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Quiescent center formation in maize roots is associated with an auxin-regulated oxidizing environment

Keni Jiang, Yu Ling Meng and Lewis J. Feldman*

Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720, USA
*Author for correspondence (e-mail: feldman@nature.berkeley.edu)

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SUMMARY

Embedded within the meristem of all Angiosperm roots is a population of slowly dividing cells designated the quiescent center (QC). In maize roots the QC can constitute upwards of 800-1200 cells, most of which spend an extended period of time (180-200 hours) in the G_1 phase of the cell cycle. How the QC forms and is maintained is not known. Here we report that cells of the QC are characterized by their highly oxidized status. Glutathione and ascorbic acid occur predominately in the oxidized forms in the QC. This is contrasted with the status of these redox intermediates in adjacent, rapidly dividing cells in the root meristem, in which the reduced forms of these two species are favored. Using a redox sensitive fluorescent dye we were able to visualize an overall oxidizing environment in the QC, and we also made comparisons with the adjacent, rapidly dividing cells in the root meristem. Altering the distribution of auxin and the location of the auxin maximum in the root tip activates the QC, and cells leave G_1 and enter mitosis. Commencement of relatively more rapid cell division in the QC is preceded by changes in the overall redox status of the QC, which becomes less oxidizing. We discuss how the position of the auxin maximum may influence the redox status of the QC and thereby modulate the cell cycle.

Key words: Auxin, Root, Quiescent center, Redox regulation, Maize

INTRODUCTION

Organization of root meristems is hypothesized to depend on the formation and maintenance of a population of slowly dividing cells collectively termed the quiescent center (QC) (Fig. 1A) (Kerk and Feldman, 1995). In maize roots, cells of the QC preferentially accumulate at G_1/S, and divide on average about every 180-200 hours, in contrast to the adjacent, more rapidly cycling cells of the proximal meristem (PM), which divide on average every 20 hours, and serve as the source of most new derivatives in a growing root (Fig. 1D) (Clowes, 1961). Depending on the species and the age of the individual root, the QC may vary in size from 4 cells in *Arabidopsis*, to 800-1200 cells in maize. In some cultivars of maize it is possible to dissect out QCs (Fig. 1B-D).

How a QC forms is not known, but it is suggested that it may arise as a consequence of polar auxin transport (Kerk and Feldman, 1995; Kerk et al., 2000). The availability of many *Arabidopsis* mutants perturbed in their auxin signaling, such as *mp*, *ett*, *aux1* and *pin1*, all point to a central role for auxin in the establishment and maintenance of organization in root meristems (Sabatini et al., 1999). The recent characterization of AtPIN4, a member of the PIN family of putative auxin efflux carriers, suggests a model in which QC establishment occurs because of the development of an auxin sink (Friml et al., 2002). According to this model, root meristems organize because of a 'sink-driven morphogenetic auxin gradient'. Thus, QC formation can be viewed as a consequence of a sink-dependent gradient in auxin. What is not certain, however, is the mechanism (intermediate steps) by which auxin specifies QC formation. In this regard, it has recently been proposed that ascorbic acid (AA) may serve as an intermediate linking auxin and QC establishment (Kerk and Feldman, 1995). Since this suggestion, a number of investigators have provided data supporting a role for AA and/or glutathione (GSH) in root meristem establishment. As in the case of ascorbate, which was shown to be at low levels in the QC of maize (Kerk and Feldman, 1995), measurements of glutathione in intact roots of *Arabidopsis* similarly showed reduced levels in the QC (Fricker et al., 2000; Sánchez-Fernández et al., 1997). Additional evidence for a role for GSH in root meristem organization comes from *Arabidopsis* mutated in the gene *ROOT MERISTEMLESS* (*RML1*), which encodes for the first enzyme of glutathione biosynthesis (Vernoux et al., 2000). Mutations in this gene lead to a reduction in adequate levels of GSH and plants are unable to form an active postembryonic root meristem. But these mutants can be rescued (an organized root meristem reforms) by providing seedlings with GSH (Vernoux et al., 2000). The mechanism(s) by which AA and/or GSH influence the development of root meristems is not certain, though attention has focused on the possible roles of these compounds as redox intermediates. The absence of detectable AA in the QC led to experiments in which roots were treated with AA, which stimulated QC cells to divide, and resulted in the suggestion that the QC arises, and/or is maintained, because of a localized depletion of AA (Kerk and Feldman, 1995; Liso et al., 1988).

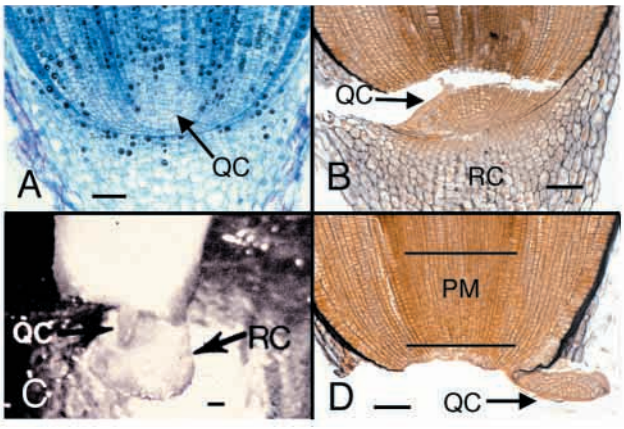

Fig. 1. Views of maize root quiescent center (QC).
(A) Autoradiograph of a median longitudinal section (MLS) of a maize root supplied with [^{3}H]thymidine for 8 hours. Note prominent QC and root cap (RC). (B) MLS showing QC and RC detached together from root proper. (C) Whole maize root with QC and RC dissected free of the root proper. (D) MLS showing detached QC; the RC has already been excised. The extent of the proximal meristem (PM) is also shown. Scale bar, 100 μm.

A link between polarly transported auxin and AA was proposed based on the observation that: (1) auxin stimulates the synthesis of ascorbic acid oxidase (AAO), the enzyme that converts AA from the reduced to the oxidized form; and (2) that AAO is able to degrade auxin (Kerk et al., 2000). Taken together, these two observations formed the basis for the hypothesis that the QC develops as a consequence of the root's requirement to lower endogenous auxin levels (Kerk et al., 2000). In this scenario polarly transported auxin increases AAO levels, resulting in a turnover of auxin and a localized depletion of AA, with a concomitant formation of a QC. Thus, in mutants with perturbed auxin transport, one would predict that alterations in the distribution of redox intermediates, such as AA and GSH, would be associated with changes in the QC. In this paper we explore further the proposed role of redox intermediates in linking auxin and QC formation.

