pooled and analysed (Figure 3B). We observed a significantly higher IAA synthesis rate in shr root tips compared to wild-type suggesting that the higher IAA concentration that we observe in the root apex is in part due to higher root specific IAA synthesis, and not necessarily caused by accumulation of IAA coming from the aerial part of the seedling.

We conclude that shr primary root determinancy is not due to either a reduced level or synthesis of auxin. Instead, mutant root apical meristem tissues exhibit a higher rate of biosynthesis of auxin and transient accumulation of this hormone during the first week after germination.

Root determinacy is associated with a loss of PIN auxin carrier accumulation
In order to understand why auxin accumulates in the root apex during the first 10 DAG, we monitored the expression patterns of AUX1 and PIN classes of auxin influx and efflux carriers in wildtype versus shr root apical tissues (Fig. 4).

In wildtype root apical tissues, PIN1 was detected predominantly in stele and endodermal cells and its pattern of expression did not change significantly during root development between 2 and 10 DAG (Figure 4A). In shr root apical tissues, the PIN1 signal was observed only in stele cells since this mutant lacks an endodermis (Figure 4A). Most strikingly, PIN1 abundance progressively decreased in shr mutant seedlings from 4 DAG and the signal had almost completely disappeared by 10 days (Figure 4A).

In the case of PIN2, its signal was significantly reduced from 4 DAG in shr versus wildtype (Figure 4B). However, the polarity of the PIN2 protein in epidermal cells was the same in shr as in wild type, i.e. shootward/basipetal (pointing away from the root apex) in the membrane of epidermal cells and rootward/acropetal (pointing towards the root apex) in the membrane of cortical cells until the transition to the elongation zone where PIN2 switches to a basipetal orientation in the cortex (Supplemental Figure S3). Nevertheless, the switch in PIN2 orientation in the cortex was shifted toward the root apical meristem in shr (data not shown). This shift is consistent with the reduction of root meristem size in the shr mutant (Figure 2).

In wild type root apical tissues from 2 to 10 DAG, PIN4 was expressed in the quiescent centre, the surrounding initials and their abutting daughters with a polar localization towards the quiescent centre (Figure 4C) consistent with previous reports (Friml et al., 2002; Blilou et al., 2005). In contrast, PIN4 was absent in the quiescent centre of shr seedling roots 2DAG, but was detected in initials and their daughter cells plus the stele (Figure 4C). However, PIN4 was no longer detectable in these shr root tissues from 4 DAG (Figure 4C). Immunolocalization (Figure 4D) revealed that PIN7 was one of the first PIN proteins to be down-regulated in the shr background, being almost undetectable at 2 DAG. By day 10, neither PIN7 (nor PIN3) proteins were detectable (Figure 4D & E).

In addition to PINs, we monitored the expression of an AUX1pro::AUX1-YFP translational fusion in the shr background. Contrary to PINs, AUX1-YFP expression was maintained in the shr root apex (Figure 4F). The only significant difference detected was the absence of AUX1-YFP signal in the vascular strand and a smaller domain of expression in the lateral root cap (reflecting the loss of protophloem cells and smaller size of the meristem, respectively).
In summary, *shr* root apical tissues exhibit a progressive loss of PIN (but not AUX1) auxin transporter expression, which is likely to negatively impact meristem function (Blilou et al, 2005) and may contribute towards the loss of primary root indeterminancy.

The *scr* mutant must clearly reinitiate RAM activity based on its ability to grow to 50% of the wildtype root length (see new figure S2). Intriguingly, unlike *shr*, the *scr* mutant does not lose PIN protein expression (Fig S7), consistent with a link between cell proliferation and PIN protein abundance.

**SHR is not required for the initiation and radial patterning of anchor root primordia**

We next investigated the impact of the loss of SHR and primary root meristem activity on root architecture. The majority of *shr* seedlings exhibit a distinctive “tripod” like root phenotype, with nearly all (89% at 15 DAG, n=394) forming at least one anchor root originating at the root-hypocotyl junction (Figure 1E). In contrast, wild-type *Arabidopsis* seedlings rarely activate anchor root primordia at the root-hypocotyl junction (Figure 5B). Indeed, only 10% (n=40) of wild-type seedlings had formed one or more anchor root by 8 days after germination (Figure 5A). Anchor root activation in the *shr* seedlings may represent a generic mechanism to compensate for loss of root apical meristem activity. To test this, we tested the impact of loss of the RAM in wild-type seedlings by excising the bottom 2-3mm of the wild-type root at 3 DAG, then scoring numbers of anchor roots at 8 DAG (Figure 5C). The percentage of seedlings that formed one or more anchor root by 8 DAG increased dramatically from 10% (n=40) in non-excised wild-type seedlings to 97% (n=39) in RAM-excised wild-type seedlings (Figure 5C). A representative image is shown in Figure 5B. We conclude that this represents a generic mechanism present in wild-type *Arabidopsis* to compensate for the loss of growth potential in the primary root. In the case of *shr*, the progressive loss of root apical meristem activity most likely results in the activation of anchor root primordia. Hence, unlike the primary root apical meristem, SHR is clearly not required for the post-embryonic activation of anchor root primordia, based on its mutant phenotype (Fig. 1E).

We went on to investigate whether SHR was necessary for radial patterning of anchor roots. Confocal imaging of anchor roots in wild-type revealed a radial patterning identical to that
observed in primary and lateral roots (Figure 5D). Surprisingly, shr anchor roots also contained a wildtype number of layers, as revealed by the presence of two cell layers between the AUX1-YFP-marked epidermis and pericycle layers in shr AUX1pro::AUX1-YFP seedlings (Figure 5E). This observation contrasts the radial organisation of mutant primary root tissues, where loss of SHR results in the loss of a cell layer (Benfey et al., 1993, Scheres et al., 1995). Hence, activation and radial patterning of anchor root primordia appears not to be dependent on SHR function. Nevertheless, shr anchor root growth behaviour is determinate, where the new organ ceases growing at approximately the same length as the primary root (Fig. 1E). This implies that, as in the case of primary root meristem, SHR also functions as an indeterminacy factor for anchor root development.

