RESEARCH ARTICLE

Precise control of plant stem cell activity through parallel regulatory inputs

Tom Bennett1,*,‡, Albert van den Toorn1,2,†, Viola Willemsen1,2 and Ben Scheres1,2,§

ABSTRACT

The regulation of columella stem cell activity in the Arabidopsis root cap by a nearby organizing centre, the quiescent centre, has been a key example of the stem cell niche paradigm in plants. Here, we investigate interactions between transcription factors that have been shown to regulate columella stem cells using a simple quantification method for stem cell activity in the root cap. Genetic and expression analyses reveal that the RETINOBLASTOMA-RELATED protein, the FEZ and SOMBRERO NAC-domain transcription factors, the ARF10 and ARF16 auxin response factors and the quiescent centre-expressed WOX5 homeodomain protein each provide independent inputs to regulate the number of columella stem cells. Given the tight control of columella development, we found that these inputs act in a surprisingly parallel manner. Nevertheless, important points of interaction exist; for example, we demonstrate the repression of SMB activity by non-autonomous action of WOX5. Our results suggest that the developmental progression of columella stem cells may be quantitatively regulated by several more broadly acting transcription factors rather than by a single intrinsic stem cell factor, which raises questions about the special nature of the stem cell state in plants.

KEY WORDS: Stem cell, Stem cell niche, Root cap, Plant development

INTRODUCTION

The regenerative capacity of stem cells (SCs) in animal systems has generated immense attention. SC activity has predominantly been conceptualised as resulting from intrinsic and extrinsic ‘stemness’ factors that are necessary and sufficient to confer SC fate (Morrison et al., 1997). However, it has proved difficult to identify a firm molecular basis for SC identity that goes beyond the operational definition of SCs, and SC behaviour may be best understood as a property of whole-tissue systems (Lander, 2009). SCs have also been described in plants, generally located within the proliferative zones (meristems) located at growing shoot and root apices. Clonal analysis has identified ‘initial’ cells that continuously produce daughter cells while remaining undifferentiated themselves – which are therefore operationally defined SCs – but there are few of these truly long-lived SCs in plant meristems (Stewart and Derman, 1970; Scheres et al., 1994). Intrinsic stemness factors have not been described in plants (Aichinger et al., 2012), and plant SCs have mostly been discussed in the framework of ‘stem cell niches’ (SCN), where SCs are ‘protected’ from differentiation by ‘organizing’ cells (Scheres, 2007).

In seedling root meristems of Arabidopsis thaliana, approximately four cells located near the root apex divide infrequently (‘quiescent centre’, QC; Fig. 1A) (Dolan et al., 1993). Cells surrounding the QC act as initials for one or more cell files that form the root (Fig. 1A), and clonal analysis confirms that these cells behave as SCs (Dolan et al., 1994; Scheres et al., 1994). The main root ‘meristem zone’ lies on the proximal (shootward) side of the QC, and spans ~30 cells within each file. The root cap (RC), a specialized structure (reviewed by Barlow, 2003), resides on the distal side of the QC. The RC consists of a central ‘columella’ region, and the larger and more-complex lateral root cap (LRC). Ablation of QC cells leads to differentiation of adjacent columella stem cells (CSCs), suggesting that the QC signals to the SCs to prevent their differentiation (van den Berg et al., 1997) as in a classical SCN (Scheres, 2007). The QC-expressed transcription factor WUSCHEL-RELATED HOMEOBOX5 (WOX5) might be or control this signal, as CSCs differentiate prematurely in wox5 mutants (Sarkar et al., 2007). WOX5 is closely related to WUSCHEL (WUS), which is expressed in the shoot meristem organizing centre and moves into over-lying cells (Yadav et al., 2011, 2013). As WUS and WOX5 are functionally interchangeable (Sarkar et al., 2007), WOX5 may also be a mobile QC-derived signal. Other transcription factors acting in RC development have been identified; the NAC-domain protein FEZ is required for CSC activity (Willemsen et al., 2008), whereas another NAC domain protein, SOMBRERO (SMB) drives cell-cycle exit and differentiation in CSC daughter cells (Willemsen et al., 2008; Bennett et al., 2010; Fendrych et al., 2014). The cell cycle and differentiation regulator RETINOBLASTOMA-RELATED (RBR) also drives cell cycle exit in CSC daughters and promotes differentiation (Wildwater et al., 2005). In addition, two negatively acting auxin response factor (ARF) proteins, ARF10 and ARF16, that do not appear to directly interact with auxin signalling (Vernoux et al., 2011) influence the number and location of SC divisions in the RC (Wang et al., 2005).

The columella allows the regulation of higher-level coordination of division and differentiation, as it contains four apparently distinct cells types (including the QC) in a regular and stable arrangement (Fig. 1A,B). The QC undergoes periodic periclinal divisions (Ortega-Martinez et al., 2007), in which rootward daughter replenish existing CSCs, which in turn differentiate (Cruz-Ramirez et al., 2013). It is important to note that cell position, behaviour (e.g. division/differentiation) and identity are separable concepts in colunmella development, even though in wild-type roots there is usually a highly predictable relationship between the three. Columella development has previously been described through changes in cell identity and differentiation state: QC → CSC → differentiated cells (DC) → border-like cells (BC). However, no factors that confer distinct cell identities
have been identified so far in the columella, and in various developmental mutants, correlations between position, behaviour and apparent cell identity breakdown. It is not yet clear whether CSCs are defined intrinsically at a molecular level, or whether their behaviour is maintained through a combination of cues from a highly regular cellular environment. To address this issue, we assessed or reassessed the functions and interactions of the known RC transcription factors FEZ, SMB, RBR, ARF10/ARF16 and WOX5. We demonstrate that none of these regulatory factors fully dictates CSC state. Instead, CSC-like behaviour emerges by modulation of division and differentiation through mostly parallel pathways. We show that WOX5 controls CSC-activity by non-cell autonomously repressing expression of SMB – providing a genetic entry into a long-known interaction between organizer and SCs.

