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# The NAC Domain Transcription Factors FEZ and SOMBRERO Control the Orientation of Cell Division Plane in *Arabidopsis* Root Stem Cells

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## SUMMARY

**Because plant cells do not migrate, cell division planes are crucial determinants of plant cellular architecture. In *Arabidopsis* roots, stringent control of cell divisions leads to a virtually invariant division pattern, including those that create new tissue layers. However, the mechanisms that control oriented cell divisions are hitherto poorly understood. Here, we reveal one such mechanism in which FEZ and SOMBRERO (SMB), two plant-specific NAC-domain transcription factors, control the delicately tuned reorientation and timing of cell division in a subset of stem cells. *FEZ* is expressed in root cap stem cells, where it promotes periclinal, root cap-forming cell divisions. In contrast, SMB negatively regulates FEZ activity, repressing stem cell-like divisions in the root cap daughter cells. *FEZ* becomes expressed in predivision stem cells, induces oriented cell division, and activates expression of its negative regulator, SMB, thus generating a feedback loop for controlled switches in cell division plane.**

## INTRODUCTION

Stem cells divide to self-renew and to create daughter cells that can differentiate. When a stem cell divides symmetrically, the stem cell pool is enlarged. When the division is asymmetric, one of the daughter cells self-renews and the other differentiates. In *Arabidopsis*, root stem cells are maintained by a small group of mitotic-inactive cells, the quiescent center (QC), by means of as yet unidentified short-range signals (van den Berg et al., 1997). The ''organizing'' QC cells and the surrounding stem cells together form a stem cell niche reminiscent to the microenvironment that maintains stem cells in animals (Spradling et al., 2001). The root stem cell niche is positioned by the activity of two patterning pathways that provide combinatorial input to specify the niche within a larger mitotic cell pool. The first input involves PLETHORA proteins, members of the AP2 transcription factor family required for stem cell niche maintenance, the expression of which is regulated by distal accumulation of the plant growth regulator auxin (Aida et al., 2004; Blilou et al., 2005; Galinha et al., 2007). The second input is defined by SHORTROOT (SHR) and SCARECROW (SCR) GRAS family transcription factors that confer competence for QC specification to a single layer of cells (Sabatini et al., 2003). Downstream of SCR, the plant RETINOBLASTOMA-RELATED (RBR) protein and the WUSCHEL homolog, WOX5, have been implicated in stem cell maintenance (Wildwater et al., 2005; Sarkar et al., 2007).

Asymmetric stem cell divisions can be highly oriented, such as in the *Drosophila* germ line (Yamashita et al., 2004; Wallenfang and Matunis, 2003) and in the *Arabidopsis* root tip (Dolan et al., 1993). However, neither the transcription factors required for QC-mediated stem cell maintenance nor the RBR-pathway, which has roles in other aspects of development, can explain the highly specific asymmetric cell divisions of specific root stem cells. One of these stem cell-specific division patterns is the alternation of the cell division plane in the epidermal (Epi)/lateral root cap (LRC) stem cell, which coordinates stem cell renewal and the formation of two different tissue layers. In plants, oriented cell divisions are pivotal for proper organ architecture, because cell walls restrict cell migration and divisions in the direction of growth create new layers. However, the mechanisms by which the orientation of cell division planes is controlled have remained elusive.

Some of the founding members of the NAC domain transcription factor family, No Apical Meristem (NAM) and CUP-SHAPED COTYLEDONS (CUC), are involved in organ boundary specification in the shoot. These factors have been postulated to regulate orientation of cell division or cell division and expansion (Souer et al., 1996; Aida et al., 1999; Aida and Tasaka, 2006). Here, we report on the identification of the *FEZ* and *SOMBRERO* (*SMB*) genes, which encode nuclear NAC domain proteins. We demonstrate that both are intrinsically required for correct execution of the root cap developmental program, and are active in root cap stem cells and their immediate daughters. FEZ and SMB antagonistically control the division frequency of columella (COL) root cap cells and the cell division plane orientation of the COL and Epi/LRC stem cells. Our data demonstrate that the frequencies and orientations of stem cell divisions crucial for plant morphogenesis are under the control of nuclear factors, the activity of which is restricted to specific stem cells and their immediate daughters.

## RESULTS

### Identification of the *FEZ* and *SMB* Genes Involved in Root Cap Development

In the *Arabidopsis* root, tissue layers which originate distally to the organizing QC form the root cap, a protective layer which is constantly sloughed off during development. The root cap consists of the central COL (Figure 1A, pink), which contains starch granules, and the LRC (Figure 1A, dark purple). COL cells are generated by stem cells adjoining the distal face of the QC (Figure 1A, magenta). In the COL stem cells, each asymmetric division results in the regeneration of one stem cell and the formation of a daughter cell that ceases to divide and differentiates (Figure 1C), as visualized by the accumulation of starch granules (Figure 1H). LRC cells are produced by stem cells located distolaterally to the QC (Figure 1A, mid-purple; adjacent to COL stem cells). These cells also produce Epi cells, and hence are known as Epi/LRC stem cells. LRC layers are generated by periclinal divisions in the Epi/LRC stem cells (Figure 1B, "PC"), at a comparable rate to the production of COL layers. LRC daughter cells undergo anticlinal divisions to lengthen the root cap layer. Epi cells are formed by anticlinal divisions in the Epi/LRC cells (Figure 1B, "AC"). The Epi/LRC stem cells must therefore be able to switch between division planes in order for the root to develop normally.

We identified genes specifically involved in root cap development by screening for expression changes in COL/LRC markers using a line double homozygous for the enhancer traps J1092 and ET244 (Malamy and Benfey, 1997; Figures 1E and 1F, which was subjected to ethyl methane sulphonate (EMS) mutagenesis. Candidate lines with changes in J1092 and ET244 expression patterns were then rescreened for alterations in root cap structure that might indicate stem cell defects. One line with reduced J1092 activity possessed fewer root cap layers (Figures 1I–1M) and was named *fez*. Two lines showed reduced ET244 activity associated with additional root cap layers (Figures 1N–1R); complementation crosses revealed allelism, and we named these mutations *sombrero-1* (*smb-1*) and *smb-2*.

*FEZ* (At1g26870) and *SMB* (At1g79580) were identified by map-based cloning (Figure 2A) and encode NAC domain transcription factor proteins containing a conserved N-terminal NAC domain (Figure 2B (shaded areas) and Figure 2C). In addition, the FEZ protein has a predicted leucine zipper at the C terminus. Although members of the same protein family, FEZ and SMB do not group into particularly closely related NAC protein subfamilies (Ooka et al., 2003). In the case of *FEZ*, 5′RACE-PCR revealed a translational start different from the annotated translational start indicated in the TAIR database (Figure 2B; see also Supplemental Data available online).