**SHR is required for lateral root initiation and patterning of new primordia**

We next investigated whether SHR plays a role during lateral root (LR) development. We initially determined whether SHR was expressed during LR formation employing a GUS reporter regulated by 2.5 kilobases (kb) of the native SHR promoter (SHRpro::GUS). SHRpro::GUS was expressed exclusively in the central zone of the LR primordia throughout the initiation and emergence processes (Supplemental Figure S2A-E). Upon emergence, reporter expression extended into the root cap, in addition to the stele and the base of the LR (Supplemental Fig. S4E-J). SHRpro::GUS expression in the lateral root then resumed a pattern similar to that observed in the primary root (i.e. stele exclusive; Supplemental Fig. S4K-L). Thus SHR was expressed during all stages of lateral root development.

As the SHR protein regulates primary root radial patterning in a non-cell autonomous manner by migrating from the stele to the quiescent centre and future endodermis tissues (Nakajima et al., 2001), we examined whether SHR also moved to cells outside its expression domain during LR development. To do this we created a SHR-YFP translational fusion driven by 2.5 kilobases (kb) of the native SHR promoter (SHRpro::SHR-YFP) for confocal microscopy observation. The SHR-YFP fusion was functional based on its ability to fully rescue the shr mutant primary and lateral root phenotypes (Supplemental Figure S5). The LR expression of SHR-YFP in shr SHRpro::SHR-YFP was then analysed by confocal microscopy (Figure 6). SHR-YFP localisation was first observed in the lower half of young LRP (Figure 6A&B). As primordia developed, the SHR-YFP protein accumulated in the inner cell layers of the primordia, corresponding to the prospective stele and pericycle (Figure 6C-E). In addition, the
SHR-YFP protein was also targeted to the nuclei and cytoplasm of the flanking cells in the LR primordia (Figure 6E, inset). During LR emergence, the SHR-YFP protein was also targeted to the nuclei of the quiescent centre and putative endodermis (Figure 6F, inset). After emergence, the SHR-YFP protein was located in the mature lateral root as described in the primary root (Nakajama et al., 2001). However, expression was prolonged at the base of the LR following organ outgrowth and elongation (Figure 6J&K).

LR primordia originate from pericycle cells that are considered to represent part of an extended meristem which (unlike other root tissues) retain its mitotic potential once exiting the primary root meristem (Peret et al, 2009). Given the impact of the shr mutation on RAM mitotic activity, we investigated whether SHR was also required for lateral root development. The density of LR initiation in wildtype and shr seedlings was scored from 4 to 14 DAG. Less than 40% (n=49) of 14 day old mutant seedlings exhibited any LR initiation events (Figure 7A). In addition, initiation events in shr seedling that resulted in emerged LR were greatly reduced compared to the wild-type (over a 3-fold reduction, Figure 7B). Although the density of emergence events scored after one week of growth was also reduced in shr, the proportion (density of emergence event / density of initiation event) appeared higher in shr (70%) than in wild-type (40%) after two weeks of growth (Figure 7B), consistent with the existence of equilibrium between initiation and emergence (Lucas et al. 2008b). However, over 40% (n=46) of emerging LR did not develop any further following emergence (Figure 7C).

To understand why such a large proportion of LR did not develop in shr, we investigated LR development in the mutant employing scanning electron and confocal microscopy techniques (Figure 8). Electron microscopic analysis of developing LR revealed that LR emerging from shr primary roots were approximately double the thickness of wild-type LR (compare Figure 8A to B). We employed the AUX1-YFP marker (AUX1pro:AUX1-YFP) in conjunction with confocal microscopy to study the cellular organisation of LR primordia in wildtype versus the shr mutant (Figure 8C&D). In wild-type plants, primary and lateral roots are patterned in the following order of cell layers from the outside to the inside: epidermis, cortex, endodermis, pericycle, stele cylinder. In the shr mutant primary root the cortex/endodermis initials fails to divide, resulting in the cortex and endodermis being replaced by a single ground tissue layer (Scheres et al, 1995). We observed that this is also the case in shr LR primordia (Figure 8E&F). Confocal imaging revealed that LR cells in the outer tissue layers of wildtype and shr
were of equal dimension (Figure 8E&F). However, the increased thickness in developing *shr* LR appeared to be due to increased radial vascular development (Figure 8G). A similar ectopic lignification phenotype was observed in the *shr* hypocotyl (Supplemental Figure S6).

In addition to an extra-vascular development patterning defect, we observed that around half of developing *shr* LRP exhibited severe patterning defects compared to wild-type (Figure 8H&I). Prior to emergence, such LRP lacked the cellular organisation of wild-type LRP (Figure 8I) and exhibited disorganised expression of the AUX1-YFP marker (Figure 8H). Post-emergence, we distinguished two types of aborted LR. In the first category, termed “globular LRP” due to their symmetrical shape, the AUX1-YFP marker was not significantly expressed and propidium iodide rapidly stained the nuclei of LR cells (Figure 9A-D), indicating that cells were no longer viable. In the second category, AUX1-YFP expression was maintained and revealed that despite their altered outer structure (Figure 9E&H), these aborted organs retained some internal organization (Figure 9F&I). In contrast to globular LR, the second class of LR formed clusters of fused LRP (Figure 9F&I). These “cluster LRP” expressed the AUX1-YFP marker in a pattern similar to normal LRP (Figure 9G&J).

We conclude that SHR is required for the initiation, radial patterning and organisation of new LR primordia. In the absence of SHR, LR exhibit radial and vascular patterning defects. This may reflect, as observed in the primary root, that *shr* mutant cells differentiate into a state where they are no longer competent to fully form lateral roots.
Discussion:

The shr mutant was originally identified based on its radial patterning and determinate root apical meristem phenotype (Benfey et al. 1993). However, the impact of the shr mutation on other root developmental programmes is unclear. In this paper we describe the important roles played by SHR during primary, lateral and adventitious root development.

**SHR is essential for mitotic reactivation of the root apical meristem after germination**

SHR has been described to be essential for maintaining root apical meristem (RAM) activity (Benfey et al., 1993, Dilaurenzio et al., 1996; Figure 1). In mutant plants lacking SHR, primary root growth is severely reduced (Benfey et al., 1993, Scheres et al., 1995; Figure 1 & S2). Mutant roots exhibit QC defects and loss of activity of surrounding stem (initial) cells, resulting in the disruption of formative cell division events (Sozzani et al, 2010) and the depletion of proliferating cells in the RAM (Sabatini et al, 2003).

