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RESEARCH PAPER

Stem cell fate in hypoxic root apical meristems is influenced by phytoglobin expression

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Abstract

Root survival to flooding-induced hypoxic stress is dependent upon maintaining the functionality of the root apical meristem quiescent center (QC), a process that is governed by the basipetal flow of auxin leading to the formation of an auxin maximum, which is needed for the establishment of a highly oxidized environment specifying the QC niche. Perturbations in auxin flow and distribution along the root profile occurring during hypoxia can shift the redox state of the QC towards a more reduced environment, leading to the activation of the QC, degradation of the meristem, and root abortion. The maize phytoglobin gene *ZmPgb1.1* is involved in minimizing these damaging effects during hypoxia in processes that result in sustaining the PIN-mediated auxin maximum and an oxidized environment in the QC. The oxidized environment is accomplished by maintaining the activity of redox enzymes oxidizing ascorbate and glutathione. These events, compromised in QCs suppressing *ZmPgb1.1*, ensure the functionality of the QC and root meristems under conditions of low oxygen, resulting in stable root performance.

Keywords: Ascorbate, auxin, glutathione, hypoxia, nitric oxide, phytoglobin, quiescent center, redox state, root apical meristem.

Introduction

Embedded in the root tip and defining the stem cell niche (SCN), the root apical meristem (RAM) is responsible for the iterative generation of all cell types within the root. The SCN harbors two distinct types of stem cells: a slowly dividing population delineating the quiescent center (QC) and a rapidly dividing cluster of cells (initials), populating the proximal meristem (PM) and the distally located root cap (RC). The main function of the QC is to maintain the initials in an undifferentiated state (Dolan *et al.*, 1993). If ablated (van den Berg *et al.*, 1997), or activated (Jiang *et al.*, 2003), the QC cannot fulfil its task, resulting in the differentiation of the surrounding initials and root growth cessation. Thus, the retention of QC quiescence is paramount for the 'dynamic homeostasis' in the RAM, governing the rate of division and differentiation. Perturbations caused by environmental stress influence the QC to potentially modify stem cell fate. It has been suggested that phytoglobin (Pgb), through its effect on nitric oxide (NO), may alter plant cell fate (Stasolla and Hill, 2017). Here, we show that during hypoxia the level of *Pgb* is a determinant factor in the QC response to stress.

Abbreviations: Pgb, phytoglobin; PM, proximal mersitem; QC, quiescent center; RAM, root apical meristem; RC, root cap.

Conditions of hypoxia (oxygen deficiency), often experienced by plants under partially or fully submerged soils, compromise yield by interfering with basic metabolic processes (Bailey-Serres and Voesenek, 2008), often observed in the shoot (Mustroph and Albrecht, 2003), but reflecting perturbations in the root. While hypoxia-related changes in root anatomy and physiology have been mainly described in mature domains, as is best exemplified by the formation of aerenchyma (Drew *et al.*, 2000), documentations of root tip alterations are scarce, due to the difficulties in visualizing and analyzing the responses of the difference compartments harbored in the root apex. Yet, root tips are extremely sensitive to flooding stress and often die after a few hours of exposure to hypoxic conditions, compromising root survival following the re-establishment of normoxic oxygen levels (Roberts *et al.*, 1984). Maize root tips exposed to 4% oxygen for 24 h undergo massive cellular death, preceded by the accumulation of NO and reactive oxygen species (ROS) (Mira *et al.*, 2016*a*).

The state of quiescence and functionality of the QC relies on the PIN-mediated basipetal flow of auxin, contributing to the formation of an auxin maximum within the QC and the establishment of a highly oxidized ascorbate and glutathione environment (Jiang *et al.*, 2003). Experimental perturbations of auxin distribution along the root profile switch the QC redox state towards a reduced state, a condition stimulating division of the quiescent cells and causing structural abnormalities with negative repercussions on root behavior (Jiang *et al.*, 2003). Thus, factors altering auxin movement and/or the cellular redox state might influence QC functionality and overall root growth.

Pgbs are plant hemoglobin proteins and, by scavenging NO (Dordas *et al.*, 2003), regulate many NO processes, some of which are linked to auxin synthesis and distribution. This is the case for Arabidopsis *in vitro* embryogenesis (Elhiti *et al.*, 2013) and root growth under severe water stress (Mira *et al.*, 2017). The function of Pgbs has been mainly related to stress conditions, including hypoxia, which induces their expression (Taylor *et al.*, 1994). Plant response to stress is indeed influenced by Pgbs, with their constitutive expression enhancing survival to low oxygen levels in Arabidopsis, alfalfa, and maize (Hunt *et al.*, 2002; Dordas *et al.*, 2003; Igamberdiev and Hill, 2004). Root growth retardation caused by the induction of the death program observed in maize seedling subjected to 4% oxygen can be alleviated by high levels of *ZmPgb* genes, and aggravated by low levels (Mira *et al.*, 2016*a*). Hypoxic RAMs of roots suppressing *ZmPgb* genes showed anatomical abnormalities, the most conspicuous of which is the vacuolation of cells at the root apex (Mira *et al.*, 2016*a*). The ability of Pgbs to preserve the integrity of the root tip was also demonstrated in Arabidopsis plants exposed to severe drought (Mira *et al.*, 2017). Besides regulating auxin synthesis and distribution, Pgbs interfere with the cellular redox status (Igamberdiev *et al.*, 2014), and more specifically with the ascorbate–glutathione cycle through the regulation of several redox enzymes, including ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR) (Igamberdiev *et al.*, 2006).

The influence of Pgb on auxin and cellular redox state, and the relevance of both for the maintenance and functionality of the RAM are the premises of the current work, which develops a novel mechanistic model of root response to hypoxia, and possibly other types of stress. The model is centered around Pgb as a determinant factor modulating auxin distribution, changes in redox state at the QC, and ultimately QC behavior and root growth.

Materials and methods

Plant material, growth conditions, and tissue collection

The maize plants overexpressing [line ZmPgb1.1(S)] or down-regulating [line ZmPgb1.1(A)] *ZmPgb1.1* were those previously characterized (Youseff *et al.*, 2016; Mira *et al.*, 2016*a*). Relative to the wild type (WT), the expression level of *ZmPgb1.1* was increased 10 times in the ZmPgb1.1(S) line, and halved in the ZmPgb1.1(A) line (see fig. 2 in Youseff *et al.*, 2016). Hypoxic treatments were performed exactly as described (Geisler-Lee *et al.*, 2010) whereby 5-day-old maize seedlings were immersed in a liquid solution [1/2 Murashige and Skoog (MS) medium] through which air (normoxic conditions) or 4% (v/v) oxygen/96% nitrogen (hypoxic conditions) were bubbled. Seedlings were harvested at 6, 12, and 24 h during the treatments for tissue collection.

Tissues (i.e. QC, RC, and PM) were collected exactly as reported (Feldman, 1976; Jiang *et al.*, 2003). QCs were easily removed due to the weak wall junction with the proximal tissue (see Supplementary Fig. S1 at *JXB* online). Within the proximal tissue, 1 mm of the PM was harvested (Jiang *et al.*, 2003). The tissue compartments were dissected on filter paper and either frozen rapidly in liquid nitrogen (for analyses of transcript levels) or stored in extraction buffer (for enzyme assays).

Structural and localization studies

Histological examination using thin sectioning was performed (Yeung, 1990). Immunolocalization of indole-3-acetic acid (IA)A was conducted (Mira *et al.*, 2016*b*). Hypoxic root apexes were sectioned using a compresstome and pre-fixed in 4% aqueous 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride at 4 °C for 2 h. The samples were post-fixed in FAA (10% formalin, 5% acetic acid, and 50% ethanol) overnight at 4 °C, dehydrated in an ethanol series, and embedded in Paraplast Plus (Fisher). Sections were deparaffinized in xylene. After blocking with 5% BSA for 1 h, the sections were incubated with monoclonal primary IAA antibodies (1 mg ml^{-1}, Sigma) diluted 1:200 in 1× phosphate-buffered saline (PBS) containing 0.8% BSA for 4 h. Secondary antibodies [anti-mouse IgG alkaline phosphatase conjugate (1 mg ml^{-1}), Promega, USA] were applied overnight. Signal was visualized with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega).

Localization of PIN1 transcripts was conducted by RNA *in situ* hybridization (Elhiti *et al.*, 2010). Full-length *PIN1* cDNA was cloned into a pGEM-T EasyVector System (Promega) and amplified from the vector using T7 and SP6 primers. The amplification product was subsequently used for the preparation of digoxigenin (DIG)-labeled sense and antisense riboprobes, following the procedure outlined in the DIG Application Manual (Roche Diagnostics). Slide preparation, hybridization conditions, and color development were performed as documented previously (Elhiti *et al.*, 2010).

Localization of reduced ascorbic acid (AA) was carried out as previously described (Liso *et al.*, 2004).

Visualization of the oxidized redox state was conducted using the redox-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Jiang *et al.*, 2003). Mitochondrial membrane potential was estimated using MitoTracker Orange CM™Ros (Jiang *et al.*, 2003).

Detection of the S-phase cell division in hypoxic QCs was conducted using the thymidine homolog 5-ethynyl-2'-deoxyuridine (EdU) using the Click-iT® EdU Imaging Kit (Invitrogen).

Measurement of relative transcript abundance

RNA extraction from root compartments was carried out using the RNAAqueous-micro Kit (Ambion). The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis.

Quantitative reverse transcription–PCR (qRT–PCR) was performed as described (Elhiti *et al.*, 2010). All primers used for gene expression studies are listed in Supplementary Table S1 and their efficiency in Supplementary Table S2. The relative gene expression level was analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using actin as the reference gene.

Metabolite measurements, enzyme activity, and ATP/ADP measurements

Cellular AA, dehydroascorbic acid (DHA), reduced glutathione (GSH), and oxidized glutathione (GSSG) were measured according to Zhang and Kirkham (1996). Total ascorbate was determined after reduction of DHA to AA with DTT, and the concentration of DHA was calculated from the difference between total ascorbate and AA. The color was developed in the reaction with α,α'-dipyridyl and $FeCl_3$ monitored at 525 nm. The method of measurement of GSH and GSSG is based on the reaction of 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with GSH forming 5-thionitrobenzoic acid (TNB) that absorbs at 412 nm. The GSSG was measured after being reduced by glutathione reductase (GR). The amounts of reduced and oxidized ascorbate and glutathione were calculated from calibration curves.

The activities of APX (EC 1.11.1.11), MDHAR (EC 1.6.5.4), dehydroascorbate reductase (DHAR) (EC 1.8.5.1), and GR (EC 1.8.1.7) were assayed according to Murshed *et al.* (2008). APX was measured in 50 mM potassium phosphate buffer (pH 7.0) containing 0.25 mM sodium ascorbate and the extract. The reaction was started by adding H_2O_2 at a final concentration of 2.5 mM, and the reaction rate was determined spectrophotometrically at 290 nm (ε=2.8 mM^{-1} cm^{-1}). MDHAR was assayed in 50 mM HEPES buffer (pH 7.6) containing 2.5 mM AA, 0.2 mM NADH, and the extract. The assay was initiated by adding 0.4 U of ascorbate oxidase (AAO) to generate ascorbate free radical (MDHA) and the reaction rate was monitored at 340 nm (ε=6.22 mM^{-1} cm^{-1}). DHAR was measured at 265 nm (ε=14 mM^{-1} cm^{-1}). The assay buffer contained 50 mM HEPES buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH, and the extract. The reaction was initiated by adding freshly prepared DHA at the final concentration of 0.8 mM.

AAO (EC 1.10.3.3) was assessed by measuring the disappearance of ascorbate (ε=7 mM^{-1} cm^{-1}) in 0.1 M K-phosphate buffer (pH 6.0) containing 0.15 mM AA (Hayashi and Morohashi, 1993). Alcohol dehydrogenase (ADH) (EC 1.1.1.1) was measured at 340 nm (ε=6.22 mM^{-1} cm^{-1}) in 0.15 M Tris–HCl buffer (pH 9.0) containing 1 mM NAD^+, 30 mM ethanol, and enzyme extract (Hanson *et al.*, 1984). The ATP/ADP ratio was measured using the EnzyLight™ ADP/ATP Ratio Assay Kit (ELDT-100) (BioAssay Systems). QCs were collected, fixed in 2.4 M perchloric acid, and neutralized by 1 M Tris–HCl. Measurements were carried out using a luminometer (Berthold Junior LB 9509).

Experiments were performed in two biological replicates each consisting of ~80 QCs (~0.5 mg FW).

Root regeneration assays and pharmacological treatments

Root regeneration from isolated QCs was carried out following the procedure described in Feldman (1976). Freshly isolated QCs were cultured in S2M+K –2,4-D medium able to induce the formation of roots from the QCs. Root development was followed during the first 2 weeks.

For pharmacological treatments, the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) and the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) were applied to a concentration of 10 μM every other day.

Statistical analysis

Data were analyzed by one-way ANOVA using the SPSS program (SPSS Statistics for Windows, version 19.0; IBM). Treatment means were compared by Tukey HSD test (α=0.05) to differentiate the significance of differences.

Results

Pgb influences the structure of hypoxic RAMs

Root growth is inhibited by hypoxia, and this inhibition is attenuated by the overexpression of Zm*Pgb1.1* [line ZmPgb1.1(S)], and aggravated by conditions in which the same gene is suppressed [line ZmPgb1.1(A)] (Mira *et al.*, 2016*a*; Youseff *et al.*, 2016). The level of *ZmPgb.1.1* increased in WT root tips, especially in the QC, where it rose upon initiation of hypoxia before declining (Fig. 1A). Relative to WT QCs, the level of *ZmPgb1.1* was higher in the ZmPgb1.1(S) line and lower in the ZmPgb1.1(A) line (Supplementary Table S3). Regardless of the genotype, the QC niche at 0 h was composed of isodiametrical cells containing small vacuoles and retaining a quiescent state, as estimated by the absence of mitotic figures (Supplementary Fig. S2A) and the lack of incorporation of the thymidine analog EdU (Supplementary Fig. S3). In WT roots, and to a larger extent in ZmPgb1.1(A) roots, hypoxia induced the mitotic activation of the QCs, followed by their degradation after 24 h (Fig. 1B; Supplementary Fig. S3). The rapidly activated QCs of hypoxic ZmPgb1.1(A) roots often proliferated into the subtending root cap (shown by an asterisk in Fig. 1B). Hypoxia had no effects on the QCs of the ZmPgb1.1(S) lines, which remained quiescent and retained their structural characteristics typically observed under normoxic conditions (Fig. 1B; Supplementary Fig. S3).

The anatomy of the proximally located procambial initials was analyzed to discern two morphological features of cellular differentiation: a weakly stained cytoplasm and the formation of vacuoles. After 24 h of hypoxia, initials overexpressing *ZmPgb1.1* retained a densely stained cytoplasm with few small vacuoles, as also observed under normoxia (Fig. 1C; Supplementary Fig. S2B). This was in contrast to initials of hypoxic WT roots or roots suppressing *ZmPgb1.1* which had a weakly stained cytoplasm and were highly vacuolated (Fig. 1C).

As mitotic activation of hypoxic QCs was observed during the first 12 h of treatment, transcriptional and metabolic studies were conducted within this time frame.

PIN1-auxin maxima and auxin activity in hypoxic QCs are retained by the overexpression of ZmPgb1.1

Quiescence and functionality of the QC are maintained by the flow of auxin establishing an auxin maximum at the QC (Kerk and Feldman, 1995). In WT roots, the imposition of hypoxic conditions reduced the signal of PIN1 (Fig. 2A, B) and DR5 (Fig. 2C) along the root tip profile, especially within the QC. Overexpression of *ZmPgb1.1* attenuated the inhibitory effect of hypoxia on PIN1 transcripts (Fig. 2A) and IAA accumulation in the QC (Fig. 2D), while suppression of *ZmPgb1.1* abolished the PIN1 signal in the root proper, with a strong signal appearing in the columella cells (Fig. 2A), and dissipated the IAA maximum in the QC (Fig. 2D). The regulation of *ZmPgb1.1* on PIN transcripts was mediated by NO, as a rise in NO (by SNAP) in hypoxic ZmPgb1.1(S) roots [characterized by low levels of NO (Mira *et al.*, 2016*a*)] mimicked the ZmPgb1.1(A) phenotype (compare Fig. 2A with Supplementary Fig. S4). Similarly, a reduction in NO (by cPTIO) in hypoxic

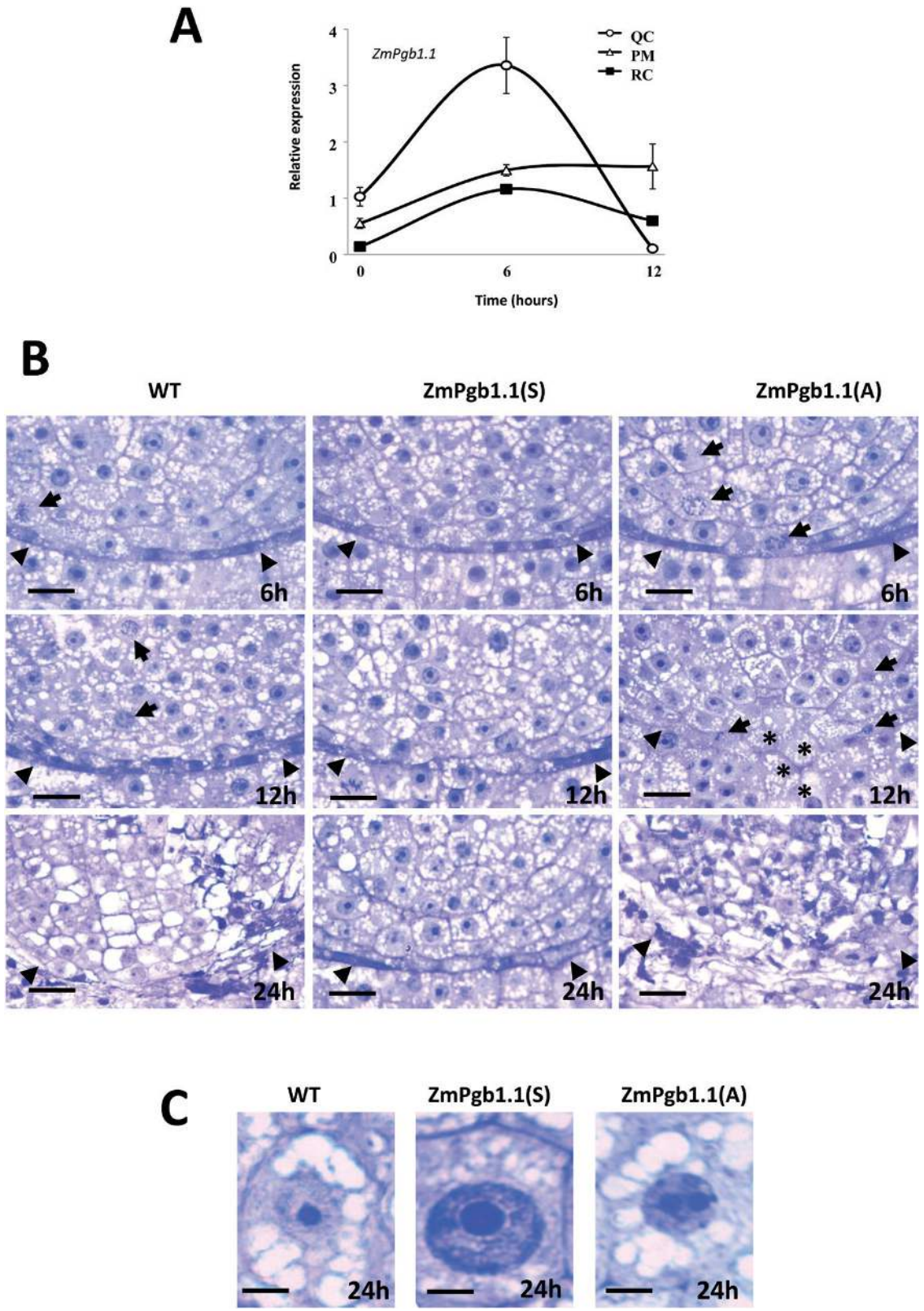

Fig. 1. Expression of *ZmPgb1.1* and morphology in hypoxic roots. (A) Transcript levels of *ZmPgb.1.1* in quiescent centers (QCs), proximal meristems (PMs), and root caps (RCs) of WT roots exposed to hypoxia for 12 h. Values are means ±SE of three biological replicates each consisting of 30 root compartments, and are normalized to the QC (0 h) value set at 1. (B) Epoxy sections showing the morphology of RAMs of WT roots and roots overexpressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1* at 6, 12, and 24 h during hypoxia. Arrows demark mitotic figures apparent in WT QCs and especially in ZmPgb1.1(A) QCs, both of which show signs of deterioration at 24 h. Proliferating cells (*) of ZmPgb1.1(A) QCs impinge into the subtending RC. Arrowheads denote the QC–RC junction. (C) Morphology of the proximal initials of hypoxic roots showing signs of vacuolation in the WT and ZmPgb1.1(A) line. Pictures are representative micrographs from >30 roots. Scale bars=30 μm (B) and 5 μm (C).