We isolated T-DNA insertions to confirm that lesions in these genes are responsible for the *fez* and *smb* phenotypes. The *fez-2* (SALK_025663) insertion is located in the second intron, and the line is phenotypically indistinguishable from the *fez-1* mutation, which creates a stop codon at the end of the first predicted exon (Figures 2B and 2C). We did not detect *FEZ* mRNA in *fez-2* by RT-PCR, indicating that it is a null allele (Figure 3A). The *smb-1* and *smb-2* mutations reside at the beginning of the first exon and are predicted to create amino acid substitutions (R » W and E » K, respectively) (Figure 2C). The *smb-3* (SALK_143526) insertion is located just after the first exon, and the line is phenotypically strongest; we could not identify transcript by RT-PCR, suggesting that it is also a null allele (Figure 3B).

### *FEZ* Regulates Periclinal Cell Division in Stem Cells

In both *fez* mutant lines there is a reduced number of COL and LRC cell layers compared with WT from late embryogenesis onward, in all individuals (Table 1; Figures 1G and 1L). Meristem length, meristem cell number, and root length in *fez* mutants is comparable to WT (Table 1), suggesting that there is not a particular defect in the production of the epidermal or other cell layers. These data are supported by time-lapse tracking of COL and Epi/LRC stem cell divisions. While the replicative divisions of Epi daughter cells occur at a WT rate in *fez*, divisions (in any plane) in the COL and Epi/LRC stem cells (taken together) only occur at 30% of the WT frequency (Figure 1S). In the Epi/LRC stem cells, periclinal (LRC-forming) divisions only occur at 23% of the WT rate, whereas anticlinal (epidermis-forming) divisions are not significantly different from WT (Figure 1U). The outer LRC layer in *fez* mutants is slightly shorter than WT, but contains similar cell numbers in circumference to WT (Table 1), suggesting that radial divisions in the LRC daughter cells are not affected. Since the organization of other cell types in the root is not affected in *fez* (Figures 1I–1M), these data suggest that the *FEZ* gene specifically stimulates periclinal divisions in the Epi/LRC and COL stem cells.

### *SMB* Promotes Daughter Cell Fate

In *smb* mutants there are additional COL and LRC cell layers in the mature embryo (Figure 1Q) and in the postembryonic state (Figure 1R). Again, meristem length and meristem cell number are comparable to WT, suggesting that the effect of *smb* is specific to the root cap. In *smb* mutants, there is an extra layer of small, stem cell-like cells, below the COL and Epi/LRC stem cells (Figure 1R). This suggests that, in the *smb* mutant, there is at least a partial failure to adopt daughter cell fate. Interestingly, time-lapse tracking shows that the frequency of periclinal divisions in the (normal) Epi/LRC stem cells in *smb* is reduced relative to WT (Figure 1T and Table 1). This suggests that periclinal stem cell divisions are "shared" between the two stem cell-like layers in *smb*, consistent with the observation that the number of extra layers in *smb* does not increase over time—*smb* has approximately one extra cell layer at any given time (Table 1). Anticlinal Epi/LRC division frequencies are unaffected in *smb* (Figure 1U), as are divisions in Epi daughter cells (Figure 1S). We conclude that the role of SMB is to promote daughter cell fate in the root cap.

### Cell-Type Specification Is Not Altered in *fez* or *smb* Mutants

The altered expression of enhancer traps in the original *fez-1* and *smb-1* alleles might reflect cell fate changes or altered differentiation progression of stem cell daughter cells. The number of cells expressing J1092-green fluorescent protein (GFP) in *fez-2* is reduced, but there is no apparent reduction in GFP levels in cells still expressing the marker (Figure S1B). The late LRC identity marker J2093 is not significantly affected in *fez* and *smb* mutants (Figures S1E and S1F), and *pWER::GFP*, visualizing the promoter activity of the *WEREWOLF* gene, which is involved in Epi cell fate decisions