SHR is also required for reinitiating mitotic activity in the RAM following germination. We report that in the absence of SHR mutant RAM cells fail to express the mitotic marker, cyclin B1:1:GUS (Figure 2B; Ferreira et al, 1994). Consistent with a role as a key root mitotic regulator, Sozzani et al (2010) recently reported that SHR controls the expression of a number of genes encoding components of the cell-cycle machinery. Direct targets of SHR include the D-type cyclin gene CYCD6;1 and cyclin-dependent kinase genes CDKB2;1 and CDK2;2 (Sozzani et al, 2010). Whilst ectopic expression of 2 of these genes in shr were able to partially restore formative divisions, they were not able to rescue mutant root growth (Sozzani et al, 2010). Hence, further genes are likely to be required. Indeed, 266 genes were identified by Sozzani et al (2010) to be direct targets of SHR, of which 65 were differentially expressed in a SHR-inducible system. In total, the expression ~ 2,500 genes were observed to change over a 12 hour time course following induction of SHR expression in a shr background. Hence, SHR appears to control the expression of a very large number of genes, consistent with its role as a key regulator linking patterning and growth (Sozzani et al, 2010).

We report that the shr mutant exhibits a progressive reduction and eventual loss of detectable PIN auxin efflux carrier protein following germination (Figure 4). PIN protein
localization was performed up to 10 days after germination on seedling roots from multiple independent shr null alleles isolated from different accessions (see materials and methods for details). Every null alleles tested was observed to exhibit a progressive loss of PIN1, 2, 3, 4 and 7 proteins (Fig. 4). Hence, PIN protein abundance appeared to be dependent on SHR activity. None of these PIN genes were identified amongst the direct targets of SHR regulation (Levesque et al., 2006; Sozzani et al., 2010), hence protein abundance was likely to be regulated indirectly by SHR. Surprisingly, RT-qPCR profiling revealed that SHR does not regulate PIN abundance at the transcriptional level since their mRNAs are still detectable (Figure S6). Our RT-qPCR results are consistent with recently published array data for shr and scr root apical tissues which detected PIN expression in both mutant backgrounds (Sozzani et al., 2010). Hence, SHR must regulate PIN abundance at the post-transcriptional level. Like wildtype, PIN abundance is maintained in the scr mutant (Figure S5), suggesting that the abundance of these proteins are regulated via a gene product(s) controlled by SHR (but not SCR). It is intriguing that PIN abundance in shr root apical cells decrease (Figure 4), despite elevated auxin abundance and biosynthesis in the mutant (Figure 3) and contrary to the observed auxin inducibility of PIN expression (Vieten et al., 2006). One simple explanation is that the loss of PIN abundance in shr (but not scr) may represent an indirect consequence of mutant RAM cells differentiating as a result of their failure to reinitiate cell division following germination.

**SHR is required for lateral root formation in a manner distinct from the primary root**

Lateral root primordia (LRP) in Arabidopsis originate from the pericycle (Malamy & Benfey, 1997). LRP initiation involves the asymmetric division of xylem pole pericycle founder cells (Dubrovsky et al., 2001). Based on data showing that the pericycle remains in G1 phase of the cell cycle (Beeckman et al., 2001) and that the xylem pole pericycle continues to cycle after leaving the RAM (Dubrovsky et al., 2000), it has been postulated that the pericycle acts as an extended monolayered meristem (Casimiro et al., 2003). Whilst pericycle division is first visible in the differentiation zone well above the dividing RAM, xylem pole pericycle founder cells first need to be primed in the basal meristem (close to the elongation zone) in order to trigger LRP initiation (De Smet et al., 2007). Despite of the loss of mitotic activity in the shr RAM, pericycle cells retain their ability to initiate new LRP (Figure 1 & 7).
Nevertheless, the number of newly initiated LRP in *shr* was significantly reduced, suggesting that SHR facilitates (rather than is essential for) LR initiation.

LRP development and patterning are generally considered to be a post-embryonic recapitulations of the equivalent processes in the primary root (Malamy and Benfey, 1997, Scheres et al., 2002). We investigated the expression of SHR employing transcriptional and translational reporter fusions during LRP development. We first observed that *SHR-YFP* moved to adjacent QC, CEI and endodermal cell layers when the dome-like structure of the LRP is formed (stages IV/V onwards; Figure 6). This expression event coincides with the restricted expression domain of *SCR* in emerging primordia and prior to LRP tissues adopting a radial organisation equivalent to the primary root (Malamy and Benfey, 1997). After LRP emergence, the expression domain of the SHR protein appears identical to that described for SHR in the RAM (Figure 6; Nakajima et al., 2001).

Our study also revealed that, in addition to its radial patterning function, SHR is required to coordinate the overall morphology of new LRP. In the absence of SHR over half of the initiated LRP exhibited severe patterning defects (Figure 9). These LRP were either classified as *cluster-LRP* or *globular-LRP*. *Cluster-LRP* were composed of multiple fused LRP, whilst globular-LRP failed to develop any properly organised inner architecture or boundaries. Consistent with such functions, in addition to being expressed in inner LRP tissues, the SHR-YFP marker was also detected in the nuclei and cytoplasm of the outermost cells flanking the LRP as early as stage III/IV (Figure 6). This novel domain of SHR expression in cells flanking the LRP could play a role in the specification of primordia boundaries, akin to its regulation of radial patterning in QC, CEI and endodermal cell layers. Hence, SHR appears to regulate lateral root patterning in an overlapping, yet distinct manner to the primary root.

**SHR is not required for correct patterning of anchor root tissues**

SHR is best known as a regulator of radial patterning in *Arabidopsis* primary root apical tissues (Benfey et al, 1993). The *SHR* gene is first transcribed in the stele and then the SHR protein moves outwards one cell layer to adjacent QC, cortex-endodermis initial (CEI) and endodermal cells (Nakajima et al., 2001). SHR triggers SCR expression, causing CEI cells to divide longitudinally and generate endodermal and cortical layers. In the absence of SHR,
mutant roots form a single layer of cells with a cortical identity (Benfey et al., 1993, Scheres et al., 1995). Hence, SHR is necessary for radial patterning of root tissues plus specifying endodermal cell fate. In contrast to defective radial patterning in the shr RAM, mutant anchor roots retain a wildtype-like tissue organisation (Figure 5). Nevertheless, SHR is required for radial patterning of lateral root primordia tissues (Figure 8). These differences are unlikely to reflect that SHR is essential for radial patterning only in root-derived organs since this gene product is also required for formation of the starch-sheath layer in shoot tissues (Fukaki et al., 1998). Instead, it is most likely that another GRAS family member(s) (Pysh et al, 1999) performs a SHR-like patterning function in this tissue.