We make measurements in the QC and adjacent meristem of the distribution of auxin (indole-3-acetic acid, IAA), of redox intermediates (glutathione, ascorbic acid and NADPH), and also of the enzymes associated with their synthesis and metabolism. We focused on these particular intermediates because they are generally believed to be major players in the control of the redox status in cells (Noctor and Foyer, 1998). In addition, by using several approaches to perturb polar auxin transport we were able to alter the redox environment of the QC, thereby providing insight into the mechanism by which auxin, via redox intermediates, may mediate the organization of root meristems.

MATERIALS AND METHODS

Plant growth conditions and tissue collection

Corn caryopses (*Zea mays* var. Merit, Asgrow Seed Co., Kalamazoo, MI) were imbibed and germinated in the dark at 25°C for 2 days. Tissues were collected by surgical removal of the root cap, and excision of the QC (Feldman and Torrey, 1976). In this cultivar the QC is separable from the proximal meristem (PM) (Fig. 1D) as a consequence of a weak, thin-walled junction, making possible routine, clean dissections of isolated QCs (Fig. 1B-D). After excision of the QC, the 0.5-1 mm stump of meristematic tissue (the PM) bordering the basal side of the QC (Fig. 1D) was also collected. As tissues were collected, they were either frozen immediately on dry ice, or, for enzyme analysis, the tissue was placed directly into the cold extraction buffer.

Ascorbate and glutathione extraction and assay

For ascorbate or glutathione analysis 60-70 QCs (0.42 mg) and 20 PMs (11 mg) were homogenized in 70 μl or 150 μl, respectively, of the appropriate extraction buffer. Extraction and analysis of ascorbate and glutathione were carried out essentially following the protocol of Zhang and Kirkham (Zhang and Kirkham, 1996), except that the total volume of the reactions was 100 μl. Spectrophotmetric measurements were made on a Shimadzu UV160U spectrophotometer using Eppendorf Uvette microcuvettes. For each assay standard curves were run simultaneously. To determine whether the extraction procedure could result in an artifactual loss (oxidation) of reduced ascorbic acid (AA) or of reduced glutathione (GSH), converting the compounds to their oxidized forms [dehydroascorbate (DHA) or oxidized glutathione (GSSG), respectively] we 'spiked' a sample of tissue at the beginning of the homogenization with a known amount of reduced AA or reduced GSH. In general, we were always able to recover 75-90% of the ascorbic acid or the glutathione in the reduced forms (data not shown). The values for endogenous AA and GSH were corrected accordingly. Each extraction was repeated at least three times with the variation indicated.

Enzyme extraction and assay

The activities of four AA/GSH cycle-associated enzymes were also measured: ascorbate oxidase (EC 1.10.3.3; AAO), dehydroascorbate reductase (EC 1.8.5.1; DHAR), ascorbate free radical reductase [EC 1.6.5.4; AFR (monodehydroascorbate reductase)] and glutathione reductase (EC 1.6.4.2; GR). They were assayed according to procedures described previously: AAO (Kerk and Feldman, 1995), DHAR and AFR (Arrigoni et al., 1997) and GR (Zhang and Kirkham, 1996). The final volumes for each assay were 100-150 μl.

Measurement of reactive oxygen intermediates

Measurements of reactive oxygen species (ROS) $O_2^{\cdot -}$ and H_2O_2 were carried out following the protocol of Schopfer et al. (Schopfer et al., 2001). H_2O_2 measurement was accomplished using a Bio-Tek FL600 microplate fluorescence reader, and readings compared to those from a simultaneously run standard curve. At least three replicates were averaged for each experiment.

Measurement of NADPH/NADP$^+$

NADPH/NADP$^+$, key regulators of the ascorbic acid/glutathione cycles, were measured following the protocol of Zhang et al. (Zhang et al., 2000). For each experiment 60 QCs and 20 PMs were extracted and a standard curve was prepared in a range from 0.02 to 0.1 mM NADPH. At least three replicates were run for each measurement. Using this protocol the limit of detectability is 10^{-3} mM NADPH.

Immunolocalization of auxin (IAA)

Tissue for immunolocalization and binding of the auxin antibody were carried out essentially as described previously (Kerk and Feldman, 1995) using alkaline phosphatase for detection (anti-mouse IgG AP conjugate; Promega, Madison, Wisc.). For visualization of the IAA distribution we used NBT/BCIP tablets for alkaline phosphatase (Roche). The monoclonal antibody used for this immunolocalization has been shown to be highly specific to free auxin in *Zea* root tips (Shi et al., 1993), and was used previously on maize roots (Kerk and Feldman, 1995). Controls were again carried out to confirm specificity.

Table 1. Effects of NPA on polar auxin transport

	Control		+NPA	
Root sections	+12*	12+48†	+12*	12+48†
Terminal mm	13 250±2 350	4 195±1 560	5 596±1 340	2 930±850
1 mm tissue directly in contact with 5[^{3}H]-IAA 'donor' block	8 272±1 670	7 405±2 100	7 430±1 900	5 270±980

Values are dpm±s.e.m.
*Samples analysed after 12 hours of treatment.
†Samples analysed after 12 hours of treatment followed by 48 hours growth without IAA. See Materials and Methods for details.
Each point is an average from 2-3 separate experiments.