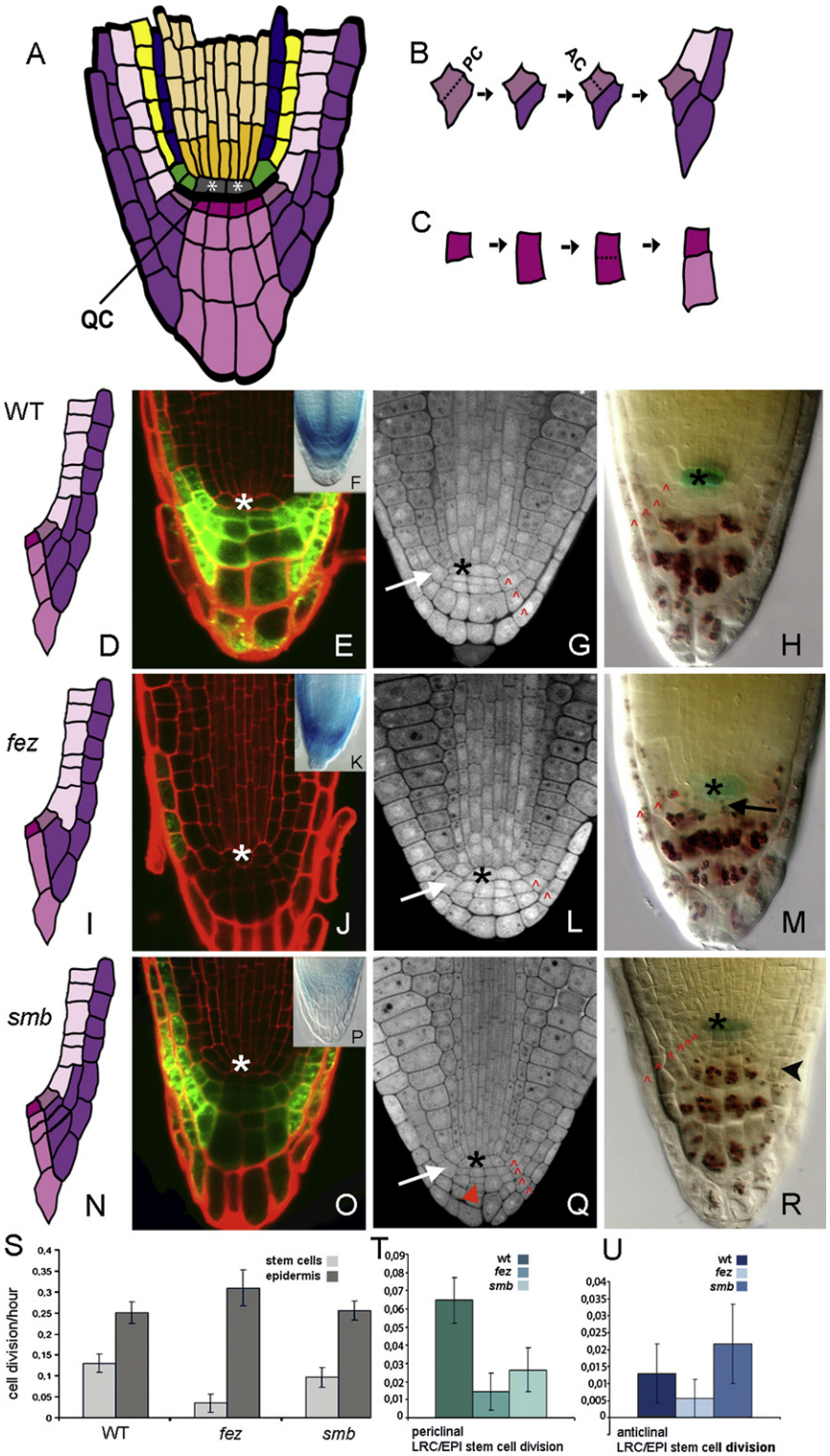


**Figure 1. Analyzes of the *fez* and *smb* Phenotype**

(A) Schematic of the *Arabidopsis* root.

(B) Schematic showing PC division in the Epi/LRC stem cell (dark pink) division that generates the LRC (purple) and anticlinal division that generates the epidermis (light pink).

(C) Schematic of anticlinal COL stem cell division, with the stem cell in red and the differentiated COL cell in pink.

(D, I, and N) Schematic of root patterning in WT, *fez*, and *smb*.

(E, J, and O) Confocal image of J1092 expression in WT, *fez-1*, and *smb-1*.

(F, K, and P) GUS expression of ET244 in WT, *fez-1*, and *smb-1*.

(G, L, and Q) Aniline-blue staining of WT, *fez-2*, and *smb-3* mature embryos; red arrowhead indicates extra COL division.

(H, M, and R) Four day postgermination root tip of WT, *fez-2*, and *smb-3*. Starch granule staining marks differentiated COL cells (brown/purple); blue staining indicates activity of the QC93 marker line; arrow marks starch granules next to the QC.

(R–T and U) Time-lapse experiments (error bars represent standard error; n = 11–14): stem cell-like divisions in COL and Epi/LRC stem cells versus replicative divisions in the epidermal layer in WT, *fez-2*, and *smb-3* (S), PCs of Epi/LRC stem cells in WT, *fez-2* and *smb-3* (T), anticlinal divisions of EPI/LRC stem cell in WT, *fez-2* and *smb-3* (U). White arrow indicates Epi/LRC stem cell; asterisk indicates QC position.

and expressed in LRC and epidermis (Lee and Schiefelbein, 1999), is expressed in *fez-2* and *smb-3*. The COL-specific marker COL93 marks a reduced region in *fez-2* and, in a larger domain, in *smb-3* (Figures S1H and S1I), consistent with the observed changes in COL cell number in these mutants (Figures 1M and 1R). Furthermore, the outer layers in the center of the root cap positively stain for starch granule accumulation in *fez* and *smb*, indicating that they have differentiated as COL cells (Figures 1M and 1R). Thus, many aspects of cell type-specific gene expression are unaltered in *fez* and *smb* mutants.

## *FEZ* and *SMB* Do Not Regulate Stem Cell Niche Patterning

Stem cell activity and maintenance in the root is regulated by patterning input from the *SHR/SCR* pathway (Sabatini et al., 2003) and the auxin-regulated *PLT* genes (Aida et al., 2004). Since FEZ mutants appear to have a defect in stem cell activity, we assessed whether FEZ is required for patterning the stem cell niche. First, we investigated whether mutations in *FEZ* and *SMB* altered the expression of the previously characterized stem cell patterning inputs. *SCR* promoter activity, which is dependent on correct activity of both SHR and SCR, is present in the endodermal layer, the ground tissue stem cells, and the QC in both WT and *fez-2* (Figures S1O–S1Q). *WOX5* transcription, which is dependent on the SHR/SCR pathway, is also unaltered in *fez-2* (Figures S1U and S1V). The auxin response

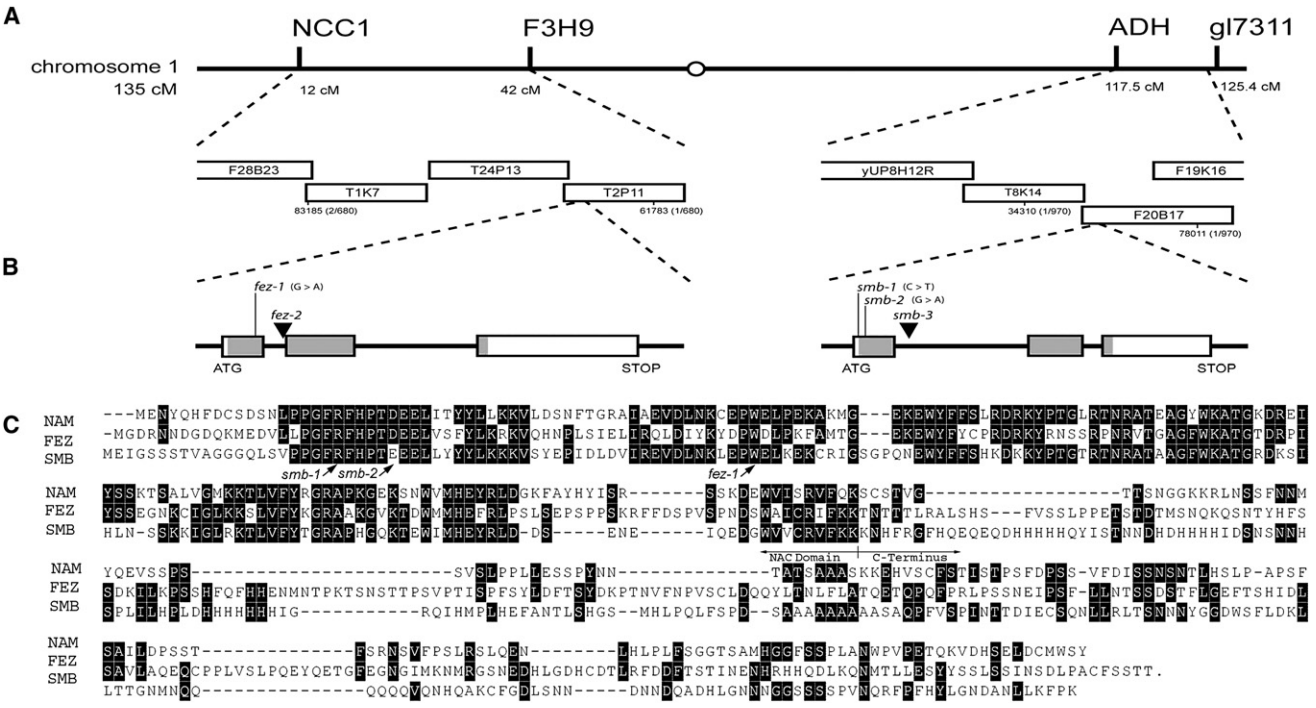


**Figure 2. Cloning and Gene and Protein Structure of FEZ and SMB**

(A) *fez* and *smb* localization relative to a contig of three BAC clones (T1K7, T24P13, and T2P11) and two BAC clones (T8K14 and F20B17), respectively; position of the markers corresponds to recombination breakpoints. The number of recombinant seedlings between the marker and the *fez* or *smb* locus are shown in parenthesis.

