

The maize root stem cell niche: a partnership between two sister cell populations

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Received: 21 August 2009 / Accepted: 5 November 2009 / Published online: 22 November 2009
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Abstract Using transcript profile analysis, we explored the nature of the stem cell niche in roots of maize (*Zea mays*). Toward assessing a role for specific genes in the establishment and maintenance of the niche, we perturbed the niche and simultaneously monitored the spatial expression patterns of genes hypothesized as essential. Our results allow us to quantify and localize gene activities to specific portions of the niche: to the quiescent center (QC) or the proximal meristem (PM), or to both. The data point to molecular, biochemical and physiological processes associated with the specification and maintenance of the niche, and include reduced expression of metabolism-, redox- and certain cell cycle-associated transcripts in the QC, enrichment of auxin-associated transcripts within the entire niche, controls for the state of differentiation of QC cells, a role for cytokinins specifically in the PM portion of the niche, processes (repair machinery) for maintaining DNA integrity and a role for gene silencing in niche stabilization. To provide additional support for the hypothesized roles of the above-mentioned and other transcripts in niche specifica-

tion, we overexpressed, in Arabidopsis, homologs of representative genes (eight) identified as highly enriched or reduced in the maize root QC. We conclude that the coordinated changes in expression of auxin-, redox-, cell cycle- and metabolism-associated genes suggest the linkage of gene networks at the level of transcription, thereby providing additional insights into events likely associated with root stem cell niche establishment and maintenance.

Keywords Quiescent center · Root · Stem cell · Stem cell niche · *Zea mays*

Abbreviations

| | |
|-------|--|
| AA | Ascorbic acid |
| AAO | ASCORBATE OXIDASE |
| ARF | AUXIN RESPONSE FACTOR |
| AGO4 | Argonaute-related gene 4 |
| AHL1 | AT-hook motif nuclear localized protein 1 |
| ATH | AT-hook protein gene |
| APX | ASCORBATE PEROXIDASE |
| ARR | ARABIDOPSIS RESPONSE REGULATOR (ARR) |
| AUX1 | Auxin influx transporter |
| CAF-1 | CHROMATIN ASSEMBLY FACTOR-1 |
| CDKA | A-type cyclin-dependent kinase |
| CKS1 | CYCLIN-DEPENDENT KINASE REGULATORY SUBUNIT |
| CYCD | D-type cyclin |
| CYCC | C-type cyclin |
| CYCL | L-type cyclin |
| DHAR | DEHYDROASCORBATE REDUCTASE |
| EZ | ELONGATION ZONE |
| FAS2 | FASCIATA 2 |
| GAPDH | GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE |

Electronic supplementary material The online version of this article (doi:10.1007/s00425-009-1059-3) contains supplementary material, which is available to authorized users.

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| | |
|-----------------|---|
| <i>GATA2</i> | GATA-type zinc finger protein 2 |
| <i>GCS</i> | γ -GLUTAMYL-CYSTEINE SYNTHETASE |
| <i>GLDH</i> | <i>L-GALACTONO-1, 4-LACTONE DEHYDROGENASE</i> |
| <i>GR</i> | <i>GLUTATHIONE REDUCTASE</i> |
| <i>GS</i> | <i>GLUTATHIONE SYNTHASE</i> |
| <i>GSH</i> | Glutathione |
| <i>KRP</i> | <i>KIP-RELATED PROTEIN</i> |
| <i>MDAR</i> | <i>MONODEHYDROASCORBATE REDUCTASE</i> |
| <i>PIN</i> | <i>PIN-FORMED</i> |
| <i>PLT</i> | <i>PLETHORA</i> |
| <i>PM</i> | Proximal meristem |
| <i>QC</i> | Quiescent center |
| <i>Rb</i> | <i>RETINOBLASTOMA</i> |
| <i>RbR</i> | <i>RETINOBLASTOMA RELATED</i> |
| <i>RC</i> | Root cap |
| <i>RHC1a</i> | <i>RINGH-2 group C protein 1a</i> |
| <i>SCR</i> | <i>SCARECROW</i> |
| <i>SCN</i> | Stem cell niche |
| <i>TF</i> | Transcription factor |
| <i>V-ATPASE</i> | Vacuolar ATP synthase |
| <i>WUS</i> | <i>WUSCHEL</i> |
| <i>Zm</i> | <i>Zea mays</i> |

Introduction

Plants maintain special microenvironments (stem cell niches [SCN]) at both the root and the shoot tips (apical meristems). As a consequence of their location within the niche, cells may display varying degrees of “stemness”, including variations in their ability to divide (or not), as well as in their states of differentiation and capacity for self-renewal (Cai J et al. 2004). Based on these features, the root SCN can be divided into two stem cell subpopulations; one characterized by cells with long cell cycles (120–200 h), and a second population characterized by more rapidly dividing cells that may also show varying degrees of differentiation. The former population comprises the quiescent center (QC) (Clowes 1961), whereas the latter, more rapidly cycling cells, populate the proximal meristem (PM). In roots of the model plant *Arabidopsis*, the mitotically active initials of the PM overlay the less active QC cells, which number 4–7 (Campilho et al. 2006; Xu et al. 2006). The root SCN in *Arabidopsis* can be contrasted with that of larger roots, such as those of corn (maize), in which the QC is composed of many more cells, perhaps 500–1,000, which show a gradation in cell activities and states of differentiation as one moves basally toward and the PM/QC border.

In addition to information on the state of differentiation of the cells populating the root SCN, and on their capacity for apparently unlimited proliferation, self-maintenance

and self-renewal (Clowes 1961), it has recently been discovered that signals move between the two sets of initials (van den Berg et al. 1995; Sarkar et al. 2007), though the identities of the signals, which may include the SHR protein (Nakajima and Benfey 2002), are not known. The expression of *WOX5*, a member of the *WUSCHEL*-related Homeobox gene family, suggests also the possible involvement of CLAVATA-like signaling (Schoof et al. 2000). The demonstration that the plant growth regulator auxin plays a central role in niche specification (Xu et al. 2006; Sena et al. 2009) points to possible physiological events underlying niche development and maintenance. Indeed, the involvement of auxin in SCN specification likely begins early in embryogenesis (Friml et al. 2003) and is most probably mediated by a number of auxin-regulated transcription factors (TF), including members of the PLETHORA protein family and the auxin response factor, MONOPTEROS (Hardtke and Berleth 1998; Sabatini et al. 1999). Evidence also points to an involvement of members of both the PIN and RETINOBLASTOMA protein families as having inputs in the designation and/or maintenance of the SCN (Aida et al. 2002; Wildwater et al. 2005; Galinha et al. 2007).

Building on what has already been established or suggested about the nature of the root SCN (Vernoux and Benfey 2005; Iyer-Pascuzzi and Benfey 2008; Sena et al. 2009), our goal in this report was to elucidate additional characteristics of plant stem cells, with particular attention directed to heretofore unreported characteristics of the stem cells that form the QC and the PM in roots of maize (Feldman and Torrey 1976). We begin this report with a consideration of the global patterns of gene expression in the maize QC and PM. These data were generated as part of a microarray data set previously used for profiling the maize root cap (RC) (Jiang et al. 2006), but these data have not been previously published. We group these QC/PM genes into categories based on putative function and then select for further analysis by real-time quantitative PCR (qPCR) specific genes hypothesized by us or others, (Blau et al. 2001; Baharvand et al. 2006) to affect “stemness”.