**Loss of SHR activity impacts root systems architecture**

We report that the loss of SHR activity leads to major changes in RSA (summarised in Figure 10). MicroCT imaging of RSA in soil revealed that the wild-type tap-root architecture is converted to a dwarfed fibrous-like root system in shr mutants (Figure 1). This dramatic change in shr RSA is caused by changes in the relative contribution of RAM, LR and anchor roots (Figure 10). We have reported that the shr RAM fails to reinitiate mitotic activity post-embryonically (Figure 2). Consequently, the mutant primary root elongates until the pool of cells at its RAM are depleted and no longer sufficient to maintain growth. Loss of RAM activity in shr promotes the initiation of anchor root primordia (Figure 7). We propose that this activation represents a mechanism to compensate for RAM death, as it could be phenocopied in wild-type following RAM excision. It remains unclear as to the underlying factors responsible for anchor root activation upon loss of the RAM. It is possible that an as yet unidentified inhibitory signal(s) is produced in the RAM that normally acts at the root-hypocotyl junction to prevent anchor root formation in the presence of an active RAM. Alternatively, anchor root activation may simply be a response to the supply of nutrients and/or proliferative signals from the aerial portions of the plant that are in excess of that able to be consumed by the determinate mutant primary root. The activation of determinate anchor roots leads to repeated ramification near the base of the root/hypocotyl junction as observed in soil-grown shr (Figure 1). Moreover, shr exhibits lower density of lateral root initiation with a significant fraction of initiated (globular and fused) lateral roots aborting post-emergence. The activation of anchor roots and lower level of lateral root initiation and development gives rise to the fibrous architecture of shr mature root system, with high
branching density near the base of the root system and low branching density along the roots (Figure 10).

In summary, our results reveal that SHR function is not restricted to the primary root. Instead, SHR is required for the initiation and patterning of LRP and for maintaining the indeterminate growth of primary, lateral and adventitious roots.
**Methods:**

**Plant Lines and Growth Conditions**

Seeds were surface-sterilised for 8 min in 50% (v/v) bleach and then washed twice in 0.1% (v/v) triton X-100. The seeds were then plated on square plates containing 0.5x Murashige and Skoog (MS) salt mixture, in 1% (w/v) bacto agar. The plates were cold treated for 2 days (d) at 4°C in the dark to synchronise germination. Plates were then incubated in a near-vertical position at 23°C in 150 µmol.m⁻².sec⁻¹ constant light with a cycle of 18 h light/6 h dark. For the analysis of the loss of the root apical meristem on Col-0 seedlings, 2-3mm of the root tip was excised with a sterile razor blade 3 d after germination, and the seedlings returned to growth conditions.

To generate the SHRp:SHR-YFP construct, 2 kb upstream sequence of the SHR gene (AT4G37650) was PCR amplified and cloned into BamHI and XhoI sites of pBluescript (stratagene) to create pBS-SHRpro. Subsequently, full length SHR gene (including 3'UTR) was PCR amplified with an in-frame XhoI restriction enzyme site at the N terminus and cloned between XhoI and KpnI sites of pBS-SHRpro to create pBS-SHR-X. Full-length EYFP sequence (excluding stop codon) was then PCR amplified using primers with in-frame XhoI restriction enzyme sites at the ends and subsequently cloned in frame at the engineered XhoI site of the SHR gene to create pBS-SHR-YFP. The SHR-YFP sequence was then cloned into a binary vector (pMOG-Mogen International) between BamHI and KpnI to create pMOG-SHR-YFP. Transformation of Agrobacterium (C58) and Arabidopsis was done as described in Swarup et al (2005).

**X-ray CT scanning**

Plants (shr & WT - col) were grown in a sandy loam soil (Dunnington Heath series; FAO class - Stagno-gleyic Luvisol) from the University of Nottingham experimental farm at Sutton Bonington (52.5ºN 1.3ºW), sieved to 2mm and packed loosely (bulk density c. 1.1 g cm⁻³) into 30mm diameter, 55mm high columns. The base of the column was covered with ‘micropore’ tape and placed in a tray to allow watering from below to prevent gas entrapment. Seeds were sown directly on the soil surface after surface watering, columns subsequently watered from below. The plants were provided with supplemental nutrients by adding 1ml of
commercial ‘Miracle-Gro’ (diluted as per instructions) to the top of the columns on two occasions - 11 days and 25 days after sowing, Plants were grown in a controlled environment room with 16hr day / 8hr night, temperature 23°C day & 18°C night. The columns with live plants were scanned in a Nanotom® X-ray micro-Computed Tomography scanner (Phoenix X-ray, Germany). Samples were scanned at 80mV and 220mA with 1440 images collected over a 50 minute period. The spatial resolution was set at 18 µm pixel⁻¹. All samples were scanned at an approximated field capacity soil moisture status. Image slices were reconstructed into 3-D volumes using DatosX Rec v.1.5 (Phoenix X-ray, Germany) with beam hardening and ring artefact reduction algorithms applied and then manipulated and analysed in VGStudioMax 2.0 (Volume Graphics, Germany). The 3-D volume was median filtered and the roots were manually segmented through a region growing tool and visual identification. Tortuosity is defined here as the ratio of the root length over the shortest distance between the two extremities of the measured root.

Phenotypic Analysis

Primary root length was determined from digital images of the plates by measuring from root tip to hypocotyl base using ImageJ 1.40 software (http://rsb.info.nih.gov/ij/). Emerged lateral and anchor roots were counted using a binocular. Two-sample unpaired t-test was used for comparison of primary root length and lateral root density, using a confidence interval of 95%.

GUS Assay and Microscopy

β-glucuronidase (GUS) activity of LAX3pro::GUS Col-0 and shr seedlings was assayed by incubating whole seedlings in a staining solution comprising 1mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) in 0.5% (v/v) dimethylformamide (DMF), 0.5% (v/v) triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA pH 8), 500 mM phosphate buffer (Na₂PO₄ pH 7) for 2-3 h at 37°C. To limit the diffusion of the blue stain and therefore maintain high staining specificity, 0.5 mM K₃Fe(CN)₆ and K₄Fe(CN)₆ were added. Both stained and unstained roots were cleared by immersion in 20% (v/v) methanol/4% (v/v) hydrochloric acid (HCl) at 57°C for 20 min, followed by immersion in 7% (w/v) NaOH/60% (v/v) ethanol at room temperature for 15 min. Roots were then rehydrated for 5 min each in 40%, 20% and 10% (v/v) ethanol,
and infiltrated in 5% (v/v) ethanol/25% (v/v) glycerol for 15 min. Roots were mounted in 50% (v/v) glycerol on glass microscope slides, and were imaged using a Nikon differential interference contrast (DIC) optics microscope.