(B) Genomic structure of *FEZ* (At1g26870) and *SMB* (At1g79580); boxes indicate coding sequence. The position of the point mutations and the resulting amino acid substitutions and insertion sites are shown. Shaded area indicates NAC domain.

(C) Comparison of protein sequences between NAM, a founding member of NAC family, FEZ, and SMB. The black areas indicate the conserved amino acids. The positions of the *fez-1*, *smb-1*, and *smb-2* mutations within the amino acid sequence are shown.

maximum, as visualized with the widely used auxin-responsive *DR5rev::GFP* marker (Figure S1R), is also unaltered in *fez-2* and *smb-3* mutants (Figures S1S and S1T). Furthermore, transcription of the *PLT1* gene is normal in these mutants (data not shown). Lastly, QC-specific enhancer trap marker line QC25, which is dependent on both *SHR/SCR* and *PLT* pathways, is expressed in the QC in both *fez-2* and *smb-3* (Figures 1M and 1R), which suggests that the patterning input needed for specification of the stem cell niche operates correctly in both mutants.

To further assess whether *FEZ* and *SMB* might affect patterning gene functions, we crossed *fez* and *smb* with *shr*, *scr*, *wox5*, and the *plt1 plt2* double mutant, all of which lose stem cells and differentiate at a characteristic rate (Sabatini et al., 2003; Aida et al., 2004; Sarkar et al., 2007). Proximal stem cell loss is not enhanced in any combination of the patterning mutants with *fez* and is not suppressed in any combination of the patterning mutants with *smb* (data not shown).

## *FEZ* and *SMB* Are Regulated Independently of Known Root Meristem Patterning Genes

Consistent with the specific roles of these genes in the root cap stem cell region, *FEZ* and *SMB* transcripts accumulate in the COL and LRC progenitors of the root stem cell domain (Figures 4A and 4G). *FEZ* transcript occupies a narrow domain including stem cells and their immediate daughters. *SMB* transcript accumulates in maturing root cap cells (Figure 4G).

We next tested whether the known stem cell niche patterning functions were required for correct expression of *FEZ* and *SMB.* Accumulation of FEZ and SMB transcript occurs in a WT manner in *wox5*, *shr*, *scr*, *plt1 plt2*, and *plt1 plt2 plt3* (Figures 4A–4D, 4G–4J, and 4M–4O). In *plt1 plt2 scr* and *plt1 plt2 shr* triple mutants, the *FEZ* and *SMB* transcripts can also be detected in the stem cell area (Figures 4E, 4F, 4K, and 4L). Reduction of *RBR* activity results in additional COL stem cells (Wildwater et al., 2005), and these extra cells all show *FEZ* expression (Figure 4P). We conclude that the initiation of *FEZ* and *SMB* transcription does not require SHR, SCR, PLT1, -2, or -3, WOX5, or RBR action.

Although the auxin-inducible *PLT1*, *PLT2*, and *PLT3* genes are not the upstream regulators of *FEZ*, the orientation of cell division in root cap cells can be influenced by accumulation of auxin upon treatment with auxin efflux inhibitors (Sabatini et al., 1999). Auxin response factors (ARFs) mediate transcriptional responses to auxin (Hagen and Guilfoyle, 2002). Aux/indole-3-acetic acid (IAA) proteins heterodimerize with ARFs and block ARF-dependent transcription. Auxin targets Aux/IAAs for proteolytic degradation via $SCF^{TIR}$-mediated ubiquitination, thereby promoting ARF protein activity (Kepinski and Leyser, 2005; Dharmasiri et al., 2005). To probe the potential involvement of ARFs in *FEZ* activation, we used a dexamethasone (DEX)-inducible line that expresses an auxin-insensitive, dominant negative version of the Aux/IAA protein IAA12/BODENLOS (BDL). This *GR:bdl* construct is expressed under control of the ubiquitous *RPS5*

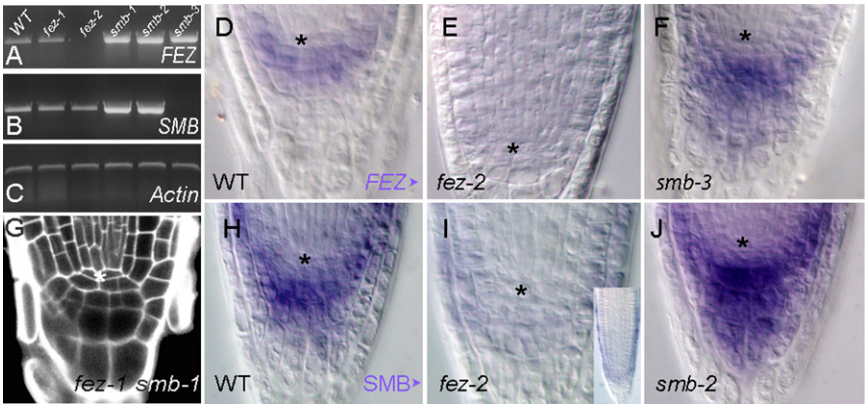


**Figure 3. Feedback Regulation of *FEZ* and *SMB* Expression**

(A–C) RT-PCR on 5 day-old WT, *fez1*, *fez2*, *smb1*, *smb2*, and *smb3* seedlings; *FEZ* transcription (A); *SMB* transcription (B); control, *Actin* transcription (C).

(D–F) *FEZ* mRNA in WT (D), *fez-2* (E), and F. *smb-3* (F).

(G) Confocal image of *fez-1 smb-1* double mutant.

(H–J) *SMB* mRNA in WT (H), *fez-2* (I), and *smb-3* (J).

Asterisk indicates position of QC.

promoter (Weijers et al., 2006). In *RPS5:GR:bdl* roots that were germinated on medium without DEX, *FEZ* transcript is detected (Figure 4Q); conversely *FEZ* transcript is lacking in roots germinated on 5 μM DEX (Figure 4R). Accordingly, a reduction in stem cell activity is suggested by the occasional appearance of starch granules in COL stem cells (data not shown). This indicates that ARF activity is required for the activation of *FEZ*.