Central to this work is an assessment of the likelihood of a linkage between the expression levels of these genes with the specification, elaboration and maintenance of the SCN. For this effort, we reasoned that if we could cause QC cells to lose their typical stem cell characteristics (i.e., stereotypically undifferentiated, with cells accumulating at the G1/S checkpoint of the cell cycle), we may also lose or at least change that gene expression characteristic of cells occupying this portion of the niche. Toward altering the status of the SCN, we surgically excised the RC (decapping) (Fig. 1a, b), which stimulates (activates) QC cells to transit the G1/S checkpoint, to divide relatively rapidly and to begin differentiating into new RC cells (Feldman 1975).

Eventually, the QC reforms and QC/PM stem cells return to their pre-decapping status. Using this technique, we identified genes, the activities of which changed (increased/decreased) coincident with an alteration in SCN size and activity. To investigate further whether specific genes with changed activities could be associated with the development/maintenance of the root SCN, we overexpressed the homologs of maize candidate genes in *Arabidopsis*. We consider results from both maize and *Arabidopsis* in terms of providing insights into the molecular and physiological basis underlying the development and maintenance of the root SCN. Additionally, we consider whether these data point to a “signature” gene expression pattern for plant (root) stem cells.

Materials and methods

Isolation of root tissues

Zea mays cv. Merit seeds were imbibed for 2 h in rapidly running water and germinated in the dark at 24°C for 48 h. For QC activation experiments, RCs were excised and QC and PM tissues either collected immediately (time 0) (as described in detail in Feldman and Torrey 1976) (Fig. 1a–c), or roots were returned to the dark at 24°C for varying periods of time (6, 20 or 32 h), and then both the QC and PM collected. As tissues were excised, they were immediately frozen in liquid N₂, pooled, and stored at –80°C until the RNA was extracted (Jiang et al. 2006). For time course experiments, root tissues (100–160 QCs or 20–25 PMs)

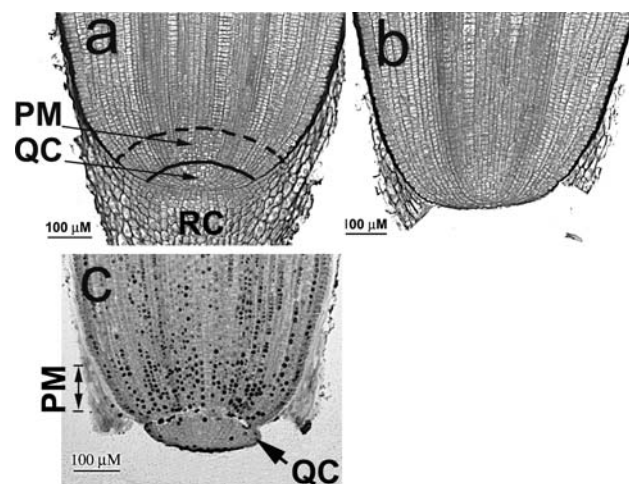


Fig. 1 Maize root apical meristems as seen in longitudinal section. **a** Intact primary root tip with the quiescent center (QC), proximal meristem (PM) and root cap (RC) (still attached). **b** A root from which the root cap has been excised leaving the QC intact. **c** Autoradiograph showing the excised (QC) and adjacent (PM)

were collected into 500 μl of RNAwiz (Ambion, Austin, TX), and stored at –80°C until use.

Microarray data analysis

Using the rice GeneChip (Zhu et al. 2003), representing 23,779 genes [approximately half of the rice genome (Benetzen et al. 2004)], we previously obtained gene expression profiles for the RC, QC and PM of maize (but to date have published only the data on RC) (Jiang et al. 2006). Previous comparative sequence analysis showed that more than 93% of full-length, nonredundant, complete coding sequences (696 in total) of maize were highly conserved with the rice genes ($E \leq -25$) (Goff et al. 2002). Zhu et al. (2003) have shown that over 90% of the maize transcripts cross-hybridize with the probes on the rice GeneChip. QC and PM microarray data were analyzed using the ANOVA one-factor F-test, followed by Fisher’s LSD and PCA. A total of 316 genes were found to be statistically enriched/reduced in their expression in the QC. Specifics for the RNA isolation, cDNA preparation and hybridization and the data transformation and statistics analysis are as described by Jiang et al. (2006). Of the 316 genes differentially expressed in the QC, 125 were reduced and 191 enriched. However, because of the cross-species nature of this methodology, we have taken a conservative approach and further filtered (using a 1.2-fold change) these two sets of genes, resulting in a reduction in the number of down- or enriched-QC genes to 70 (Supplemental Table 1) and 119 (Supplemental Table 2), respectively. Using a 1.2-fold change allowed us to capture approximately 59.8% (189) of the statistically differentially expressed QC genes, of which ten were later analyzed in more detail, and their expression levels confirmed using real-time quantitative RT-PCR (RT-qPCR) with primers selected from maize (Supplemental Table 3).

To identify the putative maize orthologs, we first searched for rice locus IDs using rice GeneChip probe region sequences in the TIGR database. Then, using the rice locus sequences, we identified the maize orthologs using the Inparanoid algorithm (O’Brien et al. 2005) (Supplemental Table 1 and 2). In addition, we also used the GeneChip rice probe region sequences for blast searches employing various maize ESTs databases (<http://tigrblast.tigr.org/tgi>, <http://www.ncbi.nlm.nih.gov/blast>), which provided either the tentative consensus (TC) number or GenBank access number for the best matching maize sequence. These identifying numbers are listed in Supplemental Tables 1 and 2. To validate the expression data, the top matching TC sequences were re-assessed, and the TC sequence(s) that contained the EST from the root tissue cDNA library was used for PCR primer design.

Real-time quantitative RT-PCR

For real-time quantitative RT-PCR, total RNA was extracted from either 400–600 RCs, 800–1,000 QCs, 300–400 PMs or 100–150 elongation zones (EZ) (a 1 mm-long segment 10 mm from the root tip), using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For each RNA sample, a 1.2-kb kanamycin RNA control was added, as previously described by Jiang et al. (2006), and then 2 μ l of this mixture was used for the cDNA synthesis.

For time course quantitative RT-PCR, RNA was extracted using 500 μ l of RNAwiz (Ambion, Austin, TX); 100 μ l of chloroform was added and the mixture shaken vigorously for 20 s. The upper aqueous phase was transferred to a new tube and 250 μ l of DEPC-treated H₂O was added. After adding 500 μ l of isopropanol, the RNA was placed at -20°C for 30 min, collected by centrifuging at 10,000 rpm for 15 min at 4°C and then solubilized in 10 μ l of DEPC-treated H₂O. At 37°C for 30 min, 4 μ g of the RNA was digested with 2 μ l of RQ1 RNase-free DNase (Promega, Madison, WI) in a 20 μ l reaction volume. The DNase was then inactivated by adding 2 μ l of RQ1 stop solution and incubated at 65°C for 10 min. After DNase digestion, 100 μ l of DEPC-treated H₂O was added to the RNA, and the concentration was measured at 260 nm. For each sample, an equal amount of RNA was used and sufficient DEPC H₂O was added to attain the same final concentration, and then 65 μ l of an approximately 10^{-7} -fold dilution of the kanamycin 1.2-kb control RNA was added to each sample as an internal standard for normalization. The RNA sample was then loaded onto a Microcon-100 column and spun 8 min at 2,500 rpm. After washing with 200 μ l of DEPC H₂O, the RNA was eluted from the column by adding 15 μ l of DEPC H₂O to the column, waiting for 5 min, and then the column was spun and inverted for 3 min at 1000 rpm. As much as 8 μ l of RNA was used for cDNA synthesis.