RESULTS

A coordinate system to monitor columella development

Depending on the exact phase of the cell cycle when imaged, the wild-type columella contains a single layer of CSCs (Fig. 1C), a partially divided set of CSCs (Fig. 1E) or two layers of cells that resemble CSCs (Fig. 1G). In the latter, the rootward cells are not CSCs and will accumulate starch and begin to expand, and become displaced to the c2 position (B). The same process can be observed by monitoring S-phase progression using incorporation and detection of the thymidine homologue F-ara-EdU (D,F,H). Incorporated F-ara-EdU is indicated in red, while all nuclei are counterstained with DAPI (blue); nuclei containing DNA that has replicated during a 24 h treatment period are thus coloured magenta. (I-M) F-ara-EdU staining showing characteristic changes in distribution and level of S-phase progression in mutant genotypes fez-2 (I), wox5-1 (J), wox5-1 fez-2 (K), smb-3 (L) and wox5-1 smb-3 (M). These changes can also be detected by quantitative analysis of cell numbers (N). (N) Analysis of cell numbers in root caps of Col-0, fez-2, smb-3, wox5-1, wox5-1 fez-2 and wox5-1 smb-3 at the positions q, c1 and c2. The total number of cells visible in each position in median sections was counted, n=25 per genotype, bars indicate s.e.m. Asterisks mark the position of q. Arrowheads indicate recently divided cells. Scale bars: 50 μm.
The use of molecular markers relies on an absolute correlation between cell identity/behaviour and the expression of the marker gene, and we know of no examples in the RC where this is the case. Indeed, the behaviour of many characterised RC marker genes changes in various mutant backgrounds (e.g. Wang et al., 2005; Sarkar et al., 2007).

To permit analysis of columella development in multiple genetic backgrounds, we have taken a simple approach that separates the effects of position, cell identity and cell behaviour. We defined the positions in the columella region as follows. The tier of cells that is contiguous with the cortical/endodermal cell layers (‘q’) forms a distinct location that normally contains QC cells. Even if QC-like behaviour is lost, q position remains clearly defined (Fig. 1A). Similarly, the c1 position is contiguous with the epidermal cell layer (including the nascent lateral root cap layer l1 if present), while each subsequent columella cell tier (c2, c3, etc.) is contiguous with one layer of LRC cells (l2, l3, etc.), and these positions can be defined irrespective of the identity and behaviour of the residing cells (Fig. 1A). For example, ectopic divisions in columella tiers can be easily identified, because more cells will be bracketed by a single LRC layer. Using this positional framework, we quantified cell numbers in different positions (c1, c2, etc.) from median optical sections through the columella. We combined this information with a qualitative assessment of differentiation based on cell shape, presence/absence of starch granules and the presence/absence of divisions – cells at q and c1 in wild type have a typical ‘brick-shaped’ morphology and no starch accumulation, whereas other columella cells are square/elongated in shape and accumulate starch.

In wild-type roots, cells at each position display stereotyped behaviour, and on this basis have been attributed different identities. At q, cells are undifferentiated and divide infrequently, and as such are described as QC cells; in wild type there are on average two cells periclinal divisions, such that on average 5.5 cells are observed at c1 position (Fig. 1N). These cells, and the cells in subsequent columella cell tier (c2, c3, etc.) from median optical sections through the columella. We combined this information with a qualitative assessment of differentiation based on cell shape, presence/absence of starch granules and the presence/absence of divisions – cells at q and c1 in wild type have a typical ‘brick-shaped’ morphology and no starch accumulation, whereas other columella cells are square/elongated in shape and accumulate starch.

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Rapid quantification of columella stem cell activity in mutants

To assess whether our quantification method can capture changes in cell division and differentiation, we described known root cap developmental mutants using this method. If CSC cell division rate is altered, we should observe a change in the average number of cells at c1, and this can indeed be observed in fez-2 (Fig. 1N), which has previously been characterised as causing a reduction in SC activity (Willemsen et al., 2008). Conversely, if differentiation is impaired, we would anticipate observing increased cell division and undifferentiated morphology at the c2 position. This can be observed at c2 in smb-3 (Fig. 1N), which we have previously characterised as causing increased SC activity (Willemsen et al., 2008). It should be noted that the number of cells at c1 and c2 is approximately the same as at c1 in Col-0 (Fig. 1N), suggesting that SMB primarily regulates differentiation and not the rate of cell division. If differentiation is accelerated, we would anticipate decreased cell division and altered morphology at the c1 position, and this is indeed the case in wox-5-1 (Fig. 1N), which has previously been characterised as promoting differentiation of CSCs (Sarkar et al., 2007).