NPA treatment

Roots were treated with NPA (sodium salt of naptalan; N-1-napthylphthalamic acid; Uniroyal Chemical Company), which is believed to inhibit polar auxin transport by binding to the auxin efflux carrier (Nemhauser et al., 2000). Using intact seedlings (with roots, 2-3 cm in length), the root was inserted through the center of a 1% agar 'collar' (1 $cm^2 \times 3$ mm thick) containing 10^{-5} M NPA, and the collar positioned at the junction of the root with the seed. The NPA-treated roots were then returned to a tray lined with moistened filter paper, with the NPA collar resting on a glass slide, and not directly in contact with the filter paper. The trays were tightly covered and the roots returned to darkness. At the end of 24 hours of NPA treatment the roots' gravity response was visibly perturbed, with roots orienting randomly (data not shown). For immunolocalization, biochemical and histological studies, the NPA collar was removed from the root after 24 hours of treatment (or for some roots, after 48 hours of NPA treatment) and subsequently these roots used as described more fully in the section detailing the respective experiments. In order to assess more quantitatively the effects of NPA on auxin transport, we used roots previously treated for 24 hours with NPA, then excised the root from the seed (root now about 3 cm in length), and at the basal cut surface of the root (proximal to the NPA collar) placed a 1% agar block (approximately 1 mm^2) containing 1×10^{-8} M 5[^{3}H]IAA (specific activity 16.7 Ci/mM; Amersham). Roots with the attached agar 'donor' blocks were returned to a moistened chamber and incubated for 12 hours in the dark. For control roots we used a plain agar collar. For most experiments 40 roots were used. Following incubation, the terminal 1 mm section (the root apex) and basal 1 mm section (the portion of the root in contact with the donor block) of the roots were excised, pooled, and then dissolved in 0.5 ml of tissue solubilizer (Amersham) for 15 hours at room temperature. Three mls of scintillation fluid (ScintiVerse, Fisher Scientific, Co.) were added to each vial and the samples counted in a scintillation counter (Beckman).

BrdU Incorporation

BrdU incorporation and detection were carried out as described previously (Kerk and Feldman, 1995).

Assaying oxidative activity (oxidative stress) in living tissues

Assaying oxidative activity (oxidative stress) in living cells was accomplished using a dye that is colorless when chemically reduced (when freshly obtained), but when oxidized, fluoresces green in UV (340 nm irradiation; 530 nm emission) light. For this work we used carboxy-H2DCFDA (C-400) dye (Molecular Probes, Eugene Oregon, catalogue no. C-6827). This dye is oxidized by a wide variety of oxidants, and hence gives a general picture of the redox environment, rather than indicating the status of a specific oxidant. At physiological pH this dye has two negative charges facilitating it passive movement through membranes during loading. Upon oxidation the fluorescent product is reportedly trapped inside the cell facilitating long term observation (Collins et al., 2000; Ha et al., 1998; Xie et al., 1999). A 10 μM solution of dye was freshly prepared in water (pH 6.8) just prior to loading into the maize root tissue. In some cases root tissues had been previously treated with NPA for 24 or 48 hours and then allowed to grow for an additional 24 hours (no NPA) prior to being exposed to the dye. In order to facilitate entrance of the dye, root caps were removed just before loading. Seedlings with decapped roots were placed in Petri dishes on moistened filter paper and the tips of the roots immersed in 50 μl of the aqueous dye. Loading typically occurred for a period of 2-3 hours, which was followed by several washes of the roots in plain water. The QCs and the adjacent meristem (PM) were then dissected free, placed in water on a microscope slide, and immediately observed and photographed (in both white and UV light) with a Leica DM microscope. It was important to photograph the tissue immediately after exposing to UV light, since prolonged illumination (in excess of 45 seconds) induced the oxidation of the dye. Although this limited our observation time, in instances where the tissues initially showed no fluorescence, this UV-inducible fluorescence allowed us to determine that dye had indeed entered the cells, but had not been oxidized, under our experimental conditions.

RESULTS

Auxin is high in columella initials and the QC

Kerk and Feldman (Kerk and Feldman, 1995) showed that the QC has relatively high levels of auxin and hypothesized that there may be a link between auxin and the establishment and maintenance of the QC. We confirmed that auxin is highest in the adjacent columella initials and QC, and diminishes in intensity in a more proximal (basal) direction (Fig. 2A-D). In order to investigate further this supposed linkage between high auxin and mitotic activity we perturbed auxin levels and distribution at the root tip, and measured both mitotic activity and the distribution of auxin. We used two approaches: (1) treating roots with the auxin transport inhibitor, NPA; and (2) excising the root cap. We monitored auxin distribution using an auxin-specific antibody and monitored mitotic activity by measuring BrdU incorporation.

NPA affects auxin distribution

In order to ascertain the effectiveness of the NPA treatment in perturbing polar auxin flow, auxin transport was assayed using radiolabelled auxin ([^{3}H]IAA), as previously described (Kerk et al., 2000). NPA treatment resulted in a 50% reduction in the amount of radioactivity transported to the tip (Table 1). A 24-hour NPA treatment resulted in an increase in auxin in the outer cortical and epidermal regions and a decrease in the amount of auxin in the columella initials. However, the QC still showed high levels of auxin (Fig. 2E). After 48 hours of NPA treatment both the QC and columella initials stained much less intensely for auxin, but the extent of staining was increased in the