The cDNA synthesis and qPCR were performed as described by Jiang et al. (2006). Target cDNAs were normalized to the kanamycin 1.2-kb control. For comparisons between tissues, the differences in transcript levels were further expressed as a ratio after setting the PM value as 1. The results are presented as the means of at least two biological replicates and are listed in Supplemental Tables 3 and 4.

To determine whether there were coherent, dominant expression patterns in either the QC or PM transcripts, we performed a tight clustering analysis of the qPCR data using the software *tightClust* (Tseng and Wong 2005). Tight clustering is a re-sampling-based approach for identifying the most informative, tight and stable clusters from the data. As it does not force all genes into clusters, it helps

avoid the contamination of tight expression patterns of biologically relevant genes from other genes with only loosely compatible patterns. In our study, we slightly modified the tight clustering algorithm by using a more reliable and sensitive gene co-expression measure *PoissonC* (Cai L et al. 2004; Kim et al. 2007) to generate the results.

Primers used for this work are listed in Supplemental Table 5. The identity of the PCR product was confirmed either by sequencing or by sizing on an agarose gel.

Transgenic *Arabidopsis* lines

Eight genes (*AHL1* [At4g12080], *GATA2* [At2g45050], *RHC1a* [At2g40830], At1g75710, At1g76510, At2g34710, At2g41310 and At3g58120), all encoding TFs, were selected for overexpression in *Arabidopsis* Col-0 under the 35S promoter. These putative maize homologs were selected based on the similarity of their protein sequences and their expression patterns in the root tissue. Briefly, the maize gene coding sequences were queried against the *Arabidopsis* database (<http://www.arabidopsis.org/>) employing the BLASTX program, and the top three matching genes were screened by comparing their expression patterns in the root tissue [using, <http://bbc.botany.utoronto.ca/efp/> (Birnbaum et al. 2003; Nawy et al. 2005), (Supplemental Table 1&2)]. Those genes with QC expression pattern in *Arabidopsis* closest to the pattern in maize were selected.

For the 35S::GATA2 construct, the entire coding sequence of At2g45050 was amplified from a full-length cDNA clone (accession NO. BT000921) and cloned into the pCAMBIA1304 (Cambia, Canberra) at the Nco I/BstE II site, downstream from the CaMV 35S promoter, replacing the mGFP5-GUS gene. The original pCAMBIA1304 was used as a control. Transgenic plants were generated by transforming Col-0 wild type (Clough and Bent 1998).

The *AHL1*, *RHC1a*, At2g34710 and At3g58120 overexpression lines are the gifts from Mendel Biotechnology Inc., Hayward, CA. Briefly, for the 35S::AHL1 construct, At4g12080 cDNA was cloned into the Sal I/Not I site of pMEN65 downstream from the CaMV35S promoter. For construction of the 35S::RHC1a, At2g40830 genomic DNA was introduced into pMEN65 downstream from the CaMV35S promoter using a Gateway genomic clone. Control lines were produced by transforming with empty pMEN65.

For the At1g76510, At1g75710 and At2g41310 overexpression lines, the CaMV 35S promoter was inserted into EcorV/SacI sites of pGII0179 and the entire coding sequences from each gene were cloned into the downstream CaMV 35S promoter. The coding sequences of At1g76510, At1g75710 and At2g41310 were amplified from cDNA that was reverse transcribed from mRNA of Col-0 wild type using RT-PCR. Subsequently, the coding sequences of

At1g76510 and At2g41310 were inserted between the XbaI/SacI sites, and the coding sequence of At1g75710 was inserted between XbaI/BamHI sites.

Overexpression for *GATA2*, *AHL1*, *RHC1a* transgenic lines was confirmed on individual plants by qPCR (Supplemental Table 6). To characterize root phenotypes, ten individual lines from each transgenic line were screened against the empty vector transgenic lines. Three lines with the short root phenotype were then selected for further analysis. T2 plants were used from either the 35S::GATA2 or 35S::RHC1a lines. T3 plants were used for 35S::AHL1 lines.

T-DNA insertion lines for At2g45050 (Salk_008743, Salk_008794 and Salk_008845), At4g12080 (Salk_024324, Salk_026823, Salk_052728 and Salk_086713, Salk_092100) and At2g40830 (Salk_127503, Salk_143110, Salk_137684, Salk_037121) were obtained from the ABRC. Lines showing the short root phenotype were genotyped by PCR using the primers listed in Supplemental Table 5.

Phenotype analysis

Arabidopsis transgenic seeds were germinated in a 1× Murashige–Skooog medium (minus sucrose) under long-day conditions (16-h light/8-h dark) at 22°C, and at an age of 2 weeks, fixed in 1 N HCl, followed by staining with Acriflavin neutral according to Ruzin (1999) and then examined by confocal microscopy.

GenBank accession numbers

See Supplemental Table 5.

Results

Global gene expression patterns in the maize root SCN

There is little difference in the number of genes expressed among the three root tissues (RC, QC, PM), irrespective of the cut-off level (data not shown). No genes were found expressed exclusively in the QC, nor did we detect any genes lacking expression in either the QC or PM. These results have been confirmed using RT-qPCR, as noted subsequently, and point to the transcriptionally active status of the QC, even though chromatin in these cells is reported to be highly condensed (Clowes 1961). Using the microarray data set (Jiang et al. 2006), we identified 316 genes with specific changed expression in the QC, and 176 with changed expression in the PM. Of the 316 genes differentially expressed in the QC, the expression levels of 125 were reduced and of 191 enriched. Further filtering of these two sets of genes using a 1.2-fold change cut-off (genes

with expression either 1.2-fold greater or lesser in the QC, as compared to both the PM and RC) reduced the number of genes with changed expression (either reduced or enriched) in the QC to 70 (Supplemental Table 1) and 119 (Supplemental Table 2), respectively.

Next, we consider the specifics of the genes in the QC showing changed expression, drawing particular attention to both differences and similarities with *Arabidopsis*, the only other root system for which the root SCN has been profiled (Nawy et al. 2005). These results form the basis of the subsequent experimental manipulations and activation of the SCN.

Major themes shown by genes with reduced levels of expression

Of the transcripts showing reduced expression, we note that the largest number (20 of 70) are from genes encoding enzymes that function in various metabolic pathways, including proteins involved in sugar metabolism/the energy production pathway (glycolysis) (Supplemental Table 1). As well, genes encoding proteins for early steps of secondary metabolism pathways are also reduced (Supplemental Table 1). Of particular interest, and not reported for *Arabidopsis*, is the detection of eight TFs with lower expression levels, of which five encode GATA types of zinc finger proteins (Supplemental Table 1).