Our analysis indicates that these mutant phenotypes cannot be described in absolute terms; for example, we still observed apparent cell divisions at c1 in wox-5-1 and fez-2, albeit at a reduced rate. Our method thus seems able to provide a more nuanced view of previously described phenotypes. To further validate our method, we assessed whether these apparent cell divisions represent recent cell division activity by incorporation and subsequent detection of the thymidine analogue F-ara-EdU. After 24 h, we detected S-phase progression in Col-0 roots that correspond to different patterns of cell numbers observed at c1, indicating that visual quantification of cell numbers detects recent cell divisions (Fig. 1C-H). Furthermore, patterns of F-ara-EdU incorporation in fez-2, smb-3 and wox-5-1 correspond to the changes in cell division inferred from cell number analysis, including reduced but not absent cell-cycle progression at c1 in fez-2 and wox-5-1, and increased S-phase progression at c2 in smb-3 (Fig. 1I-K; Table 1). We thus conclude that our method provides a reasonable assessment of dynamic developmental processes in the columella.

The columella stem cell state is not solely determined by FEZ-like proteins

We have previously shown that FEZ is a regulator of CSC activity, and it might be proposed that presence of FEZ (together with absence of SOMBRERO, see below), characterizes the CSC state. However, our current analysis demonstrates that CSC-like activity is not completely lost in fez-2 (Fig. 1N). This might be caused by

<table>
<thead>
<tr>
<th>Position</th>
<th>Replication</th>
<th>Division</th>
<th>Events/root</th>
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<tr>
<td>Col-0</td>
<td>q</td>
<td>9</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>c1</td>
<td>19</td>
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</tr>
<tr>
<td></td>
<td>c2</td>
<td>2</td>
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</tr>
<tr>
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<tr>
<td></td>
<td>c1</td>
<td>19</td>
<td>3.14</td>
</tr>
<tr>
<td></td>
<td>c2</td>
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<td>0.43</td>
</tr>
<tr>
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<td>q</td>
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<tr>
<td></td>
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<td>10</td>
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Table 1. Quantification of F-ara-EdU incorporation in root cap mutants

Seedlings of the listed genotypes were treated with F-ara-EdU for 24 h and then visualized. The number of DNA replication or cell division events that were visible in median confocal sections at the q, c1 and c2 positions was quantified. The figure in the final column is the total number of either type of event, divided by the number of roots assessed.
biochemical redundancy of the FEZ protein, a member of the large NAC-domain family, with close relatives, thus masking effects of FEZ-like proteins on CSC activity. To investigate whether such NAC-domain proteins might be required for CSC activity, we investigated three close FEZ relatives in Arabidopsis, FEZ-LIKE1 (FZL1/ANAC094, At5g39820), FZL2 (At3g12910) and FZL3/JUB1/ANAC042 (At2g43000), which are also represented in other Brassicaceae genomes (supplementary material Fig. S1). According to publically available data, FZL1 has significant expression in the root cap (http://bar.utoronto.ca/efp; Bimbaum et al., 2003; Brady et al., 2007), but FZL2 and FZL3 do not, which we confirmed by constructing FZL2:GUS and FZL3:GUS reporter lines (supplementary material Fig. S2A-D). Although we could detect FZL2 and FZL3 transcripts in the root meristems of Col-0, we did not observe any upregulation of FZL2 or FZL3 in fez-2 that might mask the lack of FEZ activity (supplementary material Fig. S2E).

Furthermore, FZL3/JUB1/ANAC042 has recently been identified as a factor controlling stress responses by regulating hydrogen peroxide homeostasis (Saga et al., 2012; Wu et al., 2012). We thus focussed our attention on FZL1, which has high identity with FEZ across the N-terminal NAC domain, but is highly divergent from and much shorter than FEZ in the C-terminal domain (supplementary material Fig. S1). In all other eurid genomes examined, we identified sequences with the FEZ C-terminal structure, but no sequences corresponding to FZL1, suggesting that the FEZ:FZL1 pair has arisen by duplication of an ancestral FEZ gene in the Brassicaceae, followed by divergence in the FZL1 lineage. FZL1:GUS and FZL1:FZL1-YFP lines confirmed that FZL1 is expressed in columella DCs – but not CSCs (Fig. 2A,B). We obtained an insertion line (fzl1-1) in the FZL1 gene and found by sequencing that the T-DNA lies 64 bp into the first exon, eliminating the production of full-length FZL1 transcript (supplementary material Fig. S3E). fzl1-1 has no root cap phenotype by itself, and when combined with fez-2 there is no enhancement in the fez-2 phenotype (Fig. 2E-I). We also made a FEZ:FZL1-GR transgenic line to test whether FZL1 can trans-complement fez-2 if expressed in SCs, but upon induction with the steroid hormone dexamethasone (DEX), we only observed no rescue of the fez-2 phenotype (Fig. 2C,D). This failure does not arise from a lack of activity in the FZL1-GR protein, as demonstrated by the very strong phenotypes seen in 35S:FZL1-GR seedlings treated with DEX (supplementary material Fig. S3A-D). We thus conclude that FZL1 does not act redundantly with FEZ in regulating CSC activity under our growth conditions. As FZL1 is structurally divergent protein that does not act redundantly with FEZ, but represents a well-conserved protein family within the Brassica family, we hypothesise that it has an as yet unidentified, neo-functional role in the RC. Our findings suggest that parallel inputs not mediated by FEZ-like proteins are required for CSC activity.