## A Feedback Loop Regulates *FEZ* and *SMB* Expression

*FEZ* and *SMB* are both expressed in root cap domain and have opposing functions in orientated cell division, suggesting that they operate in the same pathway. We created double mutants to test this hypothesis. Both *fez-1 smb-1* and *fez-2 smb-3* mutants have reduced LRC layers like *fez* (Figure 3G), demonstrating that the additional stem cell-like divisions in *smb* mutants require *FEZ* activity, and indicating that a major role of *SMB* is to negatively regulate *FEZ* activity in stem cell daughters.

Consistent with this genetic interaction, *FEZ* mRNA reaches higher levels and is distributed more broadly in *smb* mutants (Figures 3A, 3D, and 3F). *SMB* mRNA also reaches higher levels in *smb-2* and is maintained in more mature cell layers (Figures 3B, 3H, and 3J) Therefore, restriction of *FEZ* mRNA to the stem cell region is mediated by *SMB*, which also represses its own expression. *fez* mutants show similar total levels of *SMB* transcript, but fail to accumulate *SMB* transcript in the stem cell area, whereas it is still detected in more mature LRC cells (Figures 3H and 3I), indicating that FEZ specifically activates SMB in the stem cell daughters. We conclude that FEZ promotes oriented cell division in stem cells and *SMB* transcription in stem cell daughters, which then counteracts FEZ activity in the daughter cells, forming a regulatory loop.

To investigate the dynamics of FEZ and SMB protein distribution, we constructed transgenic plants carrying GFP fused with genomic *FEZ* and *SMB* fragments under control of the *FEZ* and *SMB* promoters, which rescued *fez* and *smb* mutants, respectively. FEZ and SMB translational fusions localize to the nucleus similar to other NAC domain proteins (Figures 5A–5E and 5G–5K). *FEZ* is expressed from globular stage onward in the COL progenitors after the first division of the hypophyseal cell and its expression is maintained in these cells and its descendents (Figures 5A–C). *SMB* is expressed from early heart stage onward in all basal daughter cells resulting from horizontal divisions in the COL progenitors and is maintained in these cells (Figures 5G–5I). Thus, *FEZ* is expressed before *SMB* in each cell layer, consistent with the finding that FEZ is required to activate *SMB*.

At later stages, FEZ:GFP accumulates in COL stem cells prior to division, but soon after COL stem cell division it is retained only in the daughter cells (Figures 5D and 5E, arrows). It also accumulates in the Epi/LRC stem cells and daughters, and is retained in maturing LRC layers (Figures 5D and 5E). FEZ is readily detectable in elongated stem cells (19 out of 20 cells examined) that are about to divide. In contrast, *FEZ* expression is often absent from small, postdivision, stem cells (present in 28 out of 81 examined), whereas it remains expressed in all immediate daughter cells (Figure 5F). This cyclic expression of *FEZ* in stem cells can also be observed at the mRNA level (Figure 5L).

The SMB:GFP fusion is present in stem cell daughters and accumulates invariantly in maturing root cap layers (Figures 5J and 5H).

**Table 1. Quantification of Cell Layers and Meristem Size in *fez* and *smb***

| | No. of Layers in Seedlings (n)[a] | | Seedlings with Observable Ectopic Divisions, % (n)[a] | | No. of Layers in Mature Embryos (n) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Columella | Lateral Root Cap | Columella | Lateral Root Cap | Columella | Lateral Root Cap | No. of LRC Cells in Radial Section (n) | Meristem Length, μm (n) | No. of Meristem Cells (n) |
| WT | 4.6 ± 0.2 (11) | 3.8 ± 0.1 (11) | 0 (11) | 0 (11) | 4.0 ± 0.0 (7) | 2.0 ± 0.0 (7) | 26.7 ± 0.7 (3) | 274.3 ± 6.3 (23) | 31.6 ± 0.8 (23) |
| *fez-2* | 2.9 ± 0.2 (11) | 2.0 ± 0.2 (11) | 0 (11) | 0 (11) | 3.0 ± 0.0 (7) | 1.0 ± 0.0 (7) | 25.5 ± 1.0 (6) | 237.8 ± 8.2 (21) | 27.7 ± 0.9 (21) |
| *smb-3* | 5.8 ± 0.1 (12) | 4.9 ± 0.1 (12) | 58 (12) | 75 (12) | 4.6 ± 0.2 (9) | 2.6 ± 0.2 (9) | 25.6 ± 0.8 (5) | 271.8 ± 7.7 (26) | 32.3 ± 1.0 (26) |

Data presented are mean ± SEM, unless otherwise noted. Values in parentheses represent sample sizes.

[a] Seven days postgermination.

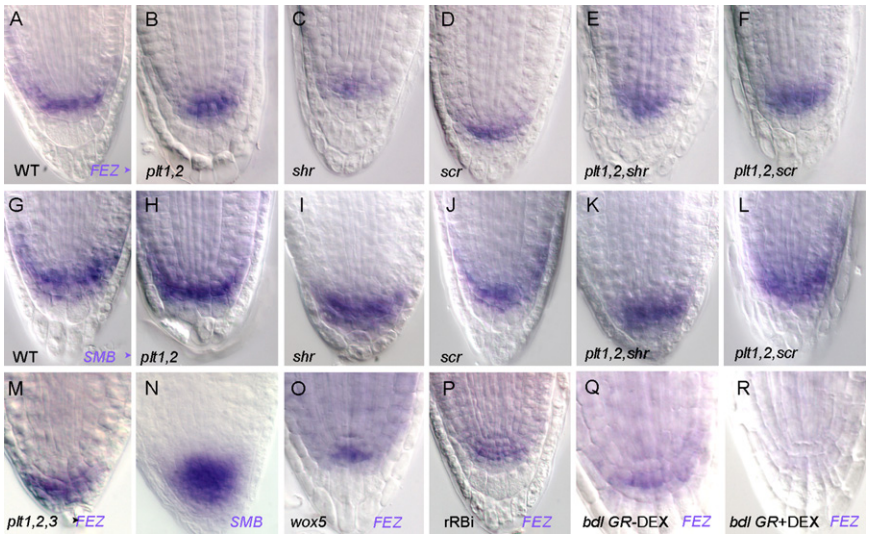


**Figure 4. *FEZ* and *SMB* Operate Independently of Patterning Genes**

(A–F, M, O, and P) *FEZ* mRNA in 2 day-old seedlings of WT (A), *plt1-4 plt2-2* (B), *shr-1* (C), *scr-4* (D) *plt1-4 plt2-2 shr-1* (E), *plt1-4 plt2-2 scr-4* (F), *wox5* (O), and *RBR rna-i* (P), or in mature embryos of *plt1-4 plt2-2 plt3-1* (M).