Major themes shown by genes with enriched levels of expression

Significantly enriched transcripts in the QC, and not reported for *Arabidopsis*, include several encoding proteins influencing membrane lipid composition, and vesicle-trafficking-associated activities (Supplemental Table 2). Proteins encoded by these genes are particularly important because they generate and regulate asymmetries, integrate multiple signals, coordinate diverse cellular pathways (Xu and Scheres 2005; Nibau et al. 2006; Teale et al. 2006; Cockcroft and Carvou 2007), affect auxin-mediated AUX/IAA proteolysis (Scherer et al. 2007), alter the auxin response (Bloch et al. 2005) and may affect mitosis and/or the cell cycle (McElver et al. 2000). We also report that six genes associated with DNA replication/repair/chromatin modifications are enriched in the QC (Supplemental Table 2), including a homolog (OS017994.1) of *Arabidopsis* *FAS2*, which is a subunit of the *CHROMATIN ASSEMBLY FACTOR-1* [*CAF-1*] complex (Supplemental Table 2). *CAF-1* is associated with the ability of stem cells to maintain genomic integrity (stemness) (Ridgway and Almouzni 2000; Kaya et al. 2001; Park and Gerson 2005; Houlard et al. 2006; Nabatiyan et al. 2006; Nijnik et al. 2007). In *fas2* mutants, the aberrant phenotype is associated with a

misexpression of *WUSCHEL* (*WUS*) in the shoot and of *SCARECROW* (*SCR*) in root meristems (Kaya et al. 2001).

From this global survey, we conclude that differences between the PM and QC are quantitative (enriched or reduced) and not qualitative (transcripts for all genes are detected), and that the QC is relatively active at the level of gene expression, with much of this activity directed at maintaining underlying metabolic machinery and the integrity of the DNA, thereby keeping the system primed to transit the G1/S checkpoint on receipt of the appropriate signal/stimulus. Nevertheless, unlike what has been reported for *Arabidopsis* (Nawy et al. 2005), we detect a reduction in the expression of a significant number of genes, many of which encode TFs.

Exploring the activities of genes hypothesized to have a role in the stem cell niche

Based on the fact that the SCN consists of populations with transcription profiles that appear to be quantitatively but not qualitatively different, we next employed qPCR, allowing greater resolution of these differences. For this effort, we selected 35 genes (10 from Supplemental Tables 1, 2) hypothesized by us, or others, as having a role in SCN establishment and/or maintenance. We compared and contrasted their activities in the QC and PM to the activities of these same genes in the root cap (RC) and elongation zone (EZ), both in the intact root and in roots in which the QC had been activated by decapping (monitored at time points 6, 20 and 32 h after decapping). Here, we report and emphasize the most significant results (Tables 1, 2, 3, 4, 5 and Figs. 2, 3, 4, 5, 6).

Regulators of transcription

The expression of a GATA-type zinc finger transcription factor (*ZmGATA*) and the A-type response regulator (*ZmRR*) are both reduced in the QC, compared to their status in the PM and RC (Table 1, Supplemental Table 3), whereas the expression of an Argonaute-related gene (*ZmAGO4*) and an AT-hook motif gene (*ZmATH*) are both enriched in the QC. With niche activation, *ZmGATA* activity increases threefold in the QC and nearly sixfold in the PM (Fig. 2 and Supplemental Table 4).

Table 1 Regulators of transcription in the maize root tip

| Gene | RC | QC | PM | EZ |
|---------------|------|------|----|------|
| <i>ZmAGO4</i> | 0.42 | 1.47 | 1 | 0.08 |
| <i>ZmRR</i> | 0.71 | 0.18 | 1 | 0.63 |
| <i>ZmATH</i> | 1.28 | 1.35 | 1 | 0.15 |
| <i>ZmGATA</i> | 1.84 | 0.37 | 1 | 0.06 |

Table 2 Auxin-associated gene expression in the maize root tip

| Gene | RC | QC | PM | EZ |
|--------------------|------|------|----|------|
| <i>ZmARF2</i> | 4.20 | 1.73 | 1 | 2.38 |
| <i>ZmARF5/MP</i> | 0.24 | 2.32 | 1 | 0.02 |
| <i>ZmARF6</i> | 1.24 | 1.34 | 1 | 0.19 |
| <i>ZmAFR7a</i> | 0.96 | 2.34 | 1 | 0.35 |
| <i>ZmARF7b</i> | 2.57 | 0.80 | 1 | 2.68 |
| <i>ZmARF7_NHP4</i> | 21.8 | 0.94 | 1 | 0.64 |
| <i>ZmARF8</i> | 3.34 | 1.32 | 1 | 0.42 |
| <i>ZmARF10</i> | 5.35 | 0.43 | 1 | 0.23 |
| <i>ZmAUX1</i> | 2.14 | 2.94 | 1 | 0.10 |
| <i>ZmPIN1a</i> | 2.00 | 0.58 | 1 | 0.23 |
| <i>ZmPIN1b</i> | 3.04 | 1.92 | 1 | 0.48 |
| <i>ZmPIN2</i> | 2.72 | 0.32 | 1 | 0.08 |
| <i>ZmPLT</i> | 0.83 | 3.8 | 1 | 0.02 |

Table 3 Cell cycle-associated gene expression in the maize root tip

| Gene | RC | QC | PM | EZ |
|------------------|------|------|----|------|
| <i>ZmCYCC</i> | 1.11 | 0.82 | 1 | 0.38 |
| <i>ZmCYCD2;1</i> | 1.63 | 0.63 | 1 | 0.21 |
| <i>ZmCYCL1;1</i> | 3.12 | 0.92 | 1 | 0.42 |
| <i>ZmCDKA</i> | 0.65 | 0.49 | 1 | 0.40 |
| <i>ZmKRP4</i> | 1.24 | 1.64 | 1 | 0.12 |
| <i>ZmRBR2;1</i> | 3.25 | 0.63 | 1 | 0.91 |
| <i>ZmCKS1</i> | 0.42 | 1.47 | 1 | 0.14 |

Table 4 Redox-associated gene expression in the maize root tip

| Gene | RC | QC | PM | EZ |
|----------------|------|------|----|----------------|
| <i>ZmGR</i> | 2.11 | 0.49 | 1 | 1.27 |
| <i>ZmMDAR</i> | 2.86 | 0.45 | 1 | 1.84 |
| <i>ZmDHAR</i> | 3.11 | 0.79 | 1 | 1.35 |
| <i>Zmγ-GCS</i> | 0.43 | 0.33 | 1 | – ^a |
| <i>ZmGS</i> | 1.43 | 0.69 | 1 | 0.59 |
| <i>ZmGLDH</i> | 0.95 | 0.78 | 1 | 0.21 |
| <i>ZmAPX</i> | 1.53 | 0.91 | 1 | – ^a |
| <i>ZmAAO</i> | 0.96 | 1.7 | 1 | 6.8 |

^a Not measured by qRT-PCR

Auxin related

We quantified the spatial expression of three genes in the auxin efflux carrier family (*ZmPINs*), one gene in the auxin influx carrier family (*ZmAUX1s*), eight genes in the *AUXIN RESPONSE FACTOR* (*ZmARFs*) family and one gene in the *PLETHORA* (*ZmPLTs*) family. Genes with increased expression in the QC, and thus overlapping in their spatial