**RBR and ARF10/ARF16 have FEZ-independent roles in columella stem cell activity**

Increased numbers of CSC-like cells are present in the arf10-2 arf16-2 double mutant, which is thought to be null for ARF10 and ARF16, and in lines lacking ARF10 and ARF16 transcript due to overexpression of mir160C, which endogenously regulates their expression (Wang et al., 2005). Similarly, the transgenic RCH1: RBR RNAi line (rRBR), which reduces RBR levels in the root meristem, and lines expressing artificial miRNAs targeted to RBR have more tiers of CSC-like cells (Wildwater et al., 2005; Cruz-Ramirez et al., 2013). We therefore questioned whether these factors might act through FEZ to reduce CSC-like activity or promote differentiation. We observed partial rescue of the rRBR phenotype in a fez-2 background (Fig. 3A,B,D,E), with fewer apparent layers of small unstained cells with CSC-like morphology, showing that FEZ activity contributes to the increased CSC activity observed upon RBR reduction, but is not absolutely required for it. The disorganised nature of rRBR root caps makes counting cell numbers in these lines impractical, so we only assessed this qualitatively. We did not observe any alteration in FEZ expression levels in rRBR, other than that more cells express FEZ, probably because the number of CSC-like cells is increased (Fig. 3C,F,J). In summary, our data reveal that FEZ activity contributes to the division-promoting effect of a reduction in RBR activity in the CSCs, but we have no evidence that RBR regulates FEZ.

Similarly, we established that FEZ contributes to the arf10 arf16 phenotype but is not absolutely required for it (Fig. 3G,H); again, the massive and disorganized root cap in arf10-2 arf16-2 makes it to accurately quantify cell numbers, so we only assessed this qualitatively. FEZ is expressed in an enlarged domain in arf10-2 arf16-2, but only at a slightly increased level of expression per cell, as a factor controlling stress responses by regulating hydrogen peroxide homeostasis (Saga et al., 2012; Wu et al., 2012).
indicating that ARF10/ARF16 is not a major regulator of FEZ expression (Fig. 3C,I,J). As RBR and ARF10/ARF16 have FEZ-independent roles and largely do not alter FEZ expression at a cellular level, they are not likely to be upstream regulators of FEZ. Taken together with the lack of redundancy between FEZ and FZL1, the most parsimonious explanation for FEZ function is that it is not necessary for CSC-like cell divisions per se, but it increases their frequency, and therefore contributes to the increase in CSC-like cell numbers in any mutant combinations that delay CSC differentiation.

SMB regulates differentiation of stem cell daughters in parallel to RBR, ARF10 and ARF16

RBR and ARF10/ARF16 do not act primarily through FEZ in controlling CSC activity, so we tested whether they function by promoting differentiation through SMB activity. We first tested genetic interactions between SMB and RBR; SMB is correctly expressed in rRBR, suggesting that RBR does not regulate SMB expression (Fig. 4C,F). The smb-3 rRBR double mutant has an additive phenotype with more tiers of small, unstained cells with CSC-like morphology relative to arf10-2 arf16-2 in 3 dpg seedlings. (C,F,I) PI-stained root caps showing expression of FEZ:FEZ-GFP in wild type (C), rRBR (F) and arf10-2 arf16-2 (I). (J) Quantification of FEZ-GFP fluorescence in rRBR and arf10-2 arf16-2, relative to wild type (100%); n=40-50 nuclei, bars indicate s.e.m.

is expressed in the correct domain (i.e. in the outermost cells of the RC), suggesting that lack of SMB protein does not explain the arf10 arf16 phenotype (Fig. 4C,I). We further tested this by making the arf10-2 arf16-2 smb-3 triple mutant; if ARF10 and ARF16 operate through SMB, the phenotype should be no more severe than arf10-2 arf16-2. However, the triple mutant phenotype was the most severe that we observed in this study. In all 7 dpg seedlings, the primary root is short and increasingly wide towards the root tip, which harbours a thick mass of cells (Fig. 4G). Instead of the extra CSC-like cells in arf10-2 arf16-2, we observe many complete layers of RC that resulted in swollen root meristems (Fig. 4G,H,J). The cells in the LRC layers are cytoplasmically dense, indicating that they have not differentiated (Bennett et al., 2010). In 12/45 seedlings, even more severe phenotypes were observed, whereby the mass of cells accumulating in the columella position organised a new root meristem, growing out of the old root. In most cases, the new root is in the same orientation as the old root (Fig. 4I), but in some cases, it grows as a tip-to-tip mirror image (Fig. 4K). Although this quasi-root meristem has no regular cellular organization comparable to wild type, there are in most cases clearly identifiable files of cells along the proximal-distal axis and root hair differentiation in distal cells, and sometimes there is an identifiable second QC. These very strong phenotypes indicate that ARF10/ARF16 and SMB act in parallel to constrain CSC-like activity and to promote differentiation in the root cap meristem. Furthermore, they indicate that columella cells retain...
multipotency, which becomes unconstrained if a combination of factors, including ARF10, ARF16 and SMB are removed.

