Cell fate specification in Arabidopsis roots requires coordinative action of lineage instruction and positional reprogramming

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Abstract
Tissue organization and pattern formation within a multicellular organism relies on coordinated cell division and cell fate determination. In animals cell fates are mainly determined by a cell lineage dependent mechanism, whereas in plants positional information is thought to be the primary determinant of cell fates (Scheres, 2001; Kidner et al., 2000; Kim et al., 2005; Scheres et al., 2002). However, our understanding of cell fate regulation in plants mostly relies on the histological and anatomical studies on Arabidopsis roots which contain a single layer of each cell type in non-vascular tissues (Scheres et al., 1994; Dolan et al., 1993; Rost, 2011). Here we investigate the dynamic cell fate acquisition in modified Arabidopsis roots with additional cell layers which are artificially generated by the mis-expression of SHORT-ROOT (SHR). We found that cell fate determination in Arabidopsis roots is a dimorphic cascade with lineage inheritance dominant in the early stage of pattern formation. The inherited cell identity can subsequently be removed or modified by positional information. The instruction of cell fate conversion is not a fast read-out during root development. The final identity of a cell type is determined by the synergistic contribution from multiple layers of regulation, including symplastic communication across tissues. Our findings underline the collaborative inputs during cell fate instruction.

Keywords: SHORT-ROOT, periclinal cell division, cell fate, cell-to-cell communication, Arabidopsis root development

Summary: Cell fate determination in Arabidopsis roots utilizes lineage-dependent mechanisms at early stages and positional-dependent signaling at later stages.

Introduction
Organogenesis in plants requires a tight spatiotemporal regulation of cell division and cell type specification (Bennett et al., 2010; Ten Hove et al., 2015; Radoeva et al., 2014; Dong et al., 2010; Abrash et al., 2009; Ten Hove, 2008). Our understanding of these two fundamental processes has been greatly advanced through using the model system, Arabidopsis roots. The Arabidopsis root is composed of concentric rings of different cell files with stele, endodermis, cortex and epidermis arranged from the inside to the outside (Benfey et al., 2000). SHORT-ROOT (SHR) was identified as a
key regulator of root radial patterning by directing the ground tissue formation
(Helariutta et al., 2000; Nakajima et al., 2001). Both cortical and endodermal cell
layers in ground tissue derive from the same cortex/endodermal initial (CEI) cells in
the stem cell niche (Petricka et al., 2012; Van Norman et al., 2011; Petricka et al.,
2008). In the CEI and CEI daughter cells (CEID), SHR activates SCARECROW (SCR),
and together both SHR and SCR turn on the expression of CYCLIN D 6;1 (CYCD6;1),
leading to the periclinal division in CEID and separating the endodermis and cortex
(Sozzani et al., 2010; Cruz-Ramírez et al., 2012) (supplemental Figure 1). Following
the periclinal division, both SHR and SCR are thought be essential for determining
the endodermal cell fate (Nakajima et al., 2001; Heidstra et al., 2004; Heo et al., 2011;
Levesque et al., 2006; Cui et al., 2007; Long et al., 2015a; Long et al., 2015b).

Recent studies have revealed a complex regulatory network involved in
SHR-mediated division and cell fate specification. However, most known regulators
in the SHR pathway, such as SCR and BIRD family of zinc finger proteins, appeared
to participate in the SHR-SCR feedback loop to restrict the functional scope of SHR
to the CEID and its derivative endodermis (Cui et al., 2007; Long et al., 2015a; Long
et al., 2015b; Welch et al., 2007; Moreno-Risueno et al., 2015). An elegant model was
also proposed to explain how SHR function is integrated with auxin signaling to
provide spatial information for the periclinal division (Cruz-Ramírez et al., 2012).
Interestingly, increased SHR movement is able to trigger extra cell division beyond
the endodermis, suggesting SHR is likely functional in outer cell layers (Cui et al.,
2007; Wu et al., 2014).

Here we examined the ability of SHR to induce periclinal cell division and determine
Cell fate in a broader developmental context in Arabidopsis. Our results indicate that a
conserved SHR-mediated regulatory network functions in most cell types outside of
the stele. Using these artificially-created supernumeral cell layers, we investigated
how cell fate commitment is spatiotemporally achieved in plants. Our results suggest
that cell fate acquisition in Arabidopsis roots is coordinately regulated by durable
positional input and transient lineage inheritance. Furthermore, stele-derived
symplastic signals participate in cell fate acquisition of ground tissues. Our findings
provide new insights into the long-standing question of cell fate adoption and
underline the crucial role of symplastic communication between tissues during the
tissue formation in the root.
Results

1. Mitotic activity is a prerequisite of conserved SHR pathways across different tissues.

To examine the ability of SHR trigger periclinal cell division, we expressed SHR in different cell types using cell specific promoters (\textit{pWER} in epidermis and \textit{pCO2} in cortex). In both epidermis and cortex, SHR triggered periclinal cell divisions (Sena et al., 2004, Figure 1B&D). When constitutively expressed under the \textit{35S} promoter, SHR induced extensive periclinal divisions in the meristem outside of the stele (Sena et al., 2004; Figure 1E). Interestingly, SHR was even able to activate periclinal division in columella stem cells (CSCs), leading to increased columella cell (CC) numbers (Figure 1F&G). In contrast, SHR direct target, \textit{SCARECROW (SCR)} was unable to influence the division pattern when mis-expressed alone (Figure 1A&C), which is consistent with the previous studies showing that SCR forms a protein complex with SHR to promote periclinal cell division (Heidstra et al., 2004; Cui et al., 2007; Long et al., 2015a; Clark et al., 2016).