Table 5 Metabolism-related gene expression in the maize root tip

| Gene | RC | QC | PM | EZ |
|-------------------|------|------|----|------|
| <i>ZmGAPDH</i> | 3.09 | 0.33 | 1 | 0.31 |
| <i>ZmV-ATPAPE</i> | 2.23 | 0.46 | 1 | 1.18 |

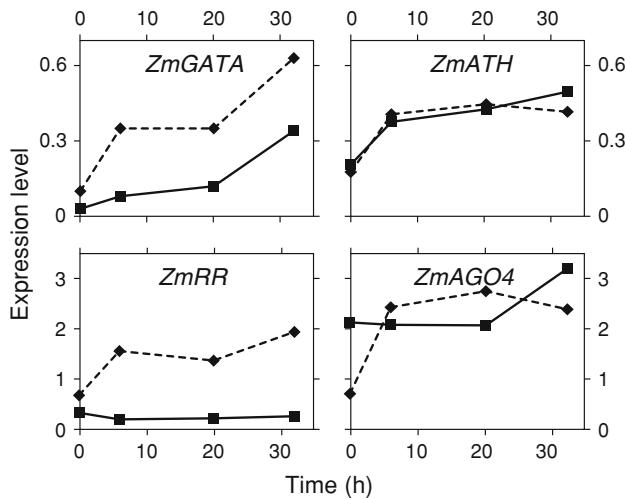


Fig. 2 Relative levels of transcription factors in the QC (filled squares on continuous line) and PM (filled diamonds on broken line) at various times following decapping (activation of the stem cell niche). Values from Supplemental Table 4

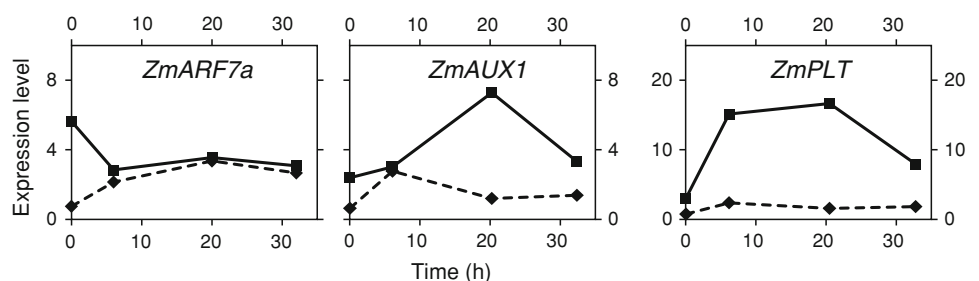
expression patterns, include *ZmARF5/MP*, *ZmARF7a*, *ZmAUX1* and *ZmPLT* (Table 2, Supplemental Table 3). This is the first report that quantitatively measures, localizes and compares the levels of these important auxin-associated transcripts in the root SCN (QC and PM).

Activation of the QC by decapping resulted in a substantial increase in the expression in both the QC (4–8-fold) and PM (2–4-fold) of two auxin-regulated genes (*ZmAUX1* and *ZmPLT*), whereas the expression of a third gene, *ZmARF7a*, is reduced in the QC portion of the niche, but increased substantially in the PM portion (Fig. 3 and Supplemental Table 4).

Cell cycle regulators

We quantified seven genes, the level of transcription of which is often associated with regulation of the cell cycle

Fig. 3 Relative levels of auxin-associated transcripts in the QC (filled squares on continuous line) and PM (filled diamonds on broken line) at various times following decapping (activation of the SCN). Values from Supplemental Table 4



(Table 3, Supplemental Table 3). In general, the levels of these transcripts are lower in the QC compared to the PM and RC, but with the two prominent exceptions: *CYCLIN-DEPENDENT KINASE REGULATORY SUBUNIT (ZmCKS1)* and cyclin-dependent kinase inhibitor *KIP-RELATED PROTEIN (ZmKRP4)* genes, both of which show enriched expression in the QC. Significantly, the transcript levels of both of these genes in the non-dividing cells of the EZ are at levels tenfold less than in the QC (Table 3). In this regard, transcripts of a *RETINOBLASTOMA (Rb)* homolog (*ZmRBR2;1*) (Rymen et al. 2007) can be detected in the QC, PM and RC (Table 3, Supplemental Table 3), and its level is significantly greater in the RC (5-fold> than in the QC, and 3-fold>than in the PM) (Table 3).

Activation of the QC resulted in an equalizing of the transcript levels in the PM and the QC, so that by 32 h after decapping transcript levels for each gene (D-type cyclin [*ZmCYCD2;1*], *ZmRBR2;1*, and an A-type cyclin-dependent kinase [*ZmCDKA*]) changed from a ratio of approximately 2:1 (PM:QC) to nearly 1:1, mostly as a consequence of an increased expression in the QC (Fig. 4 and Supplemental Table 4). Changes in expression of *ZmRBR2;1* are especially noteworthy (3-fold increase in the QC) (Fig. 4).

Redox related (ascorbate–glutathione cycle)

We observed reduced expression in both the QC and, with some exceptions, in the PM of the suite of genes encoding redox-regulating enzymes (Table 4, Supplemental Table 3). *Zmγ-GCS* and *ZmGS*, which encode proteins associated with the second to the last, and the last step, respectively, in the synthesis of glutathione, are expressed two to threefold higher in the PM than in other root tissues.

Decapping leads to an increase in the expression of six genes encoding proteins central to regulating redox status (glutathione reductase [*ZmGR*], glutathione synthetase [*ZmGSH*], γ -glutamylcysteine synthetase [*Zmγ-GCS*], monodehydroascorbate reductase [*ZmMDAR*], ascorbate oxidase [*ZmAAO*] and dehydroascorbate reductase [*ZmDHAR*]) (Fig. 5 and Supplemental Table 4). For both the QC and PM, the expression of these genes peaks 6–20 h after decapping, with levels, in general, at 32 h (the last

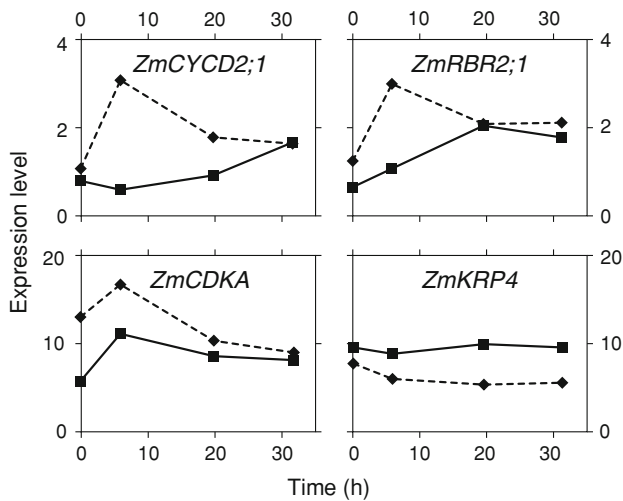


Fig. 4 Relative levels of cell cycle-related transcripts in the QC (filled squares on continuous line) and PM (filled diamonds on broken line) at various times following decapping (activation of the SCN). Values from Supplemental Table 4