For analysis of AUX1pro:AUX1-YFP and SHRpro:SHR-YFP seedlings, whole mounts were prepared on microscope slides in water, and the roots imaged either on a Leica SP2 confocal microscope using the 514 nm line of the argon laser or a Nikon eC1/TE2000U inverted confocal microscope using the 488 nm line of the argon laser. For general morphological analysis, seedlings were stained by immersion in 5 µl/ml propidium iodide for 5 min, whole mounted in water and imaged using a Nikon eC1/TE2000U inverted confocal microscope.

**IAA Quantification and Biosynthesis Measurements**

Seedlings were grown on vertical agar plates under long day conditions as described in Ljung et al., 2005. IAA quantification was performed on shr and WT seedlings at 3, 6 or 10 DAG. For IAA synthesis measurements, excised roots (cut at the hypocotyl-root junction) coming from 5-day-old shr and WT seedlings were transferred to liquid medium containing 30% ²H₂O and incubated in LD for 24 h. The most apical mm of WT and shr seedling roots was pooled for IAA quantification (50 sections/sample, 5-8 replicates) and biosynthesis measurements (100 sections/sample, 4 replicates). IAA quantifications and biosynthesis measurements were done by GC-SRM-MS (gas chromatography- selected reaction monitoring-mass spectrometry) (Edlund et al., 1995; Ljung et al., 2005) and compared using Students t test (two-sample assuming equal variances, two-tailed distribution, 0.001<p<0.01**, p≤0.001***).

**PIN localization**

Seedlings of wild type, scr and shr were sampled at different stage of development (see results). PIN localization was investigated in three different allele of shr mutants (shr1 (Ws), shr3 and tpd (Col). All alleles demonstrated similar dynamic of changes in PIN localization. Immunolocalization in roots was performed as described (Friml et al., 2002). Rabbit anti-PIN1 (Gälweiler et al. 1998), anti-PIN2 (Müller et al. 1998), affinity-purified anti-PIN3 (Friml et al. 2002), anti-PIN4 (Friml et al. 2002) and mouse anti-PIN7 (Paponov et al. 2005) antibodies were diluted 1:500, 1:400, 1:100, 1:400, 1:50, respectively. The secondary antibody, Alexa-
488-conjugated anti-rabbit or anti mouse (for PIN7) antibodies, were diluted 1:400. Solutions, during the immunolocalization procedures, were changed using a pipetting robot (Insitu Pro, Intavis).

Quantitative PCR

Plants were grown on 1/2 MS, 1% bactoagar at 23°C, 150 µmol.m⁻².s⁻¹ in long days (16 hours light). Total RNA was extracted from 6 dag roots using Qiagen RNeasy plant mini kit with on-column DNAse treatment (RNAse free DNAse set, Qiagen). Poly(dT) cDNA was prepared from 2µg total RNA using the Transcriptor first strand cDNA synthesis kit (Roche). Quantitative PCR was performed using SYBR Green Sensimix (Quantace) on a Stratagene Mx3005P apparatus. PCR was carried out in 96-well optical reaction plates heated for 5 minutes to 95°C, followed by 40 cycles of denaturation for 10 seconds at 95°C and annealing-extension for 30 seconds at 60°C. Target quantifications were performed with the following specific primer pairs for TIR1 (AT3G62980) TIR1forward 5'-ctaaactgacacgcctct-3', TIR1reverse 5'-ttggaagcaagcacctca-3'; for AFB1 (AT4G03190) AFB1forward 5'-actgatgtgctgtctgatt-3', AFB1reverse 5'-agtgaactctgtgaatagcct-3'; for AFB2 (AT3G26810) AFB2forward 5'-cttgccctgaaggaacag-3', AFB2reverse 5'-ttgagacactcaaaag-3'; for AFB3 (AT1G12820) AFB3forward 5'-tgataaacttactctacaccaacag-3' and AFB3reverse 5'-ctaaacataggtggtgcattc-3'; for PIN3 (AT1G70940) PIN3forward 5'-ccagatcactctccacacg-3', PIN3reverse 5'-ccggaacttaattgga-3'; for PIN7 (AT1G23080) PIN7forward 5'-tgggtcttgtttcctca-3', PIN7reverse 5'-tatccataacgtcgataggc-3'; for PIN1 (AT1G73590) PIN1forward 5'-ccagggagactgtgagaa-3', PIN1reverse 5'-tcatgttacgcttaac-3'; for PIN2 (AT5G57090) PIN2forward 5'-ggcagaagaagcaggaaga-3', PIN2reverse 5'-ggtggatgacggagaaca-3'; for PIN4 (AT2G01420) PIN4forward 5'-tctgctgtatcaacctgaa-3', PIN4reverse 5'-ataagacggcggatattt-3'; for IAA2 (AT3G23030) IAA2forward 5'-ggagaatctacacccctacaa-3', IAA2reverse 5'-cactgtcactgtgtttg-3'.

Expression levels were normalized to UBA (At1g04850) using the following primers UBAforward 5'-agtggagaggttgctagaaga-3' and UBAreverse 5'-ctcgggtagcacagccttta-3'. All qRT–PCR experiments were performed in triplicates and the values presented represent means ± s.d.
**Supplemental data:**

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

Supplemental Figure S1. Germination of the shr mutant is comparable to wildtype.
Supplemental Figure S2. Root length in shr is severely reduced compared to wildtype and scr.
Supplemental Figure S3. Inversion of polarity of PIN2 in the cortical layer.
Supplemental Figure S4. *SHR* gene expression pattern during lateral root development.
Supplemental Figure S5. *SHR*pro:*SHR-YFP* rescues the *shr* mutation.
Supplemental Figure S6. The *shr* mutation causes ectopic lignification in the hypocotyl.
Supplemental Figure S7. The loss of auxin carrier expression in *shr* is independent of SCR.
Supplemental Figure S8. Expression of auxin-related genes in wild-type, *shr* and *scr* seedlings.
Supplemental Movie SM1. 3D movie of 3-week old *shr* seedling grown in sandy-loam.
Supplemental Movie SM2. 3D movie of 3-week old WT seedling grown in sandy-loam.