To determine the competence of each individual cell types in response to SHR induction, we performed time-course analysis of the division pattern in \textit{pG1090-XVE::SHR} roots (Figure 1J-Q). \textit{pG1090-XVE} is an inducible promoter that allows ectopic SHR expression upon estradiol treatment (Curtis et al., 2003). After 10 hr in estradiol media, the cortex appeared to be the first cell layer that responded to SHR (Figure 1K). Interestingly, the periclinal cell division started to occur in cells located far above the stem cell niche rather than in the initial cells. In agreement with this, we observed alternated occurrence of anticlinal division and periclinal division in one cell file, shown by the directional arrangement of telophase chromosomes of \textit{pCO2:H2B-YFP} in \textit{pG1090-XVE::SHR} roots (Figure 1H&I). Thus SHR’s ability to induce periclinal cell division is not confined to the stem cell area. The epidermis, however maintained normal cell division pattern until 28hr of treatment when a few periclinal divisions became visible (Figure 1N). After 36 hr of treatment, the epidermis also exhibited extensive divisions (Figure 1O-Q). The asynchronous division suggested that different cell types possibly have distinct sensitivity to the SHR induction. However, we cannot rule out the possibility that \textit{pG1090-XVE::SHR} had distinct expression levels or SHR had dissimilar stability in different cell types.

In CEID cells, SHR activates SCR and together they upregulate \textit{CYCD6;1}, which coincides with the switch of division direction (Sozzani et al., 2010). To investigate the downstream mechanism in SHR-mediated periclinal divisions in cells outside of CEID, we observed the expression pattern of \textit{CYCD6;1} and SCR in \textit{SHR} mis-expression lines. In wild type, \textit{CYCD6;1} expression was confined in CEI and CEID (Sozzani et al., 2010; Figure 2A). When \textit{SHR} was mis-expressed in the epidermis under the \textit{WEREWOLF (WER)} promoter, we saw high level of \textit{pCYCD6;1::GFP-GUS} in the epidermis as well as its derivative cell layers (Figure 2B).
Consistent with this, 35S:SHR induced CYCD6 expression in almost all cell layers outside the stele (Figure 2C&D). In 35S:SHR/pCYCD6;1:GFP-GUS roots, we saw the clear activation of CYCD6 in CSC cells, which is in agreement with the division phenotypes in those cells (Figure 2E).
SCR, as a SHR direct target, is preferentially expressed in endodermis, CEI, CEID and QC in wild type (Di Laurenzio et al., 1996; Sabatini et al., 2003). Similar to CYCD6;1, SCR was also activated by SHR in all cells outside of stele. When SHR

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**Figure 2.** SHR mediated regulatory network is conserved across different cell types. (A-D) Expression of pCYCD6;1-GFP in different backgrounds. (E) pCYCD6;1-GUS is activated in columella stem cell (CSC) region in 35S:SHR expressing roots. White arrow heads point to the QC and asterisks represent the CSC cells. (F-H) Expression of pSCR:SCR-mCherry (SCR-R) in different backgrounds. 35S:SHR-G, 35S:SHR-GFP; pSCR:SCR-R-G, pSCR:SCR-R-GFP; pWER:SHR-Y, pWER:SHR-YFP. (I) qRT-PCR analysis of SHR targets in pG1090-XVE:SHR expressing roots at different time-points. NUC, NUTCRACKER; SCR, SCARECROW; IMP, IMPERIAL EAGLE; MGP, MAGPIE; BLJ, BLUEJAY; JKD, JACKDAW. Bars show mean ± s.e.m. of three biological replicates. (J, K) Radial patterning of roots expressing 35S:SHR in cycd6;1 and scr-4 mutants. (L) Quantitative comparison of cell layers outside of stele. 35S:GFP, p35S:SHR-GFP. Data represented are mean ± s.d. of 14–18 roots for each sample. Scale bars=20μm.
was constitutively expressed, \textit{pSCR:SCR-mCherry} was seen in all cells with SHR-GFP (Figure 2F). In multiple endodermal and epidermal layers induced by \textit{pSCR:SHR-GFP} or \textit{pWER:SHR-GFP}, SCR-mCherry exhibited almost the same pattern as SHR-GFP (Figure 2G&H). When \textit{SHR} was inducibly activated, the expression level of most known \textit{SHR} target genes including BIRD proteins was gradually enhanced, although with varied extent (Figure 2I).

To investigate whether activation of \textit{SCR} and \textit{CYCD6;1} is essential for the periclinal division, we examined the SHR-mediated division in \textit{scr-4} and \textit{cycd6;1} mutant backgrounds. In both mutants, the magnitude of ectopic divisions seemed to be affected (Figure 2J-L; supple Figure 2). The reduction of periclinal cell divisions was substantial in \textit{scr-4} but was only marginal in \textit{cycd6;1}. In accordance with the phenotypes observed in \textit{cycd6;1} mutant, loss of function of \textit{CYCD6;1} could likely be compensated by unidentified redundant genes. Removal of SCR was unable to abolish, but did lessen the extent of periclinal cell divisions upon SHR mis-expression, suggesting that SCR enhances SHR ability to induce periclinal cell division.

Although SHR activated periclinal cell divisions in most cells outside of the stele, the division appeared to be restricted to the meristem. In \textit{pG1090-XVE:SHR} roots, the induction of periclinal cell division stopped precisely at the meristem-elongation zone junction (Figure 3A-C). This is different from PLETHORA2, which has been shown to trigger division in differentiated zone (Mähönen et al., 2014). In line with this, \textit{CYCD6;1} activation induced by SHR mis-expression exhibited the similar developmental zone-confinement (Figure 3D&E). Therefore, mitotic competence appeared to be a prerequisite for SHR function.

These data reveal that SHR mediates periclinal cell division via a common pathway across different tissues. Activation of SHR can convert most mitotic active cells into periclinaly dividing cells in a cell type-independent manner.

2. Cell fate determination in plants depends on both lineage inheritance and position-based cell-cell communication.

To better understand spatiotemporal dynamics of cell specification, we next observed fluorescent markers of different cell types and traced the dynamics of cell fate transformation during extra cell layer formation induced by constitutively expressed SHR in \textit{Arabidopsis} roots. We first examined the outermost cell layer by imaging a set of markers. The WER promoter is specifically active in the epidermis, the lateral root cap and their initials (Figure 4 A&C). With extensive periclinal cell divisions triggered by \textit{35S:SHR-GFP}, we saw no noticeable changes in \textit{WER:H2B-mCherry} expression pattern (Figure 4 B&D). Consistently, enhancer trap line \textit{E3190} (specifically expressed in epidermis and lateral root cap cells) was only activated in the outermost cell layers in \textit{35S:SHR-GFP} expressing roots (Figure 4 E&F). In the root cap, \textit{E4716} specifically marked the root cap peripheral layers including border cells. There was no change in
E4716 expression pattern when extra cell layers were created in columella region by SHR mis-expression (Figure 4 G&H). These results suggest that the epidermis maintains the cell fate despite of complex and altered tissue geometry.

Root stem cell niche (SCN) is located above the root cap. In the center of the SCN is the so called the quiescent center (QC), which functions to maintain the stem cell status of the surrounding cells (supple Figure 1). When constitutively expressed, SHR induced ectopic cell divisions in SCN (Figure 1F). To determine whether QC cells maintain their identity and function properly with aberrant anatomy in SCN, we observed QC specific markers, and starch staining (Lugol’s staining) that reflects columella differentiation status. Lugol’s staining showed that the starch accumulation pattern in the 35S:SHR-GFP expressing roots is similar to that in wild type (supple Figure 3 A-F). There were extra cell layers seen in the CSC position of 35S:SHR-GFP roots. Interestingly, those extra cells showed no starch staining, suggesting that they likely resulted from CSC division (supple Figure 3 C&F). Both pWOX5:erGFP and QC25 in 35S:SHR-GFP roots exhibited wild type-like expression pattern, indicating the QC properties were well maintained in these roots (supple Figure 3 G-J). In 35S:SHR root columella, markers including pPIN3:PIN3-GFP and enhancer trap line Q1630 also displayed the correct expression pattern (supple Figure 3 K-N). Taken together, these results support the dominant role of positional information in deciding cell fate in Arabidopsis roots. The relative position of a cell type can be precisely located within the root in spite of altered tissue structure.
As the constitutive expression of SHR prevents the temporal resolution of the
potential cell fate transition, we made use of the inducible lines. Around 12 hr after
induction, \( pG1090-XVE::SHR \) started to promote the periclinal division in cortex,
resulting in two juxtaposed cells, both of which exhibited the expression of cortex
marker, \( pCO2:H2B-YFP \) (supple Figure 4A). With prolonged treatment by estradiol,
the \( pCO2:H2B-YFP \) signal in outer layers tapered off (supple Figure 4B). And
surprisingly, overall expression of \( pCO2:H2B-YFP \) in most cells became greatly
reduced after 48 hr SHR induction (supple Figure 4C). To rule out the variation among
different roots, we performed a time-course observation of \( pCO2:H2B-YFP \) in the
same root. We pre-treated \( pG1090-XVE::SHR \) expressing roots (n=10) with estradiol
for 20 hr and then monitored the fluorescent intensity in different cell layers (supple
Figure 4D). At this time-point, the extra cell layer derived from cortex retained
moderate level of \( pCO2:H2B-YFP \). After 3 hr, we saw a clear drop of YFP signal in
outer cortex layers (supple Figure 4E). This dynamic change of cell identity markers
likely resulted from the action of positional information. The similar phenomenon was
observed in the epidermis of \( pG1090-XVE::SHR \) roots. After 25 hr estradiol induction,
two juxtaposed cells expressing \( pWER:H2B-YFP \) became occasionally visible (Figure
5A). After 48 hr, many cells appeared to derive from epidermis still exhibited YFP
signal (Figure 5B). To see if cell fate conversion occurred, we performed time-course
live imaging on a single root pre-treated by estradiol for 30hr (Figure 5 C&D). At 0 hr
of the imaging, H2B-YFP stayed at a high level in the inner cell layer that derived
from periclinal cell division in epidermis. But \( pWER:H2B-YFP \) in the same cells
became markedly reduced after 3 hr while the YFP signal in the juxtaposed layers
maintained at the comparable level to that of 3 hr ago (Figure 5C&D). One possibility
is that the reduction of H2B-YFP in our observations was due to passive protein
dilution by cell division rather than active cell identity selection. However, expression
pattern of both \( pSCR:erGFP \) and \( pSCR:H2B-YFP \) roots seemed to argue against this
possibility, in which the erGFP or YFP signal was clearly visible in cortex/endodermal
initials but immediately lost in the first cortex (Figure 5E). Thus it is possible that
switch on and off of a cell specific marker is caused by an active fate conversion and
is instructed by positional information.

The expression of \( pWER:H2B-YFP \) faded away in extra cell layers originated from
the epidermis while maintained at high level in the outermost cell layer. However, it is
still difficult to know what cell types they became without additional markers. To
address this question, we introduced both epidermal marker \( pWER:H2B-mCherry \) and
cortex marker \( pCO2:H2B-YFP \) into \( pG1090-XVE::SHR \) lines. In normally patterned
roots, \( pWER:H2B-mCherry \) and \( pCO2:H2B-YFP \) have distinct expression profiles
without overlap. A 48 hr incubation in estradiol triggered periclinal cell division in
both cortex and epidermis of \( pG1090-XVE::SHR \) roots. However, we rarely detected
overlapping expression of \( pWER:H2B-mCherry \) and \( pCO2:H2B-YFP \) (Figure 5F-M).
The majority of the cells expressed the same reporters as the parental cells they
originated from, indicating plant cells still have limited ability to maintain the cell fate
lineage (Figure 5L). However, we often found that the CO2 promoter activity reduced when SHR was overexpressed (suppl Figure 4C). To overcome this obstacle caused by overexpression, we specifically created a new cell layer only from epidermis by expressing SHR under pWER promoter. To examine the cell identity of this new cell layer, we simultaneously imaged pWER:H2B-mCherry and pCO2:H2B-YFP (Figure 6A). As expected, the extra cell layers derived from epidermis that locate in the proximity of the stem cell niche expressed pWER:H2B-mCherry (Figure 6B). But this expression tapered off and as cells progress away from the position of the initial division, the cell fate was overridden by positional regulation and the extra cell file gradually adopted pCO2:H2B-YFP expression (Figure 6B). Consistent with the loss of pWER:H2B-mCherry expression, we did not observe root hair initiation in extra cell layers divided from epidermis (suppl Figure 4F-H). As a weak expression of pCO2 can also be detected in newly formed endodermis in proximal meristem, we analyzed the functional features of the extra cell layers divided from epidermis by examining the presence of Casparian strip. Both the lignin autofluorescence staining and PI penetration assay showed that there was only single layer of functional endodermis in pWER:SHR expressing roots (Figure 6C-E). In addition, PIN2-GFP in extra cell layers of 35S:SHR roots showed basal side localization, which is the same polarity as in cortex (Figure 6F).

Taken together, our results revealed that cell fate acquisition in Arabidopsis roots is a combined process in which lineage inheritance play a major role in the early stage as
a cell is produced, but the lineage determinant can be gradually overridden by
positional information. Interestingly, the fate conversion of cells outside of stem cell
niche seemed to be a relatively slow readout of positional signaling in *Arabidopsis*
roots.
It was shown in previous studies and our observations that loss of SHR function was not accompanied by loss of ground tissue identity (Sozzani et al., 2010; Carlsbecker et al., 2010). In shr-2 mutants, the mutant cell layer (which is a single ground tissue layer in shr-2) still maintained the expression of J0571, a widely used ground tissue marker (Figure 7A&B). To determine whether SHR function is sufficient to confer ground tissue identity, we examined J0571 expression in root tissues ectopically expressing SHR. Unexpectedly, we only detected the J0571 expression in a limited number of extra cell layers created by 35S:SHR (Figure 7C&I). We further visualized
another ground tissue-specific marker, \( E1839 \), and observed the expression was also
restricted to part of the extra cell layers in mature zone (supple Figure 4I&J). To
understand the cell identity of these cells locating between \( J0571 \)-expressing layers
and the epidermis, we visualized \( pPIN2:PIN2\text{-}GFP \), which is usually expressed in
both epidermis and cortex cells (Figure 7D&E). In the supernumery cell layers
produced in \( 35S:SHR \) roots, PIN2-GFP was only visible in a few cell layers that are
adjacent to the epidermis (Figure 7F&G). Interestingly, both \( J0571 \) and
\( pPIN2:PIN2\text{-}GFP \) displayed a gradient pattern horizontally, with \( J0571 \) expression
tapered off outward and PIN2-GFP declined inward. This suggests that there might be
signals transmitted across the tissue to direct the cell fate of neighboring cells. To test
this hypothesis, we utilized \( pWOL:icals3m \) system, which can block plasmodesmata
(PD) within the stele upon estradiol induction (Vatén et al., 2011). As a result, the

Figure 7. SHR integrates stele-derived signals to foster ground tissue identity.
(A-C) Expression of \( J0571 \) in the root tip of \( shr^{-2} \), wild type (WT) and \( 35S:SHR \) (35SS).
(D-F) Expression of \( pPIN2:PIN2\text{-}GFP \) in the root tip of \( shr^{-2} \), WT and 35SS.
(G) Quantification of fluorescent cell layers in 35SS roots expressing \( J0571 \) or \( pPIN2:PIN2\text{-}GFP \). Data
represented are mean±s.d. of 16–20 roots for each sample.
(H) Expression of \( J0571 \) with the activation of \( pWOL:icals3m \) in stele by the treatment of estradiol for 48 hrs.
(I) Comparison of \( J0571 \) fluorescent intensity in WT control (shown in B) and \( pWOL:icals3m \) expressing
roots (shown in H) after 48h estradiol treatment.
(J) Expression of \( J0571 \) in the root expressing 3SSS and \( pWOL:icals3m \) with 48h estradiol treatment.
(K, L) Time-course quantification of \( J0571 \) with activation of \( pWOL:icals3m \) in the root of WT (K) and
35SS (L). N=24–25 roots. Sample minimum, lower bar; lower quartile, box; median, middle cross line; upper
quartile, box; sample maximum, upper bar; green dot, excluded outliers data. The difference
between the 0h and other time-points is significant (\( P=0.00052 \) for 48h, 0.01374 for 72h and 0.00203 for
96h in K) (\( P=0.00176 \) for 48h, 1.0922E-11 for 72h and 1.97324E-13 for 96h in L). Scale bars=20\( \mu \)m.
Symplastic movement of signaling molecules out of the stele through PD was prevented in these roots. Once we blocked PD in stele, J0571 expression appeared much weaker (Figure 7H&I, supple Figure 5A&B). Quantification of fluorescence intensity of J0571 indicated that the reduction of J0571 expression occurred in both endodermis and cortex. Compared to the wild type, J0571 fluorescence intensity dropped by 48% (n=24, P = 5.81E-14; Student’s t test; supple Figure 5C) in endodermis, and reduced by 72% (n=25, P = 1.78E-20; Student’s t test; supple Figure 5D) in the cortex of pWOL:icals3m roots after 45h estradiol induction. We further crossed pWOL:icals3m into 35S:SHR lines that also express J0571 marker. Compared to untreated lines, J0571-expressing cell layers in 35S:SHR roots narrowed down upon estradiol induction of pWOL:icals3m (Figure 7J).

However, J0571 expression was not entirely abolished in the ground tissue with occluded stele, suggesting there might be gradient or dose-dependent effect of symplastic signals from the stele. To obtain the temporal information of J0571 expression change with disrupted symplastic signaling from the stele, we performed time-course analysis of J0571 in both pWOL:icals3m and pWOL:icals3m/35S:SHR backgrounds. Although J0571 exhibited certain level of varied expression, the reduction trend appeared to be similar in both genetic contexts (supple Figure 6&7). The quantification indicated a significantly depleted J0571 fluorescence intensity after the symplastic communication between stele and outer cell layers was blocked (Figure 7K&L).

Together, our results suggest that stele-derived symplastic signaling contributes to the expression of J0571, the mostly used ground tissue marker. But the acquisition of the ground tissue cell fate presumably involves multiple layers of regulation. Although SHR is able to activate a group of downstream factors involved in specifying ground tissue, additional positional cues that are independent of SHR and mediated though PD, need to participate in the regulation to confer the full ground tissue identity.
Discussion

Cell fate determination has long been a central question in development. In animals, lineage-based mechanisms for cell fate determination play a major role in organogenesis. In plants, it is widely accepted that position-dependent cell fate regulation is dominant. The evidence supporting this mostly derived from early surgical experiments and clonal analyses (Scheres, 2001; Kidner et al., 2000; Kim et al., 2005; Scheres et al., 2002). Since Arabidopsis was used as the model system, a large number of molecular and genetic tools, as well as non-intrusive techniques of cellular observation, have been developed. However, Arabidopsis has simplified structure in most organs. For example, a typical Arabidopsis root has only a single cortex layer and most cell fates are specified in the root stem cell niche. This limits the dissection of cell-fate regulation under a more complex tissue context. In this paper, we examined the spatiotemporal regulation of cell fate in Arabidopsis roots with multiple cell layers formed in the epidermis and ground tissue. Our results provide an alternative perspective on cell fate specification within a complex tissue beyond stem cell niche. This may reflect the situation in many other crop species, in which periclinal cell division in roots repeatedly occurs in meristem and the cell fate needs to be precisely determined.

SHR is a good example that asymmetric cell division and cell fate determination are coordinated by a critical regulator. In the current prevailing model, SHR function in asymmetric cell division was only restricted in CEID and endodermis by the synergistic action of a group of interacting transcription factors (Cui et al., 2007; Long et al., 2015a; Long et al., 2015b; Welch et al., 2007; Moreno-Risueno et al., 2015). A previous report also proposed that the competence to respond to SHR lies in the Epidermal/LRC initials through induction of SCR. However, monocot SHR homologs seemed to move beyond the endodermis when expressed in the stelle of Arabidopsis roots, inducing multiple layers of ground tissues (Wu et al., 2014). Thus, SHR could also function broadly in promoting periclinal cell division in various developmental contexts in addition to the CEI initials and stem cell region. This is confirmed in our mis-expression of SHR analysis in which SHR can broadly activate periclinal cell division in cells outside of its normal functional domain. When SHR was inducibly activated, the expression level of most known SHR target genes including BIRD family was gradually enhanced. Although the sensitivity to SHR induction appeared to vary in epidermis and cortex, SHR likely promotes periclinal cell division through a conserved pathway that includes most important components involved in SHR functions in CEID.

It is still unclear which components are indispensable for SHR-induced periclinal cell division. Removal of SCR was unable to abolish but did lessen the extent of periclinal division, suggesting that SCR participates in SHR regulation outside of endodermis. However, without SCR function, SHR failed to promote periclinal cell division in epidermis. This is consistent with the previous result showing blocked periclinal cell
division induced by SHR in epidermis of scr-4 (Sena et al., 2004). SCR function seems to be essential for SHR ability to induce periclinal cell division only in epidermis. In scr-4 (the seedlings were less than 5-day old), SHR still induced periclinal cell division in the mutant cell layer. In addition to SCR, the mitotic competence appeared to be a prerequisite for the SHR function as the periclinal cell division triggered by SHR was restricted to the meristem.

Based on our results, part of a plant cell's identity can be passed on from the parental cells to their progeny which resembles the lineage-based mechanism. Our observations in the epidermis and ground tissues outside of the stem cell niche suggest that the cell fate conversion is not a fast read-out. In CEID, both endodermis and cortex appeared to adopt the respective fates immediately after periclinal division. Longer fate conversion time in epidermis and ground tissues suggests that position-based regulation could first remove the inherited identity of a cell to override the cell fate and promote the fate conversion. Thus the actual cell fate acquisition in Arabidopsis roots could be a dimorphic process in which lineage inheritance play an important role in the early stage once a cell is produced, but the lineage determinant can be gradually overridden by the positional information. The hierarchical cellular states during cell fate specification is not a unique case in plants. In fly and worm development, the expression of cell-specifying transcription factors is dictated by positional signaling in very early stages, but the stable lineage is achieved through the cell-autonomous regulation by polycomb group genes (Scheres, 2001). The order of regulatory cascade in animals appears to be opposite to our observation of cell fate acquisition in plants. But in both cases, the existence of intermediate cells with mixed identity is possible. One interesting but unsolved question here in plants is how the positional information orchestrates cell-type specification by removing the inherited properties of the non-stem cell and conferring the new characteristics. In lineage-determined animal cells, cell identity conversion can be promoted via forced expression of lineage-specific transcription factors in various differentiated cell types (Chin, 2014). However, the efficiency of cell fate reprogramming in a given population was low and the completeness of fate conversion is still being questioned. Thus, the fundamental cellular principals and molecular mechanisms in both animals and plants are still awaiting elucidation.

The mobile transcription factor, SHR has been proposed as one of the regulators of endodermal identity. But in this study we found that SHR, while necessary, was not sufficient to confer endodermal cell fate. Expression of SHR in cells outside of endodermis failed to establish Casparian strip, a functional feature of endodermis. In addition to endodermis, we also have little knowledge of the specification of cortex identity. In this study, we found that stele-derived symplastic signaling contributes to the cell fate determination in ground tissues. However, restricted expression of J0571 in 35S:SHR roots indicated that SHR alone was not sufficient to confer the ground tissue identity. Thus it is possible that additional factors regulated by symplastic signaling from stele play roles in this process. Recently, two stele-derived peptides

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(CASPARIAN STRIP INTEGRITY FACTORS, CIF1 and 2) were identified to move from stele outward to induce Casparian strip formation. Although CIF1/2 is unlikely the regulator of $J0571$ due to the blocked apoplastic path by Casparian strip, there might exist other uncharacterized factors from the stele that affect $J0571$ expression (Doblas et al., 2017; Nakayama et al., 2017). Interestingly, disruption of symplastic communication between stele and the outer cell layers did not entirely abolish $J0571$ expression. Since cells divided from epidermis can gradually adopt cortex fate, it is possible that signaling from epidermis also participates in this specification and jointly promotes ground tissue identity with signals from stele. In addition, signals that are independent of symplastic transport could also be involved in this process. But no matter how complex the tissue geometry is, the root appeared to precisely locate the epidermis and endodermis. Hence, the entire regulatory network that provides positional information for cell fate determination in plants presumably involves multiple layers of regulation.

**Experimental Procedures**

**Plant materials and growth condition.**

The *Arabidopsis thaliana* Columbia ecotype (Col-0) was used as the wild type throughout the experiments. The following marker lines $CO2$:H2B-YFP, CYCD6:1:GFP-GUS, SCR:SCR-mCherry, WER:H2B-mCherry, E3190, E4716, QC25:GUS, WOX5:erGFP, PIN3:PIN3-GFP, Q1630, WER:H2B-YFP, SCR:H2B-YFP, J0571, PIN2:PIN2-GFP, E1839 were crossed into different transgenic plants or mutants. Homozygous lines were screened based on fluorescence, PCR genotyping, and the root phenotypes. After sterilization, the seeds were germinated after incubated for 2 days at 4°C in the dark. All plants were grown vertically on 1/2 Murashige and Skoog (MS) medium containing 0.05% (wt/vol) morpholinoethansulfonic acid monohydrate (pH5.7), 1.0% (wt/vol) Sucrose, and 1.0% agar in a growth chamber at 23°C under a 16/8h light/dark cycle. Plants were analyzed 6-7 d after plating unless otherwise stated.

**Plasmid construction and plant transformation.**

The 1596bp full-length cDNA of *AtSHR* was cloned into pDONR221 (Invitrogen) using BP recombination based on standard protocol (Invitrogen/Life Technologies, http://www.thermofisher.com/us/en/home/life-science/cloning/gateway-cloning.html). The destination vectors were modified from the previously reported pGreenBarT vector (Lee et al., 2006) according to traditional restriction digestion method described before (Wu et al., 2014). All expression vectors were generated through LR Gateway reaction and the resulting plasmids were transformed into Agrobacterium strain GV3101-pSouppMP. *Arabidopsis* (Col-0) was transformed following the floral dip method. Transgenic plants were screened based on the resistance to glufosinate-ammonium (Basta) in soil. For all of the transgenes discussed, at least three independently transformed lines were analyzed and one of them were chosen for further analysis.

**Confocal Microscopy imaging**

Roots were mounted in 0.01μg/mL propidium iodide (PI) in water. Roots tips were then examined using a 40×water-immersion lens on a Zeiss LSM 880 laser scanning confocal microscope with...
dual-channel setting of YFP and mCherry. Image quantification was performed using ImageJ 1.4.3 software. For fluorescence intensity analysis, we used the region selection function of ImageJ to create a region of interest where the fluorescence is typically seen. The average intensity of fluorescence was calculated by ROI manager and then used to calculate the ratio of relative fluorescence intensity. Representative images were collected from 10-25 roots with three biological replicates.

Staining and chemical treatments
For β-Glucuronidase (GUS) staining, 5-7-day old seedling were incubated in the GUS (0.5 mg/mL) staining solution for 8h at 37°C followed by the clearing in 70% ethanol. For starch staining, root tips were incubated in a 1:1 dilution of Lugol’s solution (Sigma-Aldrich) for 1 min, then briefly washed with water and mounted in the HCG solution for microscopy visualization. Samples were viewed using Nikon ECLIPSE Ni-U microscope connected to a Nikon DS-Ri2 digital camera. Visualization of PI penetration and lignin auto fluorescence was performed according to (Alassimone et al., 2010).

For estrogen induction, five-six day old seedlings were transferred to 0.5x MS (Caisson) agar (Difco-BBL) plates containing 10 μM estradiol (Sigma), and the same medium containing the estradiol carrier (DMSO) as controls.

Statistical analysis
The Arabidopsis seedlings from three biological replicates were randomly chosen from each treatment. The data analysis were carried out by using spss17.0, and independent-samples t-test were used to determine the significance between the treatment and control group (P < 0.05). The boxplot were exported by SPSS Data Editor.

Quantitative Real-time RT-PCR (qRT-PCR) analysis
cDNA was prepared from the total RNA extracted from the root tip (~1cm) of 6-d-old pG1090-XVE:SHR seedlings after 0h, 12h, 24h and 48h estradiol incubation. qRT-PCR were performed on a Stratagen Mx3005P (Agilent Technologies) with the TransStart Top Green qPCR SuperMix (Transgen), according to the manufacturer’s instruction. In each run, three technical replicates were performed for each sample. The presented results are based on three biological replicates and error bars represent standard deviation of the mean of biological replicates. The primers of interested genes were listed as follows: MGP (5′-AAAGCAGAGGAGCAAGGAGCAAGGAAAG-3′ and 5′-GGGTGAATGATCCTTCCAGTCAG-3′), NUC (5′-AGCTGCTGAAATTGGCGCTA-3′ and 5′-GAATGAGCCTTCCAGTCAG-3′), LMP(5′-CAGTCACAAGCAACGACCCAT-3′ and 5′-CCATAATTCGTCCTCCACCAAA-3′), BLJ (5′-GTCCCCTAGCCTTTTCCCTAC-3′ and 5′-CGGTGCTCACAATTCCTCCACCAAA-3′), SCR (5′-TTGAGAGCTGGAGGAAAAGAAGAAGAAGAAA-3′ and 5′-CGTCCAAGCTGAGCTGGAGGAAAAGAAGAAGAAGAAA-3′), ACT2 (5′-CGCTGACCGTATGAGCAAAG-3′ and 5′-GAGATCCACATCTGCTGGAATG-3′) was used as the reference gene.

Author Contributions
S.W., Q.Y designed research; Q.Y., P.L., N.L., H.W., M.X., and S.W. performed experiments; Q.Y.
and S.W. analyzed data; and S.W. wrote the paper.

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Figure Legends

Figure 1. Periclinal cell division triggered by SHR is independent of cell types.

(A-F) Mis-expression of SHR (B, D, E and F) and SCR (A and C). Extra cell layers in collumella was marked by bracket in (F).

(G) Quantification of cell layers in columella in wild type (WT) (n=10) and 35S:SHR-GFP (35SSG) expressing roots (n=12). Error bars indicate the standard deviation from the mean. T-tests indicate that there is a significant difference (P<0.01) in columella cell layers between WT and 35SSG.

(H, I) pCO2:H2B-YFP in pG1090-XVE:SHR expressing roots after 24 hrs of estradiol induction. Yellow arrows point to anticlinal cell division and white arrows represent periclinal cell division.

(J-Q) Time-course imaging of the root radial structure in pG1090-XVE:SHR expressing roots with estradiol treatment for different time length (as labelled). Ep, epidermis; C, cortex; E, endodermis. Black arrow heads in (K) and white arrow heads in (N) and (P) point to periclinal cell divisions. (P) is a zoomed view of boxed region in (O). Scale bars=20μm.

Figure 2. SHR mediated regulatory network is conserved across different cell types.

(A-D) Expression of pCYCD6;1-GFP in different backgrounds.

(E) pCYCD6;1-GUS is activated in columella stem cell (CSC) region in 35S:SHR expressing roots. White arrow heads point to the QC and asterisks represent the CSC cells.


(I) qRT-PCR analysis of SHR targets in pG1090-XVE:SHR expressing roots at different time-points. NUC, NUTCRACKER; SCR, SCARECROW; IMP, IMPERIAL EAGLE; MGP, MAGPIE; BLJ, BLUEJAY; JKD, JACKDAW. Bars show mean ± s.e.m. of three biological replicates.

(J, K) Radial patterning of roots expressing 35S:SHR in cycd6;1 and scr-4 mutants.

(L) Quantitative comparison of cell layers outside of stele. 35SSG, p35S:SHR-GFP. Data represented are mean±s.d. of 14–18 roots for each sample. Scale bars=20μm.

Figure 3. Mitotic competence is a prerequisite for SHR induction.
(A-C) pG1090-XVE:SHR expressing roots incubated in estradiol for 24 hrs (A&B) or 36 hrs (C). Note the periclinal cell division stopped at the junction of meristem zone (MZ) and elongation zone (EZ). This junction is marked by yellow arrow head. Blue double headed arrow indicates MZ and yellow double headed arrow represents EZ in (B).

(D, E) Comparison of pCYCD6:GUS expression between WT root (D) and 35S:SHR-GFP root (35SSG, E). Scale bars=20μm.

**Figure 4.** Epidermal cells maintain specialized fate despite of complex and altered tissue geometry.

(A-D) pWER:H2B-mCherry (pWER:HC) expression in roots of WT (A and C) and 35S:SHR-GFP (35SSG) (B and D). (C and D) are zoomed view of boxed area in (A) and (B) respectively.

(E, F) E3190 expression in roots of WT (E) and 35SSG (F).

(G, H) E4716 expression in roots of WT (G) and 35SSG (H). Scale bars=20μm.

**Figure 5.** Cell fate specification in roots with extra cell layers.


(C, D) Time-course observation of the same root expressing pG1090-XVE::SHR and pWER:HY at 30hr estradiol induction (C) and 3 hrs after (D). White arrow heads point to the expression of pWER:HY in epidermis and yellow arrow heads point to the expression of pWER:HY in the cell divided from epidermis.

(E) Expression of pSCR:erGFP (left) and pSCR:HY (right) in WT roots. White arrow heads point to cortex/endodermal initial (CEI) and its daughter cells (CEID) in which pSCR is still active. Yellow arrow heads point to the first cortex cell derived from the CEID in which pSCR activity is not seen.

(F-K) Expression of pWER:HC and pCO2:HY in pG1090-XVE:SHR expressing roots after 48 hrs in estradiol. Note the separated expression zone of pWER:HC and pCO2:HY, marked by brackets in (H) and occasionally overlapped expression zone marked by white arrow heads in (I-K). White brackets indicate the expression of pCO2:HY and yellow brackets indicate the expression of pWER:HC.

(L) Scheme describing the separated expression zone shown in (F-H). Epi, Epidermis; Epi’, extra cell layer derived from epidermis; Cor, cortex; Cor’, extra cell layer derived from cortex.

(M) Quantification of the percentage of cells expressing different markers in pG1090-XVE:SHR expressing roots after 48 hrs in estradiol. Scale bars=20μm.

**Figure 6.** Lineage inheritance in the early stage of pattern formation is modified by positional information.

(A, B) Simultaneously imaging the expression of pWER:H2B-mCherry (pWER:HC) and pCO2:HY in roots of wild type (A) and pWER:SHR expressing line (B). Yellow arrow heads point to cells losing pWER:HC expression and white arrow head marks the starting cell that adopts the expression of pCO2:HY.

(C, D) Lignified Casparian strips visible as green autofluorescence of cell walls after clearing in pWER:SHR expressing roots (marked by the white arrows). Double headed arrows represent cell layers outside of endodermis.
(E) PI penetrated into all cell layers outside of endodermis (marked by the double headed arrow). White arrow head points to the position of endodermis where PI penetration was blocked.

(F) pPIN2:PIN2-GFP in the root expressing 35S:SHR. Ep, epidermis; Ex, extra cell layers. Red lines depict the basal side of PIN2-GFP in extra cell layers and yellow lines depict the apical side of PIN2-GFP in epidermis. Scale bars=20μm.

Figure 7. SHR integrates stele-derived signals to foster ground tissue identity.
(A-C) Expression of J0571 in the root tip of shr-2, wild type (WT) and 35SS:SHR (35SS).
(D-F) Expression of pPIN2:PIN2-GFP in the root tip of shr-2, WT and 35SS.
(G) Quantification of fluorescent cell layers in 35SS roots expressing J0571 or pPIN2:PIN2-GFP. Data represented are mean±s.d. of 16~20 roots for each sample.
(H) Expression of J0571 with the activation of pWOL:icals3m in stele by the treatment of estradiol for 48 hrs.
(I) Comparison of J0571 fluorescent intensity in WT control (shown in B) and pWOL:icals3m expressing roots (shown in H) after 48h estradiol treatment.
(K, L) Time-course quantification of J0571 with activation of pWOL:icals3m in the root of WT (K) and 35SS (L). N=24-25 roots. Sample minimum, lower bar; lower quartile, box; median, middle cross line; upper quartile, box; sample maximum, upper bar; green dot, excluded outliers data. The difference between the 0h and other time-points is significant (P=0.00052 for 48h, 0.01374 for 72h and 0.00203 for 96h in K) (P=0.00176 for 48h, 1.0922E-11 for 72h and 1.97324E-13 for 96h in L). Scale bars=20μm.

SUPPLEMENTAL DATA
Supple Figure 1. Cartoon summarizing the prevailing model for ground tissue patterning mediated by SHR pathway.
Supple Figure 2. The ability of SHR to trigger periclinal cell division relies on SCR.
Supple Figure 3. QC function and stem cells were well maintained in SHR ectopic expression lines.
Supple Figure 4. Examination of cell fate with mis-expressed SHR.
Supple Figure 5. Blocked symplastic communication between stele and endodermis led to reduced J0571 fluorescent intensity in ground tissues.
Supple Figure 6. Time-course observation of J0571 with the activation of pWOL:icals3m.
Supple Figure 7. Time-course observation of J0571 in 35S:SHR roots with the activation of pWOL:icals3m.
Supple Figure 3. QC function and stem cells were well maintained in SHR ectopic expression lines.

(A-F) Lugol’s staining in roots of WT (A and D) and p35S:SHR-GFP (35SSG) (B, C, E and F).

Yellow arrow heads point to QC and red arrow heads represent columella stem cells.

(G-I) pWOX5:erGFP expression in the root of WT (G) and 35SSG (H and I). (H) is splitted green channel of (I).

(J) QC25:GUS expression in the root of 35SSG. The bracket represent columella stem cells.

Arrows point to differentiated columella cells.

(K, L) pPIN3:PIN3-GFP expression in roots of WT (K) and 35SSG (L). White arrow heads point to columella stem cells.

(M, N) Q1630 expression in roots of WT (M) and 35SSG (N). White arrow heads point to columella stem cells. Scale bars=20μm.

Supple Figure 4. Examination of cell fate with mis-expressed SHR.


(D, E) Time-course observation of the same root expressing pG1090-XVE::SHR and pCO2:HY at 20hr estradiol induction (D) and 3 hrs after (E).

(F-H) Cross-section of pWER:SHR expressing roots. Root hair cells are marked by red asterisks.

(I, J) Expression of E1839 in root mature zone of wild type and 35S:SHR (35SS).

Supple Figure 5. Blocked symplastic communication between stele and endodermis led to reduced J0571 fluorescent intensity in ground tissues.

(A, B) Comparison of J0571 fluorescent intensity in WT control (shown in A) and pWOL:icalsm expressing roots after 48h estradiol treatment (shown in B). Blue arrows in the scheme depict the direction of symplastic communication and red crosses represent the situation of blocked symplastic communication.

(C, D) Quantitative measurement of J0571 fluorescent intensity of endodermis and cortex shown in (A) and (B). Scale bars=20μm.

Supple Figure 6. Time-course observation of J0571 with the activation of pWOL:icalsm.

All seedlings were treated by estradiol for 0d, 2d, 3d and 4d (as marked). The variation of J0571 fluorescent intensity was shown from the highest level to the lowest level (from left to right) for each treatment. The gradient of green color above each panel shows the trend of varied fluorescence intensity in each treatment.

Supple Figure 7. Time-course observation of J0571 in 35S:SHR roots with the activation of pWOL:icalsm.

All seedlings were treated by estradiol for 0d, 2d, 3d and 4d (as marked). The variation of J0571 fluorescent intensity was shown from the highest level to the lowest level (from left to right) for each treatment. The gradient of green color above each panel shows the trend of varied fluorescent intensity in each treatment. In 3d and 4d treatment, both uncontrasted images and images with
enhanced brightness were shown (as marked).