(G–L, N) *SMB* mRNA in 2 day-old seedlings of WT (G), *plt1-4 plt2-2* (H), *shr-1* (I), *scr-4* (J), *plt1-4 plt2-2 shr-1* (K), *plt1-4 plt2-2 scr-4* (L), or in mature embryos of *plt1-4 plt2-2 plt3-1* (N).

(Q and R) *FEZ* mRNA in 2 day-old *RPS5:bdl:GR* seedlings, without DEX induction (Q) and with DEX induction (R).

We conclude that a cross-regulatory feedback loop regulates *FEZ* and *SMB* expression, where FEZ activates *SMB* in the root cap daughter soon after division, and SMB in turn represses *FEZ* expression in these cells, thereby preventing further stem cell divisions.

## FEZ Regulates Division Plane Orientation

The *fez* mutant phenotype suggests that FEZ may be involved in controlling the orientation of the division plane in root cap stem cells. However, other interpretations are possible. FEZ might generally stimulate cell division; the mutant phenotype could be interpreted as a reduction in cell division rates in both the Epi/LRC and COL stem cells. In addition, the COL stem cells only ever divide periclinally, even in *fez* mutants, so in these stem

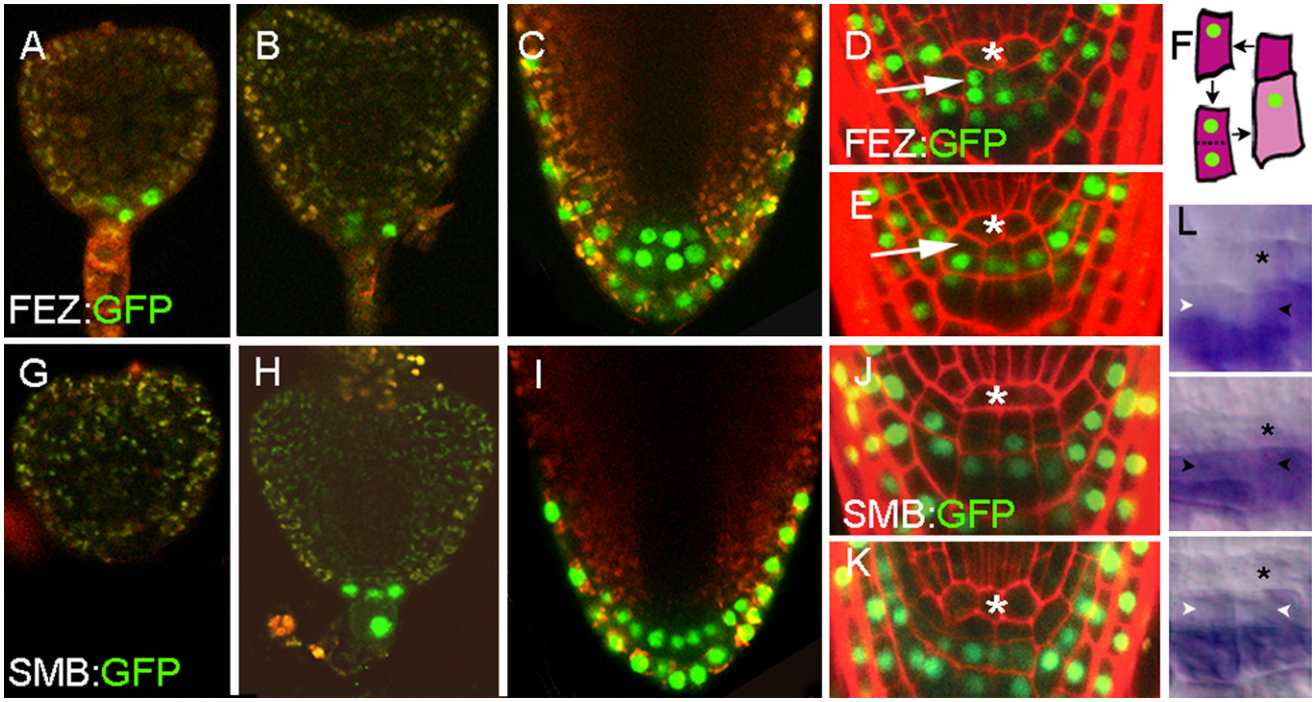


**Figure 5. Stem Cell Oscillations in FEZ Levels**

(A–C) FEZ:GFP in WT embryos.

(D and E) FEZ:GFP in 5 day-old seedling. White arrows indicate oscillation of FEZ:GFP.

(F) Model of spatial expression of FEZ.

(G–I) SMB:GFP in WT embryos.

(J and K) SMB:GFP in 5 day-old seedling.

(L) *FEZ* mRNA expression in dividing COL stem cells. White arrowheads indicate absence of expression; black arrowheads indicate presence of expression. Asterisk indicates position of QC.

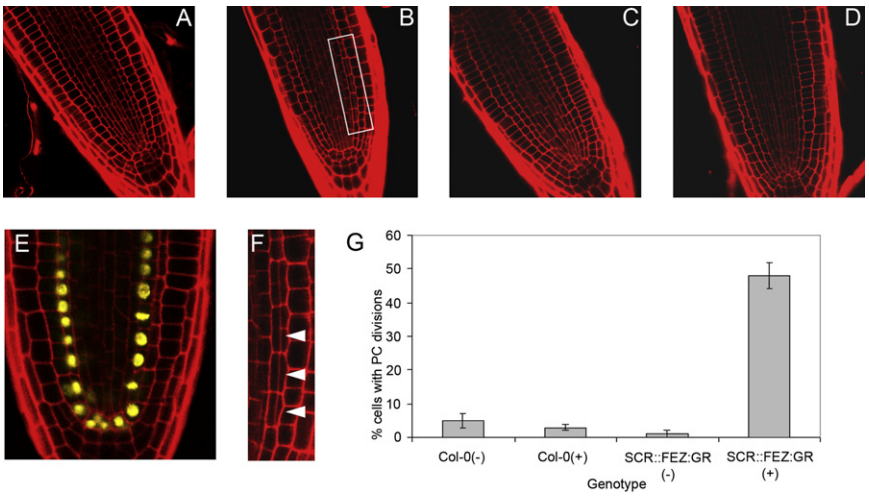


**Figure 6. Effects on Cell Division of Ectopic *FEZ* Expression**

(A and B) *SCR::FEZ:GR* roots after 2 day control treatment (A) or DEX treatment (B).

(C and D) Col-0 roots after 2 day control (C) and DEX (D) treatment.

(E) Expression pattern generated by the SCR promoter (SCR::H2B:YFP).

(F) Inset from (B), white rectangle. White arrowheads indicate periclinal divisions in the endodermis.