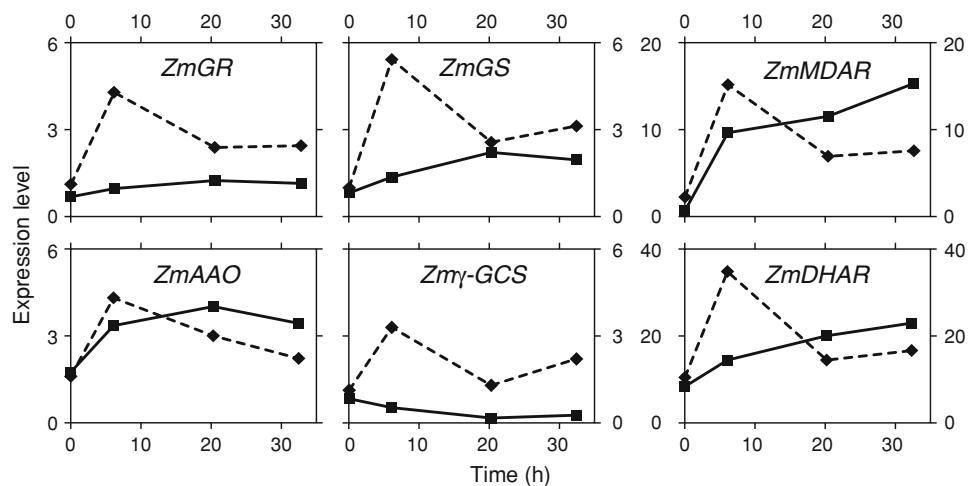
time point) 2–22-fold higher than at time 0, immediately after RC removal.

Energy/metabolism related

The expression of two metabolism-/energy-associated transcripts encoding glyceraldehyde-3-phosphate dehydrogenase (*ZmGAPDH*) and a vacuolar ATP synthase (*ZmV-ATPASE*) is reduced in the QC (5–9-fold less) and in the PM (2–3-fold less), in comparison to the adjacent RC (Table 5 and Supplemental Table 3). Compared to the QC, cells of the EZ have similar levels of expression of *ZmGAPDH*, but more than twofold the level of expression of the *ZmV-ATPASE*.

Following decapping, the expression of *ZmV-ATPASE* and *ZmGAPDH* increases markedly (5–10-fold) with QC activation (Fig. 6 and Supplemental Table 4).

Fig. 5 Relative levels of redox-associated transcripts in the QC (filled squares on continuous line) and PM (filled diamonds on broken line) at various times following decapping (activation of the SCN). Values from Supplemental Table 4



Tight clustering results

By performing the tight clustering analysis on the pooled qPCR data of the 19 selected genes from PM and QC (30 expression profiles in total), we obtained one tight cluster consisting of 12 expression profiles (Supplemental Fig. 1). This is also the only significant tight cluster we found in the data. In this cluster, the identified 12 expression profiles, showing a very strong coherent expression pattern, correspond to 12 different genes in the PM.

Overexpression in *Arabidopsis* of *GATA2*, *AHL1*, *RHC1a* and other TF genes

Because of a central role for TFs in linking transcriptional networks (Iyer-Pascuzzi and Benfey 2008), we selected for further analysis four TF genes showing reduced expression, and four TF genes showing enriched expression in the maize QC (each from different TF gene families), and overexpressed their sequence homologs in *Arabidopsis* (Supplemental Table 1 and 2). Overexpression in *Arabidopsis* of the *GATA2* (At2g45050), *AHL1* (At4g12080) (AT-hook protein) and *RHC1a* (At2g40830) (RING-H2) TF genes under the control of the 35S-promoter (overexpression confirmed by qPCR, Supplemental Table 6) resulted in alterations ranging from subtle to profound in the development of the root SCN and of the adjacent root cap (RC). Apical meristems of roots overexpressing the *GATA2* TF gene were disorganized with no distinct boundary between the RC and the root proper. Cap cells were often enlarged and their shapes distorted (Fig. 7b–d). In one overexpressing line, a new root meristem formed adjacent to the enlarged cap cells (Fig. 7d). Roots overexpressing the *AHL1* gene displayed the most variability in apical meristem architecture, varying in apical patterning from relatively typical to grossly abnormal (Fig. 7e–g). The region occupied by the QC often penetrated into the RC, destroying the RC initials,

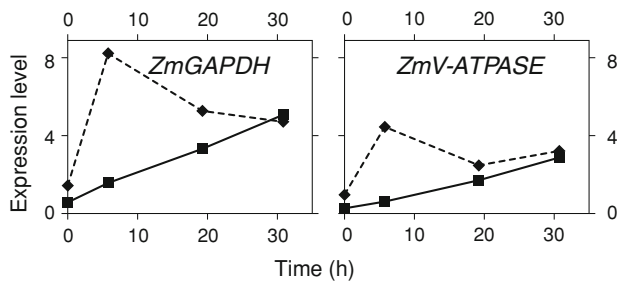


Fig. 6 Relative levels of metabolism-associated transcripts in the QC (filled squares on continuous line) and PM (filled diamonds on broken line) at various times following decapping (activation of the stem cell niche). Values from Supplemental Table 4

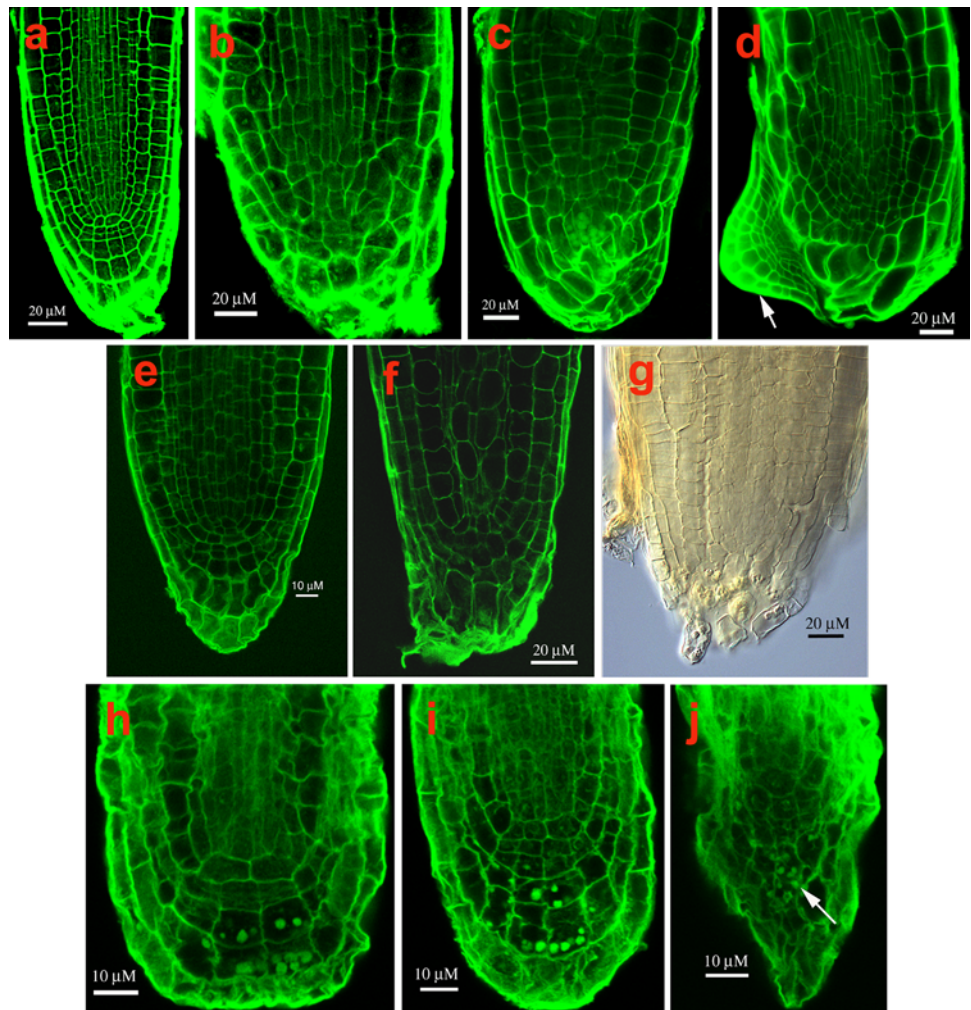
resulting in a decrease in size and often disappearance of the RC (Fig. 7f, g). In roots overexpressing *RHC1a*, apical architecture is also altered, first in the cells of the QC that become atypically enlarged, followed by changes in the shapes and activities of the RC initials, resulting in a highly disorganized RC and in the root becoming determinate (Fig. 7h–j). No root phenotypes were observed in the