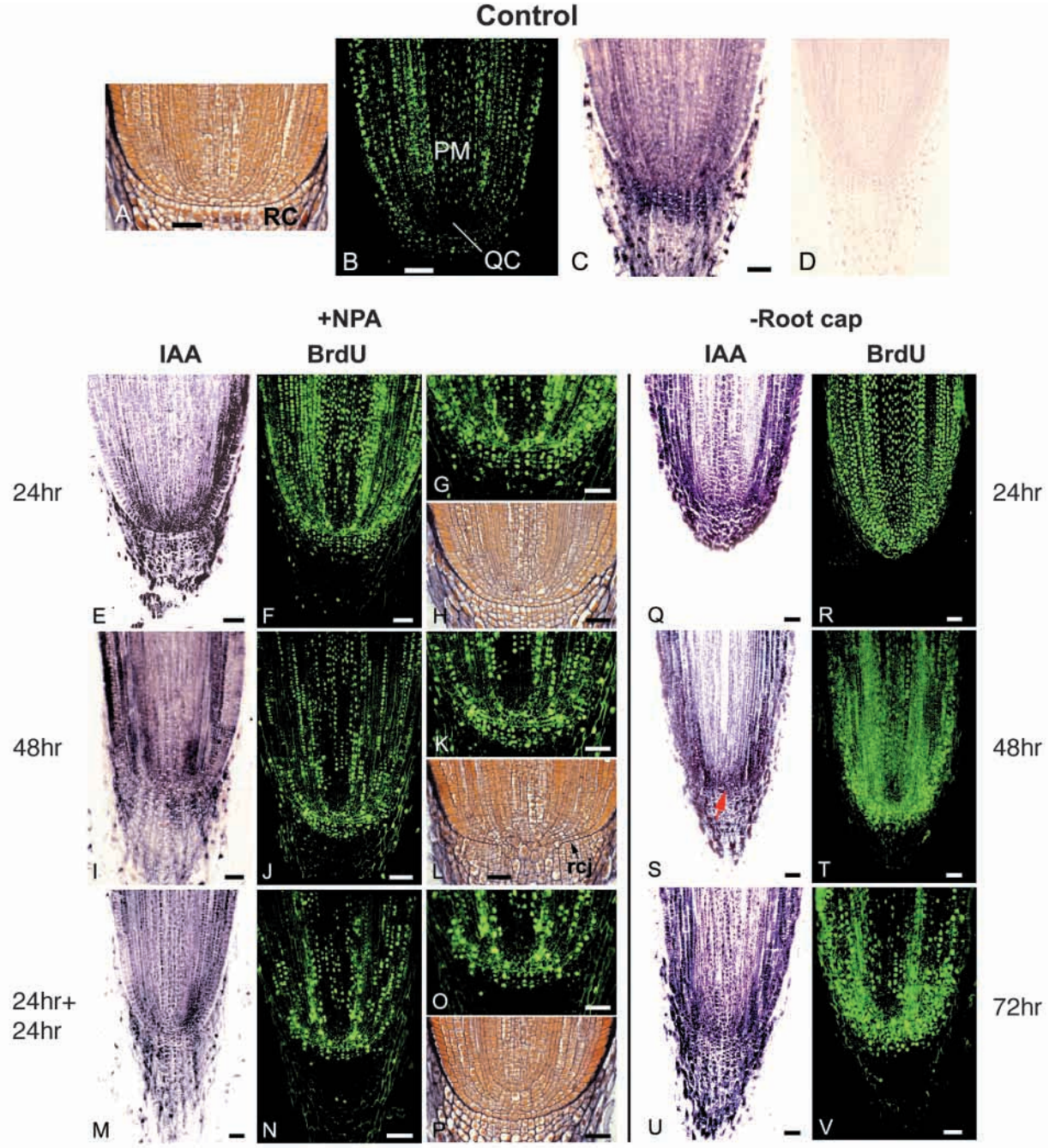

Fig. 2. Localization of auxin and cell division activity in the maize root apex. Scale bar, 100 μm. (A-D) Control roots, intact, no treatments, stained for (A) histology (Orange G and Safranin), (B) BrdU and (C) auxin. Note that the auxin maximum (dark purple color) localizes in the columella initials/QC. (D) Control, as in C, but without incubation with the primary antibody. QC, quiescent center; PM, proximal meristem; RC, root cap. (E-P) Roots treated with NPA for (E-H) 24 hours; (I-L) 48 hours; (M-P) 24 hours NPA followed by no NPA for 24 hours and stained for auxin (E,I,M) and BrdU (F,G,J,K,N,O). G,K,O are enlargements of F,J,N respectively. H,L and P show the histology. rcj, root cap junction. (Q-V) Roots from which the root cap has been excised (Q,R) 24 hours, (S,T) 48 hours and (U,V) 72 hours earlier. Arrow in S points to heavily stained, auxin-rich region of the developing columella. (Q,S,U) auxin; (R,T,V) BrdU.

proximal cortical and procambial files (Fig. 2I). Finally, in roots treated with NPA for 24 hours, and then allowed to grow for an additional 24 hours without NPA (24+24), the region of most intense staining included cortical and procambial initials (Fig. 2M), though this staining was not as extensive as in roots treated for 48 hours with NPA (compare Fig. 2I with 2M). In the 24+24-hour treated roots the QC and columella initials showed relatively little staining (Fig. 2M). Therefore, at the cellular level, the most pronounced effects of the NPA treatments are in lowering the levels of auxin in the QC and root cap initials, and in increasing auxin proximally, in the cells constituting the typically mitotically active proximal root meristem (PM) (Fig. 1D, Fig. 2B). Thus, NPA treatments lead to a proximal relocation of the auxin maximum. Monitoring of

Table 2. Ascorbic acid in the quiescent center and proximal meristem

	Tissue	
	PM	QC
Reduced (AA)		
48-hour-old control seedling	10.2×10^{-4} ($\pm2.6\times10^{-4}$)	1.95×10^{-4} ($\pm1.3\times10^{-4}$)
+NPA, 24 hours	11.1×10^{-4} ($\pm1.6\times10^{-4}$)	6.25×10^{-4} ($\pm0.7\times10^{-4}$)
+NPA, 48 hours	4×10^{-4} ($\pm1.1\times10^{-4}$)	17×10^{-4} ($\pm3.4\times10^{-4}$)
Oxidized (DHA)		
48 hour-old control seedling	3.4×10^{-6} ($\pm1.0\times10^{-6}$)	34×10^{-4} ($\pm7.0\times10^{-4}$)
+NPA, 24 hours	4.8×10^{-4} ($\pm0.9\times10^{-4}$)	11.4×10^{-4} ($\pm3.8\times10^{-4}$)
+NPA, 48 hours	1.7×10^{-4} ($\pm0.4\times10^{-4}$)	8.5×10^{-4} ($\pm2.3\times10^{-4}$)
RATIO (reduced:oxidized)		
48-hour-old seedling	300:1	1:18
+NPA, 24 hours	2.3:1	1:1.8
+NPA, 48 hours	2.3:1	2:1

Amount (μmoles/mg tissue) of reduced (AA) and oxidized (DHA) ascorbate in apical tissues of maize roots, either untreated (control), or treated with the auxin transport inhibitor NPA, for 24 or 48 hours.

QC, quiescent center; PM, meristematic zone, approx, 0.5-1 mm in length, bordering on the proximal (basal) face of the QC. Values are averages of at least 3 independent measurements (±s.e.m.).