**WOX5 and ARF10/ARF16 act in parallel in root cap development**

It has previously been suggested that the extreme arf10-2 arf16-2 phenotype (Fig. 5D) arises through ectopic expression of WOX5, because in the absence of these ARFs, auxin can no longer downregulate WOX5 (Ding and Friml, 2010). However, WOX5 is not quickly downregulated in response to auxin treatment (Ding and Friml, 2010), and while WOX5 expression is diminished after 24 h of 1 µM NAA treatment (Ding and Friml, 2010) (Fig. 5A,B) it is conceivable that this effect is part of the general collapse of the root meristem that occurs under such treatments. We also did not observe any alteration in the domain of WOX5 promoter activity in the arf10-2 arf16-2 background (Fig. 5C) that would be consistent with WOX5 being repressed by ARF10 and ARF16 in the columella. It was also previously suggested that the arf10-2 arf16-2 phenotype is rescued by the null wox5-1 allele (Ding and Friml, 2010). However, although we confirmed that the arf10-2 arf16-2 wox5-1 phenotype is less severe than arf10-2 arf16-2 at 3 dpg (though not wild type) (Fig. 5E,F), we detected a much more severe phenotype in the triple mutant at later time points (Fig. 5G,H), showing that although WOX5 activity contributes to the severity of the arf10-2 arf16-2 phenotype, wox5-1 is not epistatic to arf10-2 arf16-2. Taken together, these data show that ARF10/ARF16-mediated repression of WOX5 is not an important feature of CSCs, and that these factors also act in parallel to regulate RC development.

**WOX5 cell-autonomously restricts SC-like activity in the QC**

Deficiency in WOX5 results in differentiation of the CSCs at c1 (Sarkar et al., 2007), but we also observed that the cells at q are frequently divided in wox5-1 (Figs 1N and 6B), suggesting that WOX5 cell-autonomously represses QC divisions, which occur at low frequency in wild-type roots to replenish differentiating CSCs (Cruz-Ramirez et al., 2013). Since the size of the wox5-1 RC does not appreciably decrease over time, these ectopic divisions at q must also re-generate the CSCs that are lost through ectopic differentiation. We have previously shown that FEZ drives CSC activity in the columella, so we examined whether FEZ:FEZ-GFP expression is increased at q in the wox5-1 mutant background, and this is indeed the case (Fig. 6E,F). We then tested whether FEZ was responsible for the increased divisions at q in wox5-1, but observed that wox5-1 fez-2 has on average the same number of cells as wox5-1 (Figs 1N and 6A-D). This suggests that FEZ does not contribute to these ectopic divisions in wox5, and that other genes are the targets of WOX5 in the QC. We tested whether FEZ was likely to be a direct target of WOX5 activity using the inducible 35S::WOX5-GR line (Sarkar et al., 2007). Upon DEX treatment, FEZ-GFP expression is gradually lost from c1 over the course of 48 h (Fig. 6G-L), but even accounting for fusion protein stability, it seems unlikely that reduction in FEZ is a direct response, and FEZ is therefore likely an indirect target of WOX5. The most parsimonious explanation for these data is that FEZ becomes expressed in wox5-1 as a consequence of the activation of CSC-regenerating divisions at q. These divisions at q in wox5-1 also indicate that WOX5 is not absolutely required for cell division in the RC per se, but rather for the correct timing or positioning of those divisions.

**WOX5 non-cell-autonomously promotes stem cell activity by regulating SMB expression in the columella**

To gain further insight into the role of WOX5 in regulating CSC activity, we re-analysed the 35S::WOX5-GR phenotype (Sarkar et al., 2007). Treatment of 7 dpg roots with DEX for 48 h results in the grossly expanded columella phenotype previously described (Fig. 6L). As there is only a slight increase in the numbers of LRC layers produced during this time (−DEX: 4.17±0.11; +DEX:4.67±0.18; n=12) (Fig. 6L), and no obvious phenotype in the proximal root meristem, it seemed that WOX5 has a particularly specific role in the columella. To assess this further, we followed the establishment of the 35S::WOX5-GR phenotype in a time course after induction; after 8 h, we detected ectopic divisions in the differentiated parts of the columella (supplementary material Fig. S4A,F), and by 16 h, the majority of cells in the columella undergo ectopic divisions (Fig. 6J). By 24 h, much of the starch is lost from previously differentiated columella cells (Fig. 6K), and a second round of ectopic divisions is initiated in each tissue layer; both these phenotypes become more severe by 48 h (Fig. 6L). Overexpression of WOX5 thus results in de-differentiation of columella cells with concomitant divisions across the whole columella. The phenotype is therefore not only caused by failure of CSC daughters at c1 to differentiate, but by re-entry of previously differentiated cells from c2 and beyond into the cell cycle. We examined whether the positive effects of WOX5 on CSC-like activity were mediated by regulation of FEZ activity. FEZ is still expressed in the c1 position in wox5-1 mutants (Fig. 6F), and wox5-1 and fez-2 mutants have a slightly additive effect on the reduction in cell numbers at c1 (Fig. 1N). Furthermore, FEZ expression does not mark all CSC-like cells in 35S::WOX5-GR,
instead becoming progressively restricted to the outer columella layers (Fig. 6I,J,L). The phenotype of fez-2 35S:WOX5-GR after DEX induction shows that, although FEZ contributes to the severity of the WOX5 overexpression phenotype, it is not required for it (Fig. 6G,H). Taken together, these data show that WOX5 and FEZ act in parallel to regulate columella development. These data also suggest that WOX5 does not simply prevent premature differentiation of CSCs, but may also promote their division.