**Acknowledgements:**

We thank Philip Benfey (Duke University, USA) for providing seed for *shr1* (WS) and *shr3* alleles and Laurent Laplaze (Montpellier, France) and members of our team for their helpful comments on the manuscript.

**Author contributions:**

Mikaël Lucas - performed research; analyzed data; and co-wrote the paper
Ranjan Swarup – performed research; contributed transgenic tools and analyzed data
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Kamal Swarup - performed research and analyzed data
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Alan Marchant – performed research and analyzed data
Goran Sandberg – designed the research; analyzed data
Mike Holdsworth – designed the research; analyzed data
Klaus Palme – designed the research; analyzed data
Tony Pridmore – designed the research; analyzed data
Sacha Mooney - designed the research; analyzed data
Malcolm J. Bennett - designed the research; analyzed data; and co-wrote the paper
References:


Figure 1. Root system architecture becomes determinate in the shr mutant
(A-B) 3D X-ray micro tomography analysis of root system architecture of 3-weeks old soil grown shr
(A) and wild-type (B) seedlings grown in a sandy loam soil. Scales bars = 1 mm.
(C) Quantitative description of root architecture extracted from (A) and (B).
(D) Root system architecture of 4-weeks old compost grown wild-type and shr seedlings. Scale bar =
1 cm.
(E) Root system architecture of 12-days old agar grown wild-type and shr seedlings. Scale bar = 1
cm.

Figure 2. The shr mutation blocks mitotic activity in the root apical meristem following germination.
(A) CyclinB1:1:GUS expression during wild-type (WT) and shr embryo development.
(B) CyclinB1:1:GUS post-germination expression in the root apical meristem of WT and shr at
different days after germination (DAG).
(C) LAX3pro::GUS expression in the root apical meristem of shr at different days after germination
(DAG). The red line indicates the position of the quiescent centre and the black bar indicates the start
of the elongation zone coinciding with the LAX3pro::GUS expression.

Figure 3. Auxin abundance and biosynthesis are perturbed in shr root apical tissues.
(A) IAA concentration in the root apex of WT and shr at 3, 6 and 10 days after germination.
(B) IAA biosynthesis in the root apex of WT and shr at 6 days after germination.
IAA quantifications and biosynthesis measurements were performed by GC-SRM-MS (gas
chromatography- selected reaction monitoring-mass spectrometry) after 24 hours incubation with
$^2$H$_2$O and compared using Students t test (two-sample assuming equal variances, two-tailed
distribution, 0.001<$p$<0.01**, $p$≤0.001***). Values represent means +/- s.d.

Figure 4. The shr mutation causes a progressive reduction of PIN auxin transporter abundance.
Dynamic changes of PIN1 (A), PIN2 (B), PIN4(C), PIN3 (D) and PIN7 (E) expression as shown by
imunolocalisation in WT and shr. Expression of AUX1 (F) in WT and shr at 5 days after germination.
PINs expression in (A-E) was assessed by immunolocalization while AUX1 expression in (F) was
investigated using the fusion protein AUX1-YFP.

Figure 5. The shr mutation causes activation of anchor root primordia.
(A) Percentage of Col-0 (n=40) and shr (n=394) seedlings having zero (grey) or one or more anchor
roots (black) at 8 DAG.
(B) Excision of the root apical meristem of wild-type seedlings. Seedlings were grown vertically and half the seedlings had 2-3 mm of the root (from the apex upwards) excised at 8 DAG. Representative wild-type (+RAM) and wild-type minus RAM (-RAM) seedlings are shown at 5 days after excision. Scale bar = 5 mm.

(C) Percentage of wild-type control (n=40) and excised (n =39) seedlings having zero (grey) or one or more anchor roots (black) 5 days after excision of the RAM.

(D) Root structure of wild-type anchor root. Epidermis (ep), cortex (co), endodermis (en), pericycle (pe) and stele (white) tissue structures (inset) are revealed by the LTi6a membrane marker (green) and propidium iodide (red). Scale bar = 100 μm.

(E) Root structure of shr anchor root. Epidermis (ep), cortex (co), endodermis (en) and stele (white) tissue structures are revealed by propidium iodide (red). Pericycle (pe) is revealed by the AUX1-YFP membrane marker. Scale bar = 100 μm.

**Figure 6.** SHR is expressed throughout lateral root development.

Confocal images of lateral root primordia and lateral roots of 8-days-old shr SHRp:SHR-YFP Arabidopsis. The SHR-YFP fusion protein is functional and rescues the shr mutant when expressed under the control of the SHR promoter (Supplemental Figure S5). (A & B) The SHR protein (green) is expressed in the lower half of the primordium during initial developmental stages. (C to E) As the primordium acquires its dome-shape, the SHR protein accumulates in the future stele of the primordium and nuclei of the flanking cells of the primordia (inset). (F & G) Upon emergence the SHR protein is targeted to the nuclei and cytoplasm of the quiescent centre and ground tissues (inset). (H & I) Once the lateral root is fully emerged, the SHR protein is targeted to an annulus of cells around the base of the new vascular bundle neighbouring the flanking cells of the original primordium (insets). (J & K) The SHR protein is located in the mature lateral root as it is located in the primary root. Localisation in the flanking cells of the young primordium is maintained along the ground tissue following lateral root outgrowth. Root tissue structure is revealed by a propidium iodide counter-staining (red). Scale bar = 50 μm.

**Figure 7.** The shr mutation disrupts lateral root initiation, patterning and emergence

(A) Time course of the percentage of wild-type and shr seedlings having initiated at least one lateral root (n = 49).

(B) Time course of densities of lateral root initiation and lateral root emergence events in wild-type and shr seedlings with at least one initiation event (in events per mm ; n = 20). Values represent means +/- s.d.

(C) Percentage of abnormal lateral roots after emergence in shr SHRp:SHR-YFP and shr seedlings (n=46).
**Figure 8.** The *shr* mutant exhibits radial and vascular patterning defects

(A & B) Electron microscopy images of Col-0 (A) and *shr* (B) emerging lateral roots. Emerging lateral roots in *shr* are ~2 times wider than wild-type lateral roots.

(C & D) Confocal images of Col-0 AUX1pro:AUX1-YFP (C) and *shr* AUX1pro:AUX1-YFP (D).