Table 3. Glutathione in the quiescent center and proximal meristem

	PM	QC
Reduced (GSH)		
48-hour-old control seedling	3.2×10^{-4} ($\pm0.7\times10^{-4}$)	5.6×10^{-5} ($\pm2.1\times10^{-5}$)
+NPA, 24 hours	7.0×10^{-4} ($\pm0.9\times10^{-5}$)	4.7×10^{-5} ($\pm1.1\times10^{-5}$)
+NPA, 48 hours	1.3×10^{-4} ($\pm0.8\times10^{-4}$)	9.6×10^{-4} ($\pm4.1\times10^{-4}$)
Oxidized (GSSG)		
48-hour-old control seedling	1.4×10^{-5} ($\pm0.7\times10^{-5}$)	4.8×10^{-4} ($\pm1.2\times10^{-4}$)
+NPA, 24 hours	6.5×10^{-5} ($\pm3.6\times10^{-5}$)	1.5×10^{-4} ($\pm0.6\times10^{-4}$)
+NPA, 48 hours	7.5×10^{-5} ($\pm1.8\times10^{-5}$)	7.4×10^{-4} ($\pm3\times10^{-4}$)
Ratio (reduced:oxidized)		
48-hour-old seedling	23:1	1:8.6
+NPA, 24 hours	11:1	1:3.1
+NPA, 48 hours	1.7:1	1.3:1

Amount (μmoles/mg tissue) of reduced (GSH) and oxidized (GSSG) glutathione in apical tissues of maize roots, either untreated (control), or treated with the auxin transport inhibitor NPA, for 24 or 48 hours. QC, quiescent center; PM, the meristematic zone, ~0.5-1 mm in length, bordering on the proximal (basal) face of the QC. Values are averages of at least 3 independent measurements (±s.e.m.).

these same treatment times with BrdU revealed that a 24-hour NPA treatment caused a diminution in QC size (Fig. 2F,G), but a small QC was still evident. At 24 hours the distal region of the QC showed less organization, as a consequence of cell enlargement and a limited amount of cell division (Fig. 2H). Treating roots for 48 hours with NPA results in an activation of the distal region of the QC (Fig. 2J,K) and cells of the QC grew into the root cap (Fig. 2L). However, a small, uncharacteristically elongated QC still remained (Fig. 2J). In roots treated with NPA for 24 hours, and then allowed to grow for an additional 24 hours without NPA, the distal end of the QC activated, and showed BrdU labeling, and this was accompanied by the production of several new (atypical) layers of cells in the region between the root cap and the tip of the procambial cylinder (Fig. 2N-P). A small QC was still evident (Fig. 2N).

Root cap excision affects auxin distribution

Excising the root cap causes changes in auxin distribution. Twenty-four hours after RC removal a large amount of auxin has accumulated throughout the root tip (Fig. 2Q). Forty-eight hours after excision a new, rudimentary root cap has formed, indicating that a new root cap initial layer has formed and is functioning (Fig. 2S,T). Auxin staining remains high in the procambial files and is also detected in the columella region of the regenerating cap (Fig. 2S, arrow). Excision of the root cap also activates the QC. Twenty-four hours after cap excision most cells of the root tip have incorporated BrdU and a QC is not detected (Fig. 2R). By 48 hours after excision a very small QC is beginning to reform (Fig. 2T), and by 72 hours a distinct QC is evident (Fig. 2V).

Ascorbate, glutathione, associated enzymes and NADPH/NADP$^+$ in the QC and PM

Measurements were made of the reduced and oxidized forms of ascorbate and glutathione in the QC and in adjacent meristem tissue (PM) (Tables 2, 3), and ratios were calculated. The PM tissue showed about 10× more ascorbate in the reduced form (AA) than found in the QC, but about 1000× less of the oxidized form (DHA) compared to the QC. In the QC, there is less AA than DHA (a ratio of 1:18), compared with a ratio of 300:1 (reduced:oxidized) in the PM. With regard to glutathione, here too the reduced form (GSH) is about 10× more concentrated in the PM than in the QC, whereas the oxidized form (GSSG) is about 10× higher in the QC than in the PM. For the PM, the ratio of reduced to oxidized is 23:1 and for the QC, 1:8.6. We also measured the activities of 4 key enzymes involved with regulating ascorbate and glutathione levels (Table 4). For ascorbic acid oxidase (AAO) we found approximately 4× as much activity in the QC as in the adjacent meristem. For glutathione reductase (GR) approximately 9× more activity was found in the PM than in the QC. The activity of ascorbate free radical reductase (AFR or monodehydroascorbate reductase) was about the same for both tissues. No dehydroascorbate reductase activity (DHAR) was detected in either tissue. The concentrations of NADPH and NADP$^+$ were measured for the PM and were 0.05±0.01 mM and 0.09±0.02 mM, respectively, but neither of these two species was detected in the QC (limits of assay detectabilty $>10^{-3}$ mM) (Table 5).

$O_2^{\cdot-}$ and H_2O_2 levels in the QC and PM

We also measured the levels of $O_2^{\cdot-}$ and H_2O_2 since these two species can represent major reactive oxygen species intermediates (ROS) (Table 6). While it is not possible to obtain a specific number for the concentration of $O_2^{\cdot-}$, following the protocol of Schopfer et al. (Schopfer et al., 2001) we find 15.6× more $O_2^{\cdot-}$ (on a per mg fresh weight basis) in the QC than in the PM (Table 6) (using a 25-minute incubation). For H_2O_2 34× more was measured per mg QC tissue, than for PM tissue.

NPA affects levels of ascorbate, glutathione and the activities of associated enzymes

We also measured the levels and state of ascorbate and

Table 4. Relative activities for 4 enzymes associated with ascorbate/glutathione metabolism

	PM	QC	Ratios
Ascorbic acid oxidase (AAO)			
48-hour-old control seedling	–0.036	–0.133	
+NPA, 24 hours	–0.066	–0.349	
+NPA, 48 hours	–0.076	–0.396	
QC:PM (control)			–0.133/–0.036=3.7:1
QC:PM (+NPA 48 hours)			–0.396/–0.076=5:2
QC (control)/+NPA 48 hours			–0.133/–0.396=1:3
PM (control)/+NPA 48 hours			–0.036/–0.076=1:2.1
Glutathione reductase (GR)			
48-hour-old control seedling	–0.146	–0.017	
+NPA, 24 hours	–0.201	–0.949	
+ NPA, 48 hours	–0.058	–1.032	
QC:PM (control)			–0.017/–0.146=1:8.6
QC:PM (+NPA 48 hours)			–1.032/–0.058=17.8:1
QC (control)/+NPA 48 hours			–0.017/–1.032=1:60.7
PM (control)/+NPA 48 hours			–0.146/–0.058=2.5:1
Ascorbate free radical reductase (monodehydro-ascorbate reductase) (AFR)			
48-hour-old seedling (control)	–0.241	–0.166	
+NPA, 48 hours	–0.158	–0.187	
QC:PM (control)			–0.166/–0.241=1:1.45
QC:PM (+NPA 48 hours)			–0.187/–0.158=1.2:1
QC (control)/+NPA 48 hours			–0.166/–0.187=1:1.1
PM (control)/+NPA 48 hours			–0.241/–0.158=1.5:1
Dehydroascorbate reductase (DHAR)	none detected	none detected	

Activity (OD/mg tissue) was determined individually in both quiescent centers (QC) and in the zone of rapidly dividing cells, approx. 0.5-1 mm in length, bordering on the proximal face of the QC (PM). Measurements were made in tissues from untreated roots (control) and roots treated with 10^{-5} M NPA for 24 or 48 hours.

associated enzymes in NPA-treated roots. For the QC, NPA treatment increases 10× the levels of reduced AA, and decreases by a factor of 4 the levels of the oxidized species (Table 2), resulting in a change in the ratio of reduced to oxidized forms, from 1:18 to 2:1. For the meristem (PM) tissue, NPA causes a marked shift in the amounts and ratios of AA and DHA. AA decreases about 2.5×, but DHA increases about 50×, resulting in a change in the ratio of AA to DHA from 300:1 in the untreated, to 2.3:1 in the NPA-treated roots. Thus NPA treatment results both in a change in the absolute amounts and in the ratios of the two forms of ascorbate. A similar trend holds for glutathione; NPA treatments alter the reduced:oxidized ratios in both the QC and PM (Table 3). NPA treatment also changes the activity of AAO and GR (Table 4). In general, AAO activity increases 3-4× in both the QC and meristem. In contrast, GR activity increases 60× in the QC, while at the same time decreasing about 3× in the adjacent meristem tissue. Thus, NPA treatments cause the redox status of the QC to become less oxidizing.

Visualization of the oxidized redox status of the QC

Since the biochemical data indicated that the QC and PM were possibly very different with regard to their overall redox status, we attempted to visualize these suggested differences using a redox-sensitive dye, fluorescing in an oxidizing environment and not fluorescing, or fluorescing less in relatively less oxidizing environments. We found that the QC was highly fluorescent, indicating a relatively oxidizing environment (Fig. 3A,B). This is in contrast to the adjacent PM tissue, which showed no fluorescence (Fig. 3C,D) thereby suggesting that the overall redox status of the PM is relatively less oxidizing than that in the adjacent QC. These results with the dye are consistent with our measurements of higher levels of specific reactive oxygen species (e.g., $O_2^{\cdot -}$ and H_2O_2) in the QC, compared to the PM.

Table 5. Levels of NADPH/NADP$^+$ in the quiescent center (QC) and proximal meristem (PM)

	QC	PM
NADPH/NADP$^+$	None detected*	0.05±0.01/0.09±0.02†

*Limits of detectability=10^{-3} mM.
†mM.

Table 6. Levels of $O_2^{\cdot -}$ and H_2O_2 in the quiescent center (QC) and proximal meristem (PM)

	QC	PM	QC:PM
$O_2^{\cdot -}$*	0.047	0.003	15.6:1
H_2O_2†	802±27	23±8	34:1

*OD_{470} units/mg tissue.
†nM/mg.

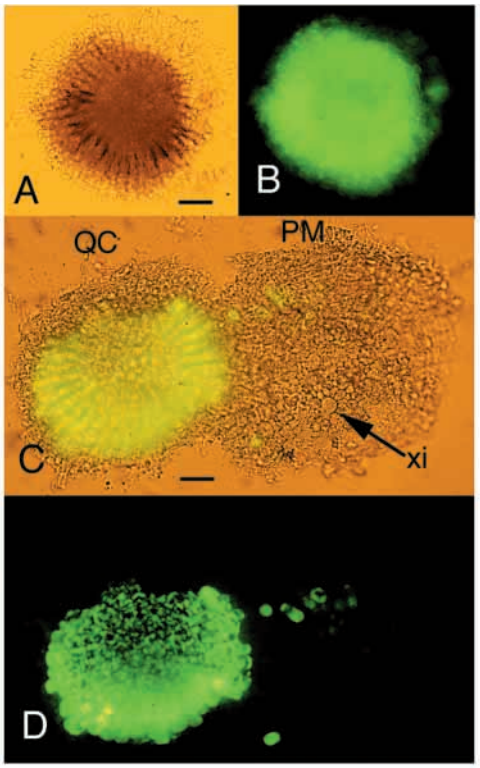

Fig. 3. Whole mounts of excised quiescent centers (QC) with or without the adjacent proximal meristem (PM); from roots pre-treated for 2-3 hours with the redox sensitive dye carboxy H2DCFDA. Fluorescence indicates a relatively oxidizing environment. (A,B) An isolated QC with its distal face showing. Photographed in white light (A) or in UV light (B). (C,D) QC and adjacent PM. Photographed simultaneously with white and UV light (C), or in UV light only (D). Note the metaxylem initial (xi arrow) on the distal face of the PM. Scale bar: 100 μm.

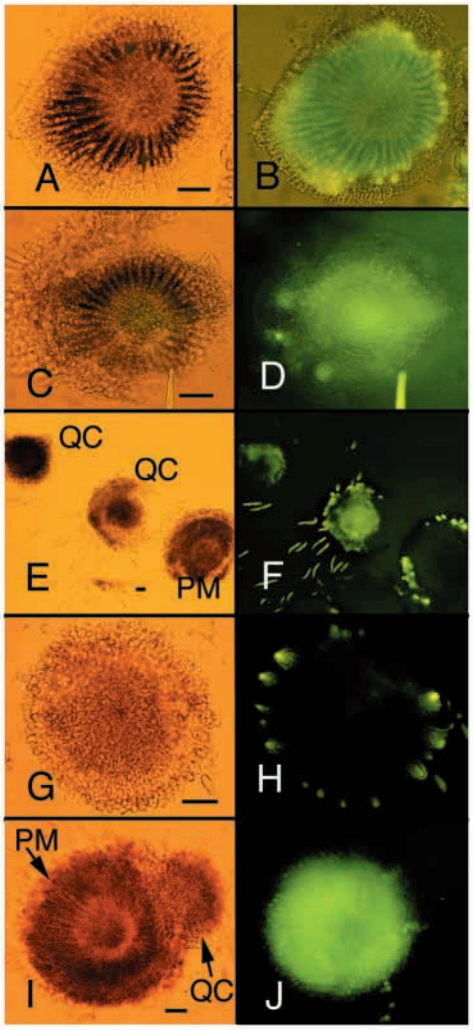

Fig. 4. Whole mounts of excised quiescent centers (QC), with and without the adjacent proximal meristem (PM), from roots pre-treated for 2-3 hours with the redox sensitive dye carboxy H2DCFDA. C-J are from roots exposed to NPA (for 24 or 48 hours), and then either treated with the dye, or allowed to grow for an additional amount of time, with no NPA, and then treated with the dye. (A,B) Control, no NPA. Photographed in white light only (A) or in white +UV (B). (C,D) QC at the end of the 24-hour NPA treatment. Photographed in white light (C) or in UV (D). The extent of fluorescence has decreased relative to the control (B). (E-H) QCs and a PM from roots allowed to grow for 24 hours after the end of the NPA treatment. Photographed in white light (E,G), or in UV (F,H). At this time QCs show variable fluorescence. The PMs essentially show no fluorescence (F). G and H are from a QC showing essentially no fluorescence. (I,J) QC and adjacent PM from roots at the end of a 48-hour NPA treatment. Photographed in white (I) or in UV light (J). Note that the PM region is now highly fluorescent, with no fluorescence in the QC. Scale bar, 100 μm.

NPA and decapping change the redox status in the QC

Using the redox-sensitive dye we also monitored the overall redox status of NPA-treated root meristems (Fig. 4A-J). Twenty-four hours after commencing NPA treatment fluorescence was noticeably less in QCs (Fig. 4C,D). Thirty-six to forty-eight hours after commencing the NPA treatment the extent of fluorescence was considerably diminished, or nearly undetectable, in QCs (Fig. 4E-H). Forty-eight to seventy-two hours after beginning NPA treatment the QC showed reduced or little fluorescence, whereas the adjacent meristem was highly fluorescent (Fig. 4I,J). This is the reverse of what was observed in untreated roots (compare to Fig. 3C,D). Thus, the NPA-induced shift in the auxin maximum from the QC to the PM is paralleled by the development of a more oxidized status in the PM, a more reduced status in the QC, and the activation of cell division in the QC. Decapping also causes changes in the redox status of the QC and meristem. Twenty-four hours after RC removal the redox status of the now activated 'QC' (Fig. 2R) was relatively more reduced (compare Fig. 5B,D). Forty-eight hours after RC removal the central portion of the regenerating root apex showed a small, reforming QC (Fig. 2T) and a central region of fluorescence, indicating the development of a region with a relatively more oxidizing status (Fig. 5F). By 72 hours a new root apex was almost completely reformed and the zone of fluorescence had enlarged (Fig. 5H).

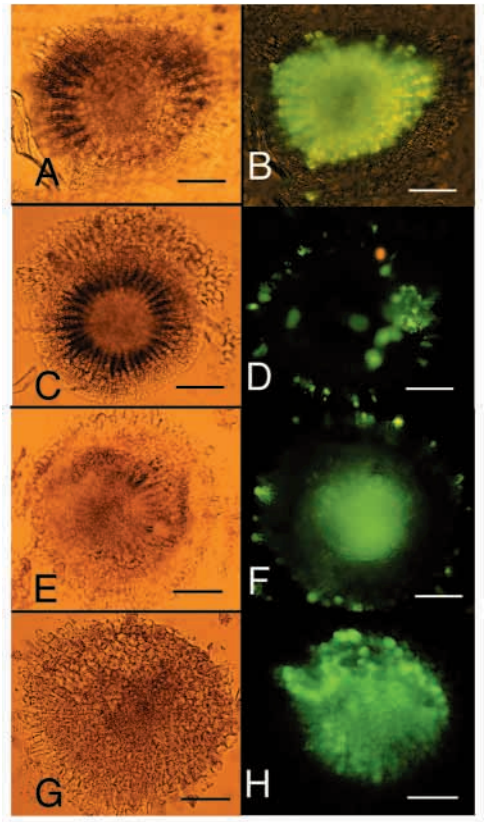

Fig. 5. Whole mounts of excised quiescent centers (QC) from roots that have had their root cap (RC) excised, and subsequent regeneration allowed to proceed for various periods of time. During the last 2-3 hours of regeneration roots are treated with the redox sensitive dye carboxy H2DCFDA. (A,B) Control, time zero; RC excised just prior to placing roots in dye. Photographed in white light (A) or white plus UV (B). (C,D) 24 hours after removal of the RC. Photographed in white light (C) or UV (D). Note the disappearance of fluorescence from the QC, indicating a less oxidizing environment. (E,F) 48 hours after removal of the RC. Photographed in white light (E) or UV (F). A low level of fluorescence is detected in the center of the tissue. (G,H) 72 hours after removal of the RC. Photographed in white light (G) or UV light (H). The fluorescence has expanded to include an area similar to that of the control (Fig. 7B). Scale bar, 100 μm.

DISCUSSION

Redox regulation of the cell cycle in the QC

Earlier we showed that reduced ascorbate (AA) was not detectable histochemically in the QC, but was observed in the adjacent PM (Kerk and Feldman, 1995). Here we show the reduced form of ascorbate (AA) is 5× lower in the QC than in the PM. But more dramatically, the levels of oxidized form (DHA) are 1000× greater in the QC than in the adjacent PM (Table 2). High levels of DHA in the QC are particularly noteworthy, since DHA accumulation is generally considered a negative event for cell metabolism (Arrigoni, 1994). Thus, in rapidly dividing cells of the PM, not only is AA higher than in the QC, but DHA is 1000× lower. Hence, both the ratios and absolute amounts of AA and DHA differ between the QC and PM. A similar trend holds for glutathione, which in the reduced form (GSH) is about 10× lower in the QC than in the PM, and in the oxidized form (GSSG) is about 10× higher in the QC, compared with the PM (Table 3). In the QC the GSH:GSSG ratio is weighted in favor of the oxidized form (1:8.6), whereas in the PM, the reverse holds (23:1). In addition, we were unable to measure NADPH (Table 5) in the QC, suggesting that the QC would not regenerate GSH from GSSG very efficiently (NADPH is required for this reaction) (Noctor and Foyer, 1998). Because in most tissues GSH is believed to be the major molecule contributing to overall cellular redox status, and because depletion of GSH reportedly traps cells in the G_1 phase (Vernoux et al., 2000), the apparent inability of the QC to regenerate GSH may, in part, account for the accumulation of QC cells at G_1/S.