We have previously reported that SMB limits cell divisions and drives differentiation in the RC, both processes that have been shown here to be affected by WOX5 overexpression. We thus tested whether SMB might be a target of WOX5 activity in the columella, by analysing SMB:SMB-GFP expression in the wox5-1 and 35S:WOX5-GR backgrounds. As observed with FEZ, SMB expression moves up one layer in the wox5-1 mutant, such that it is expressed at c1 (Fig. 7A,B). Furthermore, after induction with DEX, SMB expression in the 35S:WOX5-GR columella is greatly reduced within 4 h (Fig. 7C,D) and is completely absent by 24 h after induction (supplementary material Fig. S4E-H). However, WOX5 overexpression has no effect on SMB expression in the LRC, consistent with the phenotype of 35S:WOX5-GR. We therefore tested whether the wox5 CSC differentiation phenotype is suppressed in wox5-1 smb-3 double mutants, which is indeed the case; a wild-type level of cells is present at c1 (Fig. 1N), corresponding to increased S-phase progression (Fig. 1J,M; supplementary material Fig. S5E-H) and a more wild-type cellular appearance (Fig. 7E-H; supplementary material Fig. S5A-D). However, wox5-1 smb-3 phenotype still occurs at q in this double mutant (Fig. 1N), so we conclude that CSC differentiation in wox5-1 is not simply caused by loss of QC integrity, and that divisions at q in wox5-1 are not simply a feedback response to reduced CSC activity at c1. Taken together with the effect of 35S:WOX5-GR on SMB expression and de-differentiation of DCs in the columella, we believe that the most parsimonious explanation for the wox5-1 smb-3 phenotype is that WOX5 specifies the position of SC-like activity in the columella by restricting SMB expression, and excluding it from the c1 position. The wild-type level of CSC-like
divisions at c1 in wox5-1 smb-3 again demonstrates that WOX5 is not absolutely required for CSC activity. It is also noteworthy that wox5-1 smb-3 does not have as many CSC-like cells at c2 as smb-3 (Fig. 1L-N, Table 1, Fig. 7G,H, supplementary material Fig. S5), suggesting that WOX5 actively contributes to the ectopic CSC activity seen at c2 in smb-3. WOX5 promoter activity appeared unaltered in the smb-3 background (supplementary material Fig. S4C,D). Therefore, it is unlikely that ectopic WOX5 activity is directly responsible for the smb phenotype, which would imply that SMB in turn restricts the expression of WOX5 from DCs. However, due to difficulties in detecting WOX5 protein (Sarkar et al., 2007), we cannot rule out the possibility that WOX5 protein moves and that SMB limits this process.

**DISCUSSION**

**Precise developmental control from parallel regulatory elements**

Our results show surprisingly few epistatic interactions among characterised regulatory factors that influence SC activity in the RC; instead, these factors seem to regulate development mainly in parallel to each other (Fig. 8). This is exemplified by FEZ, which promotes, but is not strictly necessary for, cell division in the RC, such that fez mutations result in fewer divisions irrespective of the background – but never complete suppression of cell division. Indeed, we found no single decisive factor for CSC-like behaviour; neither WOX5 nor FEZ is required for cell division, although both promote it. Equally, we did not identify a unified differentiation pathway that prevents CSC-like activity, but rather at least three independent inputs that promote differentiation. Despite our failure to detect coordination between transcription factor inputs, RC development is very robust in wild-type plants (Fig. 1E). We suggest that these transcription factors may provide distinct quantitative inputs to control division and/or differentiation by interacting indirectly on the promoters of shared target genes. However, it is also possible that they each have distinct targets that converge further downstream to drive division or differentiation; equally, as yet uncharacterized factors may mediate interaction between these regulators. Further understanding of the quantitative control of RC development requires identification of the ultimate ‘effector’ genes downstream of these transcription factors.

**The role of WOX5 in columella root cap development**

The close evolutionary relationship of WUS and WOX5, together with their analogous expression in different organizing centres (Sarkar et al., 2007), and their proposed negative-feedback regulation by peptide signalling may indicate that a directly conserved regulatory system operates in both meristems (Brand et al., 2000; Stahl et al., 2009). However, several observations suggest that these systems are actually quite different from each other. The proposed feedback regulation of WOX5 by the CLE40 peptide, acting through ACR4/CLV1 receptor kinases, acts in a different way to the CLV3-WUS feedback, forming a loop between DCs and SCs, rather than a loop between organizer cells and SCs (Stahl et al., 2009, 2013). Furthermore, we show that much of the effect of WOX5 is mediated through SMB, and neither SMB nor its close relative are expressed in the shoot meristem, nor are they reported WUS targets (Yadav et al., 2013). However, one interpretation of the wox5 smb double mutant is that SMB restricts WOX5 activity in the RC; and SMB might therefore regulate WOX5 through the CLE40-ACR4 signalling module. Although acr4 and clv1 mutants are reported to have mild effects on CSC daughter differentiation (Stahl et al., 2009, 2013), quantification of their genetic interactions with the transcription factors discussed here is needed to assess the validity of this idea.