T-DNA insertion lines for *GATA2* (Salk_008743, Salk_008794 and Salk_008845), for *AHL1* (Salk_024324, Salk_026823, Salk_052728, and Salk_086713, Salk_092100), and for *RHC1a* (Salk_127503, Salk_143110, Salk_137684, Salk_037121), suggesting redundancy of gene function.

Discussion

The objectives for this work are to understand the factors underlying SCN specification and elaboration in maize roots, and to determine whether a molecular “signature” can be discerned for the root SCN. Toward addressing these questions, we first developed a baseline for gene expression in the QC and PM using a global microarray-based approach. We found that differences in gene expression between the QC and PM were quantitative and not qualitative, and using qPCR we ascertained how gene activity changed (or did not) in both SCN populations following activation and perturbation of the niche. Our working

Fig. 7 Confocal microscope views of Arabidopsis root apical meristems from plants over-expressing various transcription factor genes (TF). **a** Wild type. **b–d** *GATA2* TF; arrow in **d** points to a displaced meristem. **e–g** *AHL1* TF; **h–j**, *RHC1a* TF; arrow in **j** points to amyloplasts



hypothesis is that genes essential to and/or linked to niche specification will have their activities altered when the niche is perturbed (Xu et al. 2006). Genes that we have confirmed, or have newly hypothesized as important in SCN formation and maintenance, fall into the broad categories discussed below.

A role for ascorbate/monodehydroascorbate/dehydroascorbate cycle genes in SCN specification and maintenance

One of the emerging themes in biology is that stem cells have attributes of cells under stress (Ramalho-Santos et al. 2002; Baharvand et al. 2006). In maize, as a consequence of a highly oxidized microenvironment at the root tip, the cells comprising the QC are under oxidative stress, a condition that has been linked to shifts in the ratios of the oxidized and reduced forms of GSH and AA in the QC, favoring an oxidized redox status (Jiang et al. 2003). Thus, for roots, as for other organisms [e.g., yeast, *C. elegans* (Essers et al. 2005)], a strong correlation exists between oxidative stress and the development and maintenance of the SCN. Evidence supporting a role for redox status in niche establishment/maintenance comes from work on the *Arabidopsis* mutant, *ROOTMERISTEMLESS*, characterized by a defect in GSH synthesis (Vernoux et al. 2000), resulting in disorganization of the root meristem shortly after germination. This phenotype can be rescued by supplying GSH. Similarly, our earlier work showing niche disorganization following decapping is interpreted as occurring as a consequence of the changes in the redox status in both the QC and PM (Jiang et al. 2003). Here, we show that the oxidized status in the QC is, in part, paralleled by the relatively low levels in the QC of transcripts encoding proteins that regulate the ascorbate/monodehydroascorbate/dehydroascorbate cycle, including ZmGR, ZmMDAR, ZmDHA and other associated enzymes (Table 4). Based on the kinetics and the magnitude of transcript changes following decapping (Fig. 5), we would hypothesize that the development of the less oxidizing environment is mainly a consequence of an increase in the activity (3–30-fold) of the ascorbate/monodehydroascorbate/dehydroascorbate cycle and to a lesser degree due to the synthesis of more glutathione (GSH), the biosynthesis of which in the activated QC is probably low, judging by the low levels of expression of transcripts encoding 2 key GSH-biosynthesis enzymes (γ -glutamyl-cysteine synthetase [γ -GCS] and glutathione synthetase [GS], Fig. 5).

Regulation of cell cycle genes' transcription is associated with SCN maintenance

Stereotypically, adult stem cells divide relatively slowly, often accumulating at cell cycle checkpoints (Robert 2004).

QC cells typify this pattern (Clowes 1961). The maintenance of these characteristics is essential for meristem organization (Willemsen et al. 1998; Riou-Khamlichi et al. 1999; Wildwater et al. 2005). In contrast, PM cells that occupy the basal portion of the SCN divide relatively more frequently, about once every 20–30 h. Toward an understanding of differences in the regulation of the cell cycle between the two stem cell populations, and other non-stem cells, we focused on specific transcripts associated with core cell cycle regulators, including transcripts encoding ZmCYCD2;1, ZmCYCL1;1, ZmCDKA, ZmKRP4, ZmCKS1 and ZmRBR2;1 (Table 3). Here, we provide the first spatial measurements of the levels of cell cycle-associated gene activities in the root SCN. Low *ZmRBR2;1* transcription in the non-activated maize QC accords well with reports linking a reduction of RBR function with the maintenance of the stem cell state in *Arabidopsis* (Wildwater et al. 2005). In maize, we show that there is at least a three-fold difference (increase) in *RBR* expression when the QC is activated (Fig. 4). Thus, for maize our results suggest the possibility that re-patterning of the root SCN is associated with an increase of *ZmRBR2;1* expression in the QC.

G1-to-S transition is also governed by an increased expression of cyclin D (Menges et al. 2005), which Wildwater et al. (2005) suggests influences RBR control of root stem cell pool size. Our results with maize roots support this possibility, since activation of the niche leads to differences (increases) in *ZmCYCD2;1* expression (Fig. 4), reinforcing the notion that maintenance of the stem cell cycle hinges on a balance of the CYCD/RBR pathway.

Although the levels of most cell cycle-associated transcripts are reduced in the QC relative to the RC and PM, we found enhanced expression of two transcripts encoding negative regulators of the cell cycle, *ZmCKS1* and *ZmKRP4* (De Veylder et al. 2001; Wang et al. 2006) (Table 3, Supplemental Table 3). *ZmCKS1* proteins associate with cyclin-dependent kinases (CDKs) and play an essential role in the regulation of the cell cycle. *ZmCKS1* is abundant in non-dividing cells, and its level is reduced during G1/S transition (Menges et al. 2005). Overexpression of *CKS1* in *Arabidopsis* causes a reduction in root growth rates, which is primarily a consequence of an increase in the duration of the cell cycle and a shortening of the meristem (De Veylder et al. 2001). The increased levels of expression of both of these genes in the QC may point to an underlying mechanisms contributing to the reduced rate of cell division in the QC.