(E) Root structure of lateral root in *shr*. Lateral roots in *shr* exhibit the same radial patterning defect as the primary root with a single ground tissue layer (gt) replacing cortex and endodermis between pericycle (pe) and epidermis (ep). Stele tissues are marked in white. Root structure is revealed by confocal imaging with propidium iodide staining (red).

(F) Root structure of lateral root in Col-0. From outer to inner layer: epidermis (ep), cortex (co), endodermis (en), pericycle (pe), stele tissues (white). Root structure is revealed by confocal imaging with propidium iodide staining (red).

(G) Confocal images of *shr* AUX1pro:AUX1-YFP primary (bottom-left) and lateral (top-right) roots. The thickness gain in the developing lateral of *shr* is linked with excessive radial development of vascular tissues (blue bar), with no visible changes in the thickness of outer tissue layers (green and yellow bars).

(H & I) Confocal images of AUX1pro:AUX1-YFP in *shr* and wild-type. Propidium iodide (red) stain cells in the outer tissue layers of young lateral root primordia while the AUX1-YFP marker (green) is expressed in lateral root stele cells. Overlaying the two markers reveals that outer cells in *shr* lateral root primordia are non-viable (H - inset) compared to wild-type (I - inset). Scale bar = 100 μm.

**Figure 9.** SHR is required for lateral root patterning and viability

Upon emergence, ~50% *shr* lateral roots fail to elongate. Two types of patterning defects can be distinguished within these abnormal lateral roots.

(A – D) Globular lateral roots lack inner structure (B, D – schematic representation of the inner organization of the lateral root) and their cells appear to be non-viable based on their permeability to propidium iodide (red - strong nuclei staining). Images of *shr* AUX1pro:AUX1-YFP (green) taken by confocal microscopy with propidium iodide counter-staining.

(E – G & H – J) Fused lateral roots appeared outwardly amorphous (E, H), yet contained internal structure (F, I). Optical sectioning by confocal microscopy of *shr* AUX1pro:AUX1-YFP with propidium iodide counter-staining reveals multiple fused LRP (G, J – schematic representation of the fused LRP). The LRP comprising the fused lateral exhibit standard patterns of AUX1pro:AUX1-YFP expression (epidermis, lateral root cap, columella and stele tissues).

Scale bar = 100 μm.
**Figure 10.** Comparative summary of root system development of wild-type and short-root.
Inset: Main events of root system development impacted by the shr mutation

**Supplemental Movie SM1.** 3D movie of 3-week old shr seedling grown in sandy-loam.

**Supplemental Movie SM2.** 3D movie of 3-week old WT seedling grown in sandy-loam.

**Supplemental Figure S1.** Germination of the shr mutant is comparable to wildtype.
Seeds (n=50) were sowed on 1% agarose medium in water (A-B) or 1% agarose ½ MS medium (C-D), without chilling treatment (A-C) or after a chilling treatment (B-D). In all treatments except one, germination rate is slightly lower for shr than for the wild-type, but not significantly so.

**Supplemental Figure S2.** Root length in shr is severely reduced compared to wildtype and scr.
While scr root growth is still half of wild-type root growth and occurs with a similar rate, shr root growth is stunted.

**Supplemental Figure S3.** Inversion of polarity of PIN2 in the cortical layer.
In wild-type root the shift from apical to basal cortical localisation for PIN2 occurs at the level of the transition from division to elongation zone. PIN2 expression was assessed by immunolocalization.

**Supplemental Figure S4.** SHRpro:GUS expression pattern during lateral root development.
DIC microscope images of lateral root primordia and lateral roots of 8-days-old SHRpro:GUS Arabidopsis. (A & B) SHR is initially expressed in the lower half of the young primordium (stage II-IV). (C to E) As the primordium acquires its dome-shape, SHR expression domain is progressively restricted to the central part of the primordium, corresponding to future stele tissues. (F to J) Upon emergence, SHR expression becomes diffuse in the root cap while still being specifically expressed in the stele and anchorage domain of the young lateral root. (K & L) SHR expression in the lateral root then resumes a stele specific pattern similar to the one observed in the primary root. This change in expression pattern is not associated with a specific lateral root length but rather a certain distance from the primary root tip (change observed at 14 +/- 4mm from the root tip, n = 8). Scale bar = 50 μm.

**Supplemental Figure S5.** The SHRpro:SHR-YFP transgene rescues the shr mutation.
(A) Primary root (PR) length of wild-type (n=21) and shr SHRpro:SHR-YFP (n=23) over time.
(B) Lateral root (LR) density in wild-type (n=21) and shr SHRpro:SHR-YFP (n=23) over time.

**Supplemental Figure S6.** The shr mutation causes ectopic lignification in the hypocotyl.
Seedlings were stained for 5 min. in 0.01% basic fuchsin and then washed twice in 70% ethanol. Stained seedlings were then clarified (Acidified methanol protocol) and mounted in 10% glycerol. Confocal microscopy was used to visualize xylem lignification (red).

**Supplemental Figure S7.** The loss of auxin carrier expression in *shr* is independent of SCR. Dynamic changes of PIN1 (A), PIN2 (B) and PIN4 (C) abundance over time in *scr*. PIN expression was assessed employing immunolocalization (see materials and methods).

**Supplemental Figure S8.** Expression of auxin-related genes in wild-type, *shr* and *scr* 6 days old seedlings. Reverse transcriptase quantitative PCR was performed on RNA isolated from 6 days old seedlings using primers designed against several PIN, IAA2 and TIR/AFB gene sequences. Values represent normalized as means +/- s.d. Values represent means +/- s.d.
Figure 1. Root system architecture becomes determinate in the shr mutant
(A-B) 3D tomographic analysis of root system architecture of 3-weeks old soil grown shr (A) and wild-type (B) seedlings. Scales bars = 1 mm.
(C) Quantitative description of root architecture extracted from (A) and (B).
(D) Root system architecture of 4-weeks old compost grown wild-type and shr seedlings. Scale bar = 1 cm.
(E) Root system architecture of 12-days old agar grown wild-type and shr seedlings. Scale bar = 1 cm.
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(A) CyclinB1:1:GUS expression during wild-type (WT) and *shr* embryo development.
(B) CyclinB1:1:GUS post-germination expression in the root apical meristem of WT and *shr* at different days after germination (DAG).
(C) LAX3pro::GUS expression in the root apical meristem of wild-type and *shr* at different days after germination (DAG). The red line indicates the position of the quiescent centre and the black bar indicates the start of the elongation zone coinciding with the LAX3pro::GUS expression.
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(A) IAA concentration in the root apex of WT and shr at 3, 6 and 10 days after germination. 
(B) IAA biosynthesis in the root apex of WT and shr at 6 days after germination. 
IAA quantifications and biosynthesis measurements were done by GC-SRM-MS (gas chromatography-selected reaction monitoring-mass spectrometry) after 24 hours of incubation with $^{2}$H$_{2}$O and compared using Students t test (two-sample assuming equal variance, two-tailed distribution, 0.001<p<0.01**, p<0.001***). Values represent means +/- s.d.
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Figure 5. The shr mutation causes activation of anchor root primordia.