**The stem cell concept in plant meristems**

The dependency of CSC activity on the QC ‘organizer’ has been used as an example of the SCN concept in plants (Scheres, 2007). We demonstrate the specific validity of this idea in the RC, and show that QC-derived WOX5 does indeed protect CSCs from the differentiation promoted by SMB. However, WOX5 and SMB only interact in the columella context and, as wox5 smb double mutants have CSC-like cells, neither of them appears to serve as an intrinsic SC factor. Do intrinsic SC factors exist in the RC? In the RC, with a single layer of long-lived mitotic cells, it is relatively easy to assert that there are distinct SCs. However, in other meristems, it is currently rather more difficult to argue that SCs are fundamentally different from other meristematic cells – except by operational definitions based on clonal analysis. We have previously argued that SCs in plant meristems might not need to be actively specified, because they will emerge as an inevitable consequence of the rigid arrangement of plant cells in relation to each other, coupled with a broad specification of cell division activity (Bennett and Scheres, 2010). Essentially, cells located at the point of origin of a meristem cell file must behave like SCs, while all other cells will gradually be displaced from the meristem by new daughter cells of the apical-most cells and so cannot act as long-term SCs. Interestingly, recent analyses of individual SC fates in mammalian SCNs provides evidence for the existence of a pool of equipotent cells, among which spatial competition determines which cells act as long-term SCs (Ritsma et al., 2014; Rompalas et al., 2013). As plant SCs cannot be transplanted, it is difficult to distinguish potential from
actual SCs. Nevertheless, operationally defined SCs in plants might simply express a property present in all meristem cells because they are at the right position (Bennett and Scheres, 2010). However, SCs do have measurable properties that are different from neighboring mitotic cells, such as their cell cycle rate (Campilho et al., 2006; Mähönen et al., 2014). The identification and study of the target genes of the parallel-acting regulatory inputs analysed in this study—which might include either truly intrinsic SC factors specific to CSCs or general meristematic factors—will help to distinguish between intrinsically programmed and positionally selected SCs.

MATERIALS AND METHODS

Plant materials

The fez-2, smb-3, FEZ:FEZ:GFP, SMB:SMG-GFP (Willemsen et al., 2008), wox3-1, WOX3:GFP, 35S:WOX3-GR (Sarkar et al., 2007), RCH1:RRB RNAI (RBR; Wildwater et al., 2005), arf10-2 arf16-2 (Wang et al., 2005) lines have all been described previously. The fzl-1 (GABI-KAT O27D08) insertion line was provided by the Nottingham Arabidopsis Stock Centre (NASC). Primers used for genotyping mutant combinations are shown in supplementary material Table S1.

Plant constructs and transformations

Primers used in this study are listed in supplementary material Table S1. For FEZ:GUS, the previously described 4 kb promoter (Willemsen et al., 2008) was amplified from Col-0 genomic DNA, and fused to the β-glucuronidase coding sequence, in a pGREEN II vector. FZL1:GUS, FZL2:GUS and FZL3:GUS constructs were constructed in the same manner using 1486 bp upstream from the FZL1 start codon, 3304 bp upstream of FZL2 and 2504 bp upstream of FZL3. For FZL1:FZL1:YFP the FZL1 cDNA was amplified from Col-0 seedling cDNA, then fused between the FZL1 promoter above and the YFP tag in a pGREEN II vector. Transgenic plants were created by Agrobacterium floral dipping using these constructs, into the appropriate genetic background (Clough and Bent, 1998).

RT-PCR

RNA extraction, cDNA synthesis and RT-PCR were performed as described by Bennett et al. (2010); RNA was extracted from the distal 5 mm of roots.

F-ara-EdU staining

4 dpw seedlings were transferred to plates containing 3 μM F-ara-EdU ([2S]-2-deoxy-2-fluoro-5-ethynyluridine) for 24 h, and were then fixed in 4% paraformaldehyde. Detection of F-ara-EdU was performed using a Click-it EdU Alexa Fluor 555 Imaging Kit (Life Technologies) according to the manufacturer’s instructions. Seedlings were counterstained with 0.1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) and F-ara-EdU incorporation was assessed by confocal microscopy.

Microscopy

Staining for starch granules and β-glucuronidase activity were performed as described by Willemsen et al. (1998). Differential contrast interference microscopy was performed on a Zeiss Axio Imager, using 20× lenses or 40× lenses. Confocal microscopy was performed using either a Leica SP5 or Zeiss LSM700 imaging system using 20× lenses. For GFP/YFP imaging, excitation was performed using 405 nm (~10% laser power) and 555 nm (~6%) lasers. PI fluorescence was detected above 600 nm, and GFP/YFP fluorescence below 555 nm. Identical settings for GFP/YFP detection were used within experiments; PI detection was varied according to the staining of individual roots. For F-ara-EdU imaging, excitation was performed using 405 nm (~10% laser power) and 555 nm (~8%) lasers; Alexa Fluor 555 fluorescence was detected above 555 nm and DAPI fluorescence below 500 nm. Nuclear GFP quantification was performed on non-saturated images, using Zeiss ‘ZEN’ software. Fluorescence intensity in the GFP channel was measured in five widely spaced nuclei per root, in at least eight roots per genotype.