Other regulators of transcription related to SCN establishment and maintenance

We identified several different types of transcription factors (TFs) that are enriched or reduced in the QC (Supplemental

Tables 1, 2). Because of the central role for TFs in linking gene networks, we selected for further study four genes representing different classes of regulators of transcription, including *ZmRR*, the homolog of *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes. Ioio et al. (2007) hypothesize that members of this family interact with cytokinins to control the rate of cell differentiation in the meristem by affecting the position of the transition zone (TZ) where cells pass from being meristematic to elongating and differentiating. Our data point to a role for *ZmRR* in the PM (site of the transition zone), but not in the QC. In support of this view, we note that for the one member of the *RR* gene family that we investigated in detail (OS009399, an *Arabidopsis* homolog of *ARR8/ATRR3*), the expression in the QC is exceedingly low and unchanged by decapping and activation (Table 1 and Fig. 2), whereas decapping caused a threefold increase in *ZmRR* expression in the PM (Fig. 2 and Supplemental Table 4). Thus, we suggest that development and maintenance of the QC portion of the SCN may involve a repression of interactions between cytokinins and members of the *RR* gene family.

A second gene studied in more detail is *ZmAGO4*, the homolog in *Arabidopsis* which has been linked to endogenous siRNA, suggesting the possibility of epigenetic silencing (Baumberger and Baulcombe 2005). In maize, we report that this homolog is expressed most highly in the QC portion of the SCN and is barely detectable in the EZ (Table 1). Previous work which suggests that *AtAGO4* and long siRNAs direct chromatin modifications, including histone methylation and non-CpG DNA methylation (Zilberman et al. 2003), may point to other important mechanisms underlying development and maintenance of the SCN. Because decapping results in an increase in *ZmAGO4* expression (Fig. 2 and Supplemental Table 4), this suggests that *AGO4*-mediated processes may be associated with specification of the niche.

Finally, to obtain additional evidence for a role for TFs and their associated networks in niche specification and/or maintenance, we focused on three additional TFs, an AT-hook protein and two Zn finger proteins, (a RING-H2 type and a GATA type). For each, we overexpressed their homologs (*AHL1*, *RHC1a* and *GATA2*) in *Arabidopsis*, which resulted in mild to severe alterations in root meristem architecture (Fig. 7a–j). Of the three TF types that we overexpressed, *GATAs* have received the most attention. Recent studies also point to an important role for *GATAs* in stem cell maintenance (Zhao et al. 2004; Liu et al. 2005; Manfield et al. 2007; Naito et al. 2007). *HAN*, which encodes a GATA-3-like protein in *Arabidopsis*, controls the number and position of cells expressing *WUS* and thereby affects cell proliferation and differentiation. As for *GATA2*, Manfield et al. (2007) report that it is strong transcriptionally and co-expresses with a significant number of genes with

roles in cell wall assembly, suggesting that *GATA2* could be a possible link to proliferation/differentiation processes.

Transcript profiles from the maize and *Arabidopsis* QCs show similarities, but also differences

In 2005, Nawy et al. reported the transcriptional profile for the *Arabidopsis* QC. Using a technical approach that involved protoplasting and sorting of GFP-expressing QC cells, obtained from many roots, they found enrichment in the QC of 290 genes, which they grouped into various functional categories (their Table I). Satisfyingly, we find similarities in the expression profiles of *Arabidopsis* (a eudicot with a small QC) to the QC of maize (a monocot with a much larger QC), including a number of transcripts associated with transcriptional regulators, DNA replication/repair and auxin. Two specific groupings not reported for the *Arabidopsis* QC, but detected in maize, include a relatively large number of enriched transcripts for mitochondrial/plastid genes, and with the exception of thioredoxin reductase (Nawy et al. 2005), transcripts associated with the redox status (Table 4 and Supplemental Table 2). Additionally, we report a large number of genes with significantly reduced expression in the maize QC (Supplemental Table 1) and which were not reported in the profile data for the *Arabidopsis* QC (Nawy et al. 2005), thereby providing additional insights into the possible molecular controls of the SCN.

Activation of the maize SCN provides additional support for a role for redox-, metabolism- and cell cycle-associated transcripts in SCN specification

Xu et al. (2006) perturbed the stem cell status in *Arabidopsis* by laser ablating one or more QC cells, resulting in a loss of expression of typical QC auxin-associated markers (e.g., *PLT1*, *WOX5*). Sena et al. (2009) have likewise explored genes in relation to niche specification during meristem root regeneration in *Arabidopsis*. They excised 130 μm of the root tip (which includes the QC and PM), and then followed gene expression in the 70 μm stump region distal to the excision. Of the gene families (auxin-, redox-, metabolism- and cell cycle-associated) for which we find increased expression in the reforming maize SCN, the only overlap with the early expressed genes in the reforming *Arabidopsis* SCN is for members of the *PLT* gene family (auxin-related genes).

Activation and re-establishment of the SCN reveals similarities and differences in transcript expression in the QC and PM

While the SCN activation experiments point to similarities in the underlying molecular machinery that may be important

in the PM and QC in defining stemness, the experiments also suggest differences between these two zones. These differences are most evident from a consideration of the kinetics of changes in gene expression patterns following decapping. Taken as a group, PM transcripts generally peak at 6 h and then decrease and stabilize 20–32 h after decapping, while the expression of QC transcripts does not show a coherent pattern. When we performed a cluster analysis for all the PM and QC transcripts, we found a high expression at the second time point and a low expression at the first time point for most PM transcripts in the tightest cluster. But no QC transcripts are found to have this tightest cluster expression pattern. From this analysis, we conclude that differences in the expression patterns/levels of genes in the QC versus PM may be important in defining distinct regions of the niche.

Conclusions

We conclude that there is no specific gene expression “signature”, which distinguishes one stem cell sub-population from the other. Further, we suggest that the pattern of increased expression of auxin-regulated genes, coupled with the reduced expression of metabolism-, redox- and some cell cycle-associated genes, comes closest to a root SCN “signature” and supports the notion that “stemness” represents a state rather than a quantifiable entity (Zipori 2004; Mikkers and Frisé 2005).

Our findings not only extend the recent work on *Arabidopsis* to the specification of the maize root SCN, but also, as importantly, we have been able to add significantly to the number of genes enriched and reduced in both the QC and PM. We thereby are able to suggest additional steps and processes possibly associated with the specification/maintenance of the root SCN, including: controls of the state of differentiation of QC cells, processes for maintaining DNA integrity (repair machinery), a possible role for cytokinins specifically in the PM portion of the niche, a role for gene silencing in niche stabilization and an underlying and central role for redox. Finally, with these new results, we demonstrate that plant and animal stem cells share an increased number of molecular characteristics, and in many ways, therefore, are more alike than they are different.

Acknowledgments We thank Dr. Peter Repetti of Mendel Biotechnology, Hayward, CA for kindly providing seeds of *Arabidopsis* over-expressing lines. We thank Dr. Shibo Zhang for assisting with the analysis of the microarray data.

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