(G) Percentage of endodermal cells with periclinal divisions in SCR::FEZ:GR relative to Col-0 (±DEX). For each root sample, the percentage of cells in the median longitudinal plane of view of the meristematic zone with periclinal divisions was assessed by confocal microscopy. Bars show the mean percentage per genotype ± standard percentage error; n = 12 samples for each genotype, and 20–50 cells per sample.

cells it cannot be ascertained whether FEZ affects the rate of divisions only in a specific plane. To assess whether FEZ activity is sufficient to alter the plane of cell division, we ectopically expressed *FEZ* under the control of the *SCR* promoter, which is specifically expressed in the QC and endodermal layer (Figure 1A, dark blue; Figure 6E). We tagged the FEZ protein with the steroid binding domain of the rat glucocorticoid receptor ("GR") at the C terminus, to generate SCR::FEZ:GR, which was then transformed into the Col-0 WT background (Lloyd et al., 1994). In this system, FEZ activity is steroid-inducible, rather than constitutive, making it easier to observe the direct effects of FEZ activity. When 4 day-old *SCR::FEZ:GR* seedlings are transferred to media containing the steroid hormone DEX, a high number of periclinal divisions can be seen in the endodermal tissue layer after 2 days (Figure 6B, inset in Figure 6F, and 6G). There is a low background level of periclinal divisions in the endodermis, but there is a greater than 10-fold increase in periclinal divisions upon FEZ induction (Figure 6G). This effect is not seen in *SCR::FEZ:GR* plants transferred to control media (Figures 6A and 6G). Periclinal endodermal divisions are rarely observed in WT seedlings that are transferred to DEX for 2 days, or in WT seedlings on control media (Figures 6C, 6D, and 6G). These results demonstrate that FEZ activity is sufficient to reorient the cell division plane, even outside the endogenous region of *FEZ* expression.

## DISCUSSION

We have demonstrated that a subset of precisely oriented stem cell divisions in the *Arabidopsis* root meristem are under the control of FEZ and SMB, two related nuclear proteins of the NAC domain transcription factor family. The Epi/LRC initial cells periodically reorient their cell division plane, thereby creating two tissue layers with distinct identities. We show that FEZ promotes the periclinal divisions in this system, and also in the COL stem cells. In addition, FEZ is able to promote periclinal divisions when ectopically expressed. Conversely, SMB is required to restrict the activity of FEZ in daughter cells, and to prevent such divisions. Our data indicate that FEZ and SMB act directly in this pathway without affecting pattern formation and tissue specification of the rest of the root meristem. Even though detection limits preclude a clear demonstration of FEZ oscillations in Epi/LRC stem cells, there appears to be an association between cells in which *FEZ* alone is expressed and the asymmetric stem cell division stimulus, and the subsequent induction of *SMB* in daughter cells to repress *FEZ* activity. Therefore, these NAC domain proteins have a specialized role in the control of stem cell division plane and the coordination between LRC and COL cell production rate. The precise spatial control of cell division planes through dedicated transcription factors with local activity allows for the production of ordered tissues in the absence of cell migration.

The relatively rare tissue-forming divisions in plants are referred to as "formative divisions," in contrast to the majority of "proliferative" cell divisions that serve to increase cell numbers within a tissue. The FEZ and SMB proteins illustrate that the formative cell divisions are controlled by locally expressed nuclear factors. The expression dynamics of *FEZ* and *SMB* and the negative control of SMB on the FEZ-initiated cell divisions have the potential to create pulses of FEZ activity to initiate cell division in COL stem cells and reorientation of Epi/LRC stem cells, followed by *SMB* expression to extinguish the division-promoting effect (Alon, 2007).

It is noteworthy that the NAC domain family founding member, petunia *NAM*, and its *Arabidopsis* homologs, the *CUC* genes, promote boundary formation between shoot-derived organs (Souer et al., 1996; Aida et al., 1997). It has been speculated that boundary formation may arise from reorientation of cell division perpendicular to prospective boundaries, and it is therefore possible that more members of the NAC domain family will turn out to influence cell division orientation in different developmental contexts.

Interestingly, the SHR and SCR GRAS domain transcription factors are required for the formative cell division in the ground tissue stem cell daughter that gives rise to endodermis and cortex (Helariutta et al., 2000; Di Laurenzio et al., 1996) (Figure 1A). However, in contrast to FEZ, these factors simultaneously act as tissue identity factors—SHR for endodermis and SCR for QC. It will be interesting to investigate whether downstream factors of SHR and SCR serve specialized roles comparable to FEZ and

SMB, or whether the GRAS transcription factors influence cell division plane directly.

An intriguing question is how plant cell division planes can be precisely controlled. In animal cells, including several stem cell systems, cell division plane switches are provoked by mitotic spindle reorientation. Spindle positioning is guided by preferential interactions of one of the centrosomes with molecules localized by stem cell organizers or by intrinsic polarity determinants (Yamashita and Fuller, 2008). This system bears resemblance to spindle orientation control in budding yeast by spindle pole bodies (Liakopoulos et al., 2003), suggesting a conserved mechanism for the orientation of cell division. Plant cells, however, have no localized centrosomes, instead possessing more diffuse microtubule organizing centers. It is long known that the "proliferative" cell divisions generally occur over the shortest path across the long axis of the cell, while tissue-forming divisions are perpendicular to this plane (reviewed by Smith [2001]). A predictive feature of cell division orientation is an oriented cortical microtubule array that condenses into the preprophase band. This structure leaves a mark consisting of at least one microtubule interacting protein, which guides the orientation of the new cell plate (Walker et al., 2007). Mutations in different components of this cytoskeleton-based machinery lead to randomization of cell division planes (Torres-Ruiz and Jürgens, 1994; Traas et al., 1995; Kawamura et al., 2006; Smith et al., 1996). These phenotypes contrast with the precise division plane alterations in *fez* and *smb* mutants. Two mechanisms could be envisaged to explain this precision. First, in the absence of FEZ, the cortical microtubules (which are otherwise normal) may fail to appropriately reorient to generate periclinal divisions; or, second, the absence of FEZ may obstruct an alternative readout of division polarity independent of cortical microtubule arrays. Better tools for visualization of microtubule dynamics in small, actively dividing cells will be required to address this issue. In parallel, the identification and analysis of targets of the FEZ and SMB transcription factors, especially those which are antagonistically regulated, may clarify how the polarized cytoskeletal machinery of plant cells is regulated to accurately control division plane orientation.