Image processing

Images were processed and assembled using Adobe Photoshop. Raw images were rotated as necessary. If blank corners were created by rotation, they were ‘filled’ in a colour matching the background of the image. The images were then cropped to the region of interest. All further image processing was applied across the whole image. For confocal images, the brightness and contrast of the green/yellow channel and red channel were altered independently to produce clear images and reduce noise. Images from the same experiment were processed in the same manner. For DCIM images, the ‘lighten shadows/darken highlights’ function was used to reduce the cross-image glare caused by optical sectioning. Where necessary, images were processed using the ‘sharpen’ tool at a setting of 150%, to produce clearer images of cellular organization. Alterations to brightness/contrast, hue/saturation and white balance were also performed to make images consistent in their appearance.

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Competing interests

The authors declare no competing financial interests.

Author contributions

T.B., A.v.d.T., V.W. and B.S. conceived and designed the experiments. T.B., A.v.d.T., V.W. and B.S. wrote the paper.

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Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.110148/-/DC1

References


|   | ArathFEZ | AralyFEZ | CapruFEZ | BraraFEZa | ThehaFEZ | ArathFZL1 | AralyFZL1 | CapruFZL1 | BraraFZL1 | ThehaFZL1 | ArathFZL2 | AralyFZL2 | CapruFZL2 | BraraFZL2 | ThehaFZL2 | ArathFZL3 | AralyFZL3 | CapruFZL3 | BraraFZL3a | ThehaFZL3 |
|---|---------|----------|----------|-----------|----------|-----------|-----------|----------|-----------|-----------|-----------|-----------|----------|----------|----------|-----------|----------|----------|----------|-----------|----------|
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**Figure S1: FEZ-like proteins**

Protein pile-up of close FEZ homologues from *Arabidopsis thaliana* and other members of the Brassica family (Arath = *Arabidopsis thaliana*, Araly = *Arabidopsis lyrata*, Capru = *Capsella rubella*, Brara = *Brassica rapa*, Theha = *Thelungiella halophila*). There are four distinct protein structures represented; FEZ, FEZ-LIKE1 (FZL1), FZL2 and FZL3. FZL1 is the most similar group to FEZ in the NAC domain (marked by the grey bar above the sequences), while all groups are equally divergent in the C-terminus.
Figure S2: Characterization of FEZ-like proteins (A-B) Expression of FZL2:GUS in root tips (A) and in the hypocotyl (B) (C-D) Expression of FZL3:GUS in root tips (C) and in older root tissue (D) (E) Expression of FZL2 and FZL3 in the root tips of Col-0 and fez-2, as assessed by RT-PCR. ACTIN1 was used as a control. Cycle numbers are indicated at the right hand side.
Figure S3: Characterization of FZL1

(A-D) Phenotypes caused by overexpression of FZL1-GR with and without 10μM dexamethasone (DEX). Cotyledons in seedlings treated with DEX are white and stain for starch (C), while roots meristems are reduced in size and lose starch in the columella (D).

(E) Schematic showing transcription and translation products of the FZL1 gene. We did not detect the predicted mRNA annotated in the TAIR database (illustrated in red), but we did detect a similar, but slightly longer mRNA with an additional 9bp at the start of the third exon (illustrated in blue). Comparison with mRNAs from other NAC-domain transcription factors (including FEZ) showed that this is the canonical mRNA structure. A second partially spliced transcript was also detected in which the first intron is retained (also in blue), leading to formation of a premature stop codon (yellow text), and a predicted truncated protein. The position of the fzl1-1 insertion is shown, along with the 35S promoter in the T-DNA (purple arrow).

We observed no full length transcript in fzl1-1. Transcription from the endogenous FZL1 promoter in fzl1-1 (light blue, not actually detected) would result in a very small truncated protein due to the introduction of an in frame stop codon. However, activity of the 35S promoter in the T-DNA does lead to the production of a partial transcript that includes part of the T-DNA sequence (pink). The first two potential start codons of this transcript would produce very short proteins, while a third potential start codon in the second exon could lead to production of a truncated protein missing half the NAC domain. However, full-length FZL1 driven by the 35S promoter results in a very strong collapse of the root meristem, which is not observed in fzl1-1, and we thus conclude that no functional protein is produced from this residual transcript.
Figure S4: The role of WOX5 in root cap development
White arrowheads indicate ectopic cell divisions.
(A-B) Additional timepoints in 35S:WOX5-GR induction timecourse (see also figure 3I-L), also showing FEZ-GFP expression; (A) 4 hours post induction, (B) 8 hours post induction.
(C-D) PI stained root caps showing WOX-GFP expression smb-3 mutant (D) relative to wild-type.
(E-H) Additional timepoints in 35S:WOX5-GR induction timecourse (see also figure 3O, P) showing effect on SMB-GFP expression; (E) 4 hours post induction, (F) 8 hours post induction, (G) 24 hours post-induction, (H) no induction.
Figure S5: Additional wox5-1 smb-3 images.
(A-D) DICM images of wox5-1 smb-3 roots. Asterisks mark the q position, yellow arrows mark the position of CSC-like cells.
(E-H) S-phase progression in wox5-1 smb-3 monitored by incorporation of F-ara-EdU into DNA. Incorporated F-ara-EdU indicated in red, while all nuclei are counterstained with DAPI (blue).
**Development 141: doi:10.1242/dev.110148: Supplementary Material**

Table S1: Oligonucleotide primers used in this study.

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