(A) Percentage of Col-0 (n=40) and shr (n=394) seedlings having zero (grey) or one or more anchor roots (black) at 8 DAG.

(B) Excision of the root apical meristem of wild-type seedlings. Seedlings were grown vertically and half the seedlings had 2-3 mm of the root (from the apex upwards) excised at 8 DAG. Representative wild-type (+RAM) and wild-type minus RAM (-RAM) seedlings are shown at 5 days after excision. Scale bar = 5 mm.

(C) Percentage of wild-type control (n=40) and excised (n=39) seedlings having zero (grey) or one or more anchor roots (black) 5 days after excision of the RAM.

(D) Root structure of wild-type anchor root. Epidermis (ep), cortex (co), endodermis (en), pericycle (pe) and stele (white) tissue structures (inset) are revealed by the LTt6a membrane marker (green) and propidium iodide (red). Scale bar = 100 micrometers.

(E) Root structure of shr anchor root. Epidermis (ep), cortex (co) and endodermis (en) and stele (white) tissue structures are revealed by propidium iodide (red). Pericycle (pe) is revealed by the AUX1-YFP membrane marker. Scale bar = 100 micrometers.
Figure 6. SHR is expressed throughout lateral root development. Confocal images of lateral root primordia and lateral roots of 8-days-old shr SHRpro:SHR-YFP Arabidopsis. The SHR-YFP fusion protein is functional and rescues the shr mutant when expressed under the control of the SHR promoter (Supplemental Figure S5). (A & B) The SHR protein (green) is expressed in the lower half of the primordium during initial developmental stages. (C to E) As the primordium acquires its dome-shape, the SHR protein accumulates in the future stele of the primordium and nuclei of the flanking cells of the primordia (inset). (F & G) Upon emergence the SHR protein is targeted to the nuclei and cytoplasm of the quiescent centre and ground tissues (inset). (H & I) Once the lateral root is fully emerged, the SHR protein is targeted to an annulus of cells around the base of the new vascular bundle adjoining the flanking cells of the original primordium (insets). (J & K) The root tissue structure is revealed by a propidium iodide counter-staining (red). Scale bar = 50 micrometers.
Figure 7. The shr mutation disrupts lateral root initiation, patterning and emergence

(A) Time course of the percentage of wild-type and shr seedlings having initiated at least one lateral root (n = 49).

(B) Time course of densities of lateral root initiation and lateral root emergence events in wild-type and shr seedlings with at least one initiation event (in events per mm; n = 20). Values represent means +/- s.d.

(C) Percentage of abnormal lateral roots after emergence in shr SHRpro:SHR-YFP and shr seedlings (n=46).
Figure 8. The shr mutant exhibits radial and vascular lateral root patterning defects
(A & B) Electron microscopy images of Col-0 (A) and shr (B) emerging lateral roots. Emerging lateral roots in shr are ~2 times wider than wild-type lateral roots.
(C & D) Confocal images of Col-0 AUX1pro:AUX1-YFP (C) and shr AUX1pro:AUX1-YFP (D).
(E) Root structure of lateral root in shr. Lateral roots in shr exhibit the same radial patterning defect as the primary root with a single ground tissue layer (gt) replacing cortex and endodermis between pericycle (pe) and epidermis (ep). Stele tissues are marked in white. Root structure is revealed by confocal imaging with propidium iodide staining (red).
(F) Root structure of lateral root in Col-0. From outer to inner layer: epidermis (ep), cortex (co), endodermis (en), pericycle (pe), stele tissues (white). Root structure is revealed by confocal imaging with propidium iodide staining (red).
(G) Confocal images of shr AUX1pro:AUX1-YFP primary (bottom-left) and lateral (top-right) roots. The thickness gain in the developing lateral of shr is linked with excessive radial development of vascular tissues (blue bar), with no visible changes in the thickness of outer tissue layers (green and yellow bars).
(H & I) Confocal images of AUX1pro:AUX1-YFP in shr and wild-type. Propidium iodide (red) stain cells in the outer tissue layers of young lateral root primordia while the green marker (inset) is expressed in lateral root stele cells. Overlaying the two markers reveals that outer cells in shr lateral root primordia are non-viable (H - inset) compared to wild-type (I - inset). Scale bar = 100 micrometers.
Figure 09. SHR is required for lateral root patterning and viability

Upon emergence, ~50% shr lateral roots fail to elongate. Two types of patterning defects can be distinguished within these abnormal lateral roots.

(A – D) Globular lateral roots lack inner structure (B, D – schematic representation of the inner organization of the lateral root) and their cells appear to be non-viable based on their permeability to propidium iodide (red - strong nuclei staining). Images of shr AUX1pro:AUX1-YFP (green) taken by confocal microscopy with propidium iodide counter-staining.

(E – G & H – J) Fused lateral roots appeared outwardly amorphous (E, H), yet contained internal structure (F, I). Optical sectioning by confocal microscopy of shr AUX1pro:AUX1-YFP with propidium iodide counter-staining reveals multiple fused LRP (G, J – schematic representation of the fused lateral root proliferation). The LRP comprising the fused lateral root exhibits standard patterns of AUX1pro:AUX1-YFP expression (epidermis, lateral root cap, columella and stele tissues).

Scale bar = 100 micrometers.
1DAG: Absence of mitotic activity at root apex
3DAG: Progressive depletion of cell pool at root apex
5DAG: Complete loss of PIN protein at root apex / First LRP initiation
7DAG: End of PR elongation / Activation and emergence of anchor root
12DAG: Anchor root determinate growth / LR emerge with patterning defect
28DAG: Fibrous determinate root system

Figure 10. Comparative summary of root system development of wild-type and short-root.
Inset: Main events of root system development impacted by the shr mutation