Because the AA:DHA and GSH:GSSG ratios in the QC are skewed in favor of the oxidized forms, this suggests that the QC would be less able to rid itself of ROS and hence would have a relatively more oxidized redox state. Use of a redox-sensitive dye revealed that the QC is, relatively, under more oxidative stress than the adjacent PM cells (Fig. 3). Although we cannot precisely quantify the degree of oxidative stress in the QC, compared to the PM, it is clear from the measurements of ROS ($O_2^{\cdot -}$ and H_2O_2), that there are differences in the oxidative intermediates in these two tissues (15.6× more $O_2^{\cdot -}$ and 34× more H_2O_2 were found in the QC, compared to the PM) (Table 6). Taken together, these data point to a general inhibition in the QC of mechanisms to generate and maintain a reduced redox status.

Activating the QC correlates with changes in the redox status

Data thus far presented suggest that the QC is under oxidative stress. We therefore reasoned that if cells in the QC can be stimulated to divide, QC activation should be associated with changes in the redox status of the QC. Excision of the cap or treatment of roots with NPA activates the QC. Excision of the cap both alters QC redox status (Fig. 5D) and causes the QC to disappear (Fig. 2R). Reappearance of a QC in decapped roots (Fig. 2T) correlates with the redevelopment of an oxidized state in cells of the presumptive QC (Fig. 5F). As with root cap excision, NPA treatments not only activate the QC but also result in changes in the redox status of the QC (Fig. 2F,J,N; Fig. 4; Tables 2-4). Twenty-four-hour NPA-treated roots have a small QC (compare Fig. 2B and 2F) and also, the zone of fluorescence (indicating a relatively oxidizing status) is diminished (Fig. 4D). After 48 hours of NPA treatment, when the QC is maximally activated (Fig. 2J,K), no fluorescence is detected (Fig. 4G,H), thereby indicating a relatively more reduced redox status. Interestingly, after 48 hours of NPA treatment, it is the PM that now fluoresces (Fig. 4I,J). Thus, as with root cap removal, NPA treatments cause both an activation of the QC and a changed redox status in both the QC and PM. Taken together, these results demonstrate that not only is an oxidized redox state characteristic of the QC, but moreover, that activation of the QC correlates with the development of a less oxidized status in the QC, and the development of a new redox status (more oxidized) in the adjacent PM. Given that mild oxidative stress has been shown to impair the G_1/S transition in plants (Chen and Ames, 1994; Bijur et al., 1999; Chen et al., 1995; Logemann et al., 1995; Reichheld et al., 1999) and in animals (Russo et al., 1995) and that redox status

can be a modulator of the balance between renewal and differentiation in animal cells (Smith et al., 2000), we conclude that the lengthened cell cycle times in the QC are also likely a consequence of the relatively oxidized redox status of the QC. This correlation between redox status and the degree of quiescence further supports the suggestion that "intracellular redox homeostatis could affect cell-cycle progression by regulating key components of the G_1/S transition" (den Boer and Murray, 2000), and leads us to conclude that the establishment and maintenance of an oxidizing environment may be central to the development and elaboration of a QC.

Auxin regulates the QC via redox

Excision of the cap or treatment of roots with NPA not only causes changes in the redox status of the QC and activation of the QC, but also brings about changes in auxin levels and distribution in the root tip. These observations suggest a linkage between auxin distribution, QC formation and redox status at the root tip. In an untreated, control maize root the auxin maximum is located in the root cap columella initials/QC (Fig. 2C), mirroring very closely the auxin patterns observed in *Arabidopsis* roots (Friml et al., 2002; Sabatini et al., 1999). We show that NPA causes a shift in the auxin maximum from the columella/QC zone to the region of the PM (compare Fig. 2C and 2I), thereby confirming in maize what has earlier been reported for the effects of NPA on the position of the auxin maximum in *Arabidopsis* (Sabatini et al., 1999). But in addition, we extend this work and show that a shift in the auxin maximum correlates with a change in the redox status, which becomes more oxidizing in the region to which the auxin maximum has been shifted (the PM), and less oxidizing in the region from which the auxin maximum has been displaced (the QC) (Fig. 4G,H). Of particular note is the observation that shifting the auxin maximum to the PM results in a decrease in BrdU incorporation in the PM (compare Fig. 2B and 2J), indicating a decrease in the rate of cell division in the PM. Taken together these results provide strong evidence that QC activation and changes in the redox status are associated with a shift of the auxin maximum.

As with the NPA treatments, excising the cap leads to marked changes in auxin distribution in the remaining root tissues. Of particular significance is the appearance of auxin staining (development of a new auxin maximum) in the columella region of the new, regenerating root cap (Fig. 2S; arrow). While it is not now certain whether the increase in auxin in this region occurs before the start of reforming a new QC, it is clear that the development of a new auxin maximum in the regenerating cap occurs at least at the very earliest stages of QC reformation (Fig. 2S,T). These results thus imply a role for the root cap initial layer/columella (and perhaps the whole cap) in the development and maintenance of the QC, and further suggest that positioning of the auxin maximum in the (new) root cap may be central to QC redevelopment. Support for an hypothesized role for the auxin maximum in 'specifying' the QC comes from *Arabidopsis* that was treated for extended periods with NPA; a shift of the auxin maximum to new, more proximally located cells is accompanied by the atypical expression of QC-specific markers in these cells (Sabatini et al., 1999).

Auxin is widely involved in patterning (Friml et al., 2002; Sabatini et al., 1999), acting as a positional signal (Uggla et al., 1996). Based on our findings that location of the auxin maximum correlates with oxidative stress in the QC, we suggest that auxin can provide positional cues by virtue of its ability to influence, on a localized scale, the redox status of tissues. The challenge now is to elucidate the hypothesized link between auxin and ROS formation.

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