## EXPERIMENTAL PROCEDURES

### Plant Materials, Growth Conditions, and Mutagenesis

The enhancer trap lines J1092 (C24) and J2093 (C24) were obtained from the Nottingham *Arabidopsis* stock center (NASC), as were the *fez-2* (N525663) and *smb-3* (N643526) SALK T-DNA lines. The T-DNA insertion sites were confirmed by PCR-based genotyping. The following lines have been described elsewhere: ET244 GUS enhancer trap (Malamy and Benfey, 1997); *shr-2* (Col) (Nakajima et al., 2001); *scr-4* (WS) (Fukaki et al., 1998); *plt1-4* and *plt2-2* (WS) (Aida et al., 2004); *plt1-4 plt2-2 plt3-1* (Galinha et al., 2007); QC25 (Sabatini et al., 1999); *DR5rev::GFP* (Benková et al., 2003); *pSCR::H2B:YFP* (Heidstra et al., 2004); *RCH1::RBR* RNAi (Wildwater et al., 2005); and *RPS5A::bdl:GR* (Weijers et al., 2006).

*fez-1*, *smb-1*, and *smb-2* mutants were generated by EMS mutagenesis (Willemsen et al., 1998) of doubly marked homozygous J1092/ET244 plants. A prescreen on pools representing 4500 M1 plants were analyzed for changes in J1092 expression. The seedlings were checked 2 and 3 days after germination using a Leica MZ FLIII1 stereomicroscope equipped with a GFP filter. The candidate lines were screened for a second time on LRC244 expression. Two hundred seeds were sown in a single horizontal stripe at 2 cm from the bottom of a custom made plate. When roots reached the bottom of the plate, substrate was added to root tips and plates were incubated for 2 hr at 37°C positioned at an angle of 45°. GUS staining was evaluated with a Zeiss stemi SV6 stereomicroscope and candidate mutants with altered staining were rescued onto soil. Seeds were sterilized, plated, and seedlings grown as previously described by Willemsen et al. (1998).

### Map-Based Cloning

Homozygous *fez* and *smb* plants in C24/WS background were crossed to ecotype Col-0. In the F2, *fez* and *smb* mutants were selected and DNA was isolated according to Lukowitz et al. (1996). We initially mapped *FEZ* to chromosome 1 between marker NCC1 (12cM) and F3H9 (42cM), and *SMB* to chromosome 1 between markers ADH (117.5 cM) and gl7311 (125.4 cM). Fine mapping primers were designed using information from CEREON (http://www.arabidopsis.org) and Primer 3 software (http://frodo.wi.mit.edu/). For *FEZ*, the interval was narrowed down to 235 kb spanning 56 genes between markers T1K7snp472870 (position 81,185) and T2P11snp445957 (position 81,783). For *SMB*, the interval was narrowed down to 110 kb spanning 27 genes between BAC T8K14 snp-2 (position 34,310) and F20B17 ind-4 (position 78,011) on chromosome 1. Candidate genes were selected based on expression profiles specific for LRC (Birnbaum et al., 2003) and their genomic regions were sequenced. Allelism tests with T-DNA insertion alleles and complementation tests using FEZ-GFP and SMB-GFP were used to confirm gene identities.

### Expression Analysis

RNA of *FEZ* and *SMB* was obtained using the RNeasy-Plant mini-kit (QIAGEN). Chromosomal contamination was removed by Dnase I (Ambion) treatment. cDNA was prepared using Ready-to-Go You-Prime First-Strand Beads (Amersham BioSciences). The primers used for PCR of the full *FEZ* cDNA length were 5′ ATGGCGGCTGATCCTTCG 3′ (2F) and 5′ CCACATACATCAGGTTGTA CTGGAGAAAC 3′(cDNA1R).

The primers used for PCR of the full *SMB* cDNA were 5′ GCATGGTCAAA CCCATTCAT 3′(AF) and 5′ TCTTCGGAGAAACAGAACAGAA 3′ (FR). 5′RACE of *FEZ* was performed as previously described by Casamitjana-Martínez et al. (2003) with gene-specific PCR primers CCTGAAGCGGAAGGTTCAAAC AACCCTCT, CCCTCTCTCCATTGAGCTCATAAGACAACTC and TTGCGATG ACGGTGAAAAAGA.

### Plant Vectors and Transformations

SMB::SMB:eGFP and FEZ::FEZ:eGFP were generated by insertion of a 5.2 kbp *SMB* genomic fragment (primers 5′ GGGCCCTCGTTGAAGATGCCTGGA TTTAATACTG 3′and 5′ GGATCCCTTTGGGAACTTGAGAAGATTAGCGTCGT 3′) and a 6.9 kbp *FEZ* genomic fragment (5′ GGGCCCGTGCACCAGTAAACTA ATTAGTGAACCAG 3′and 5′ CCCGGGGGTTGTACTGGAGAAACAAGCTGGC AAAT 3′) into pGREENII-0229 (http://www.pgreen.ac.uk) with an eGFP-noster fragment. SCR::FEZ:GR was generated by fusing a SCR promoter fragment (Heidstra et al., 2004) to the FEZ cDNA (primers 5′ ATGGGAGATAGAAACAAC GACG 3′ and 5′ GGTTGTACTGGAGAAACAAG 3′), in turn fused to a GR encoding fragment (Lloyd et al., 1994), and insertion of the resultant construct into pGREENII-0225. *Arabidopsis* plants were then transformed by the floral dip method (Clough and Bent, 1998). The functionality of the GFP fusions was verified by the complementation of *smb-3* and *fez-2* mutant phenotypes.

### Root Length and Meristem Size Analysis

The root lengths of WT, *fez-2*, and *smb-3* were measured 5 days after germination, as described elsewhere (Willemsen et al., 1998). Meristem size was determined as the number of cells in cortex files in the meristem up to the zone of rapid elongation. LRC length was measured as the distance from the QC to the first detaching LRC cell.

### Microscopy

Whole-mount visualization of roots, starch granules, and β-glucuronidase stains were described by Willemsen et al. (1998). Mature embryos were stained with Aniline-blue, as described by Bougourd et al. (2000). Whole-mount in situ hybridization was performed with 2 or 5 day-old seedlings or mature embryos according to Hejátko et al. (2006). Gene-specific 700 bp cDNA fragment for *FEZ* (primers Seq 7F: 5′ TATATGCAGAATCTTCAAAAAG ACCAACAC 3′and cDNA1R: 5′ CCACATACATCAGGTTGTACTGGAGAAAC 3′) and gene-specific 1116 bp complementary DNA fragment for *SMB* (primers

BF: 5′ GCTGGAACCTTGGGAACTTA 3′ and FR: 5′ TCTTCGGAGAAACAGAAC AGAA 3′) was used. The *WOX5* probe was described by Sarkar et al. (2007). Laser ablation experiments were performed as described by van den Berg et al. (2005). Time-lapse experiments were done according to Campilho et al. (2006).

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and one figure and can be found with this article online at http://www.developmentalcell.com/supplemental/S1534-5807(08)00401-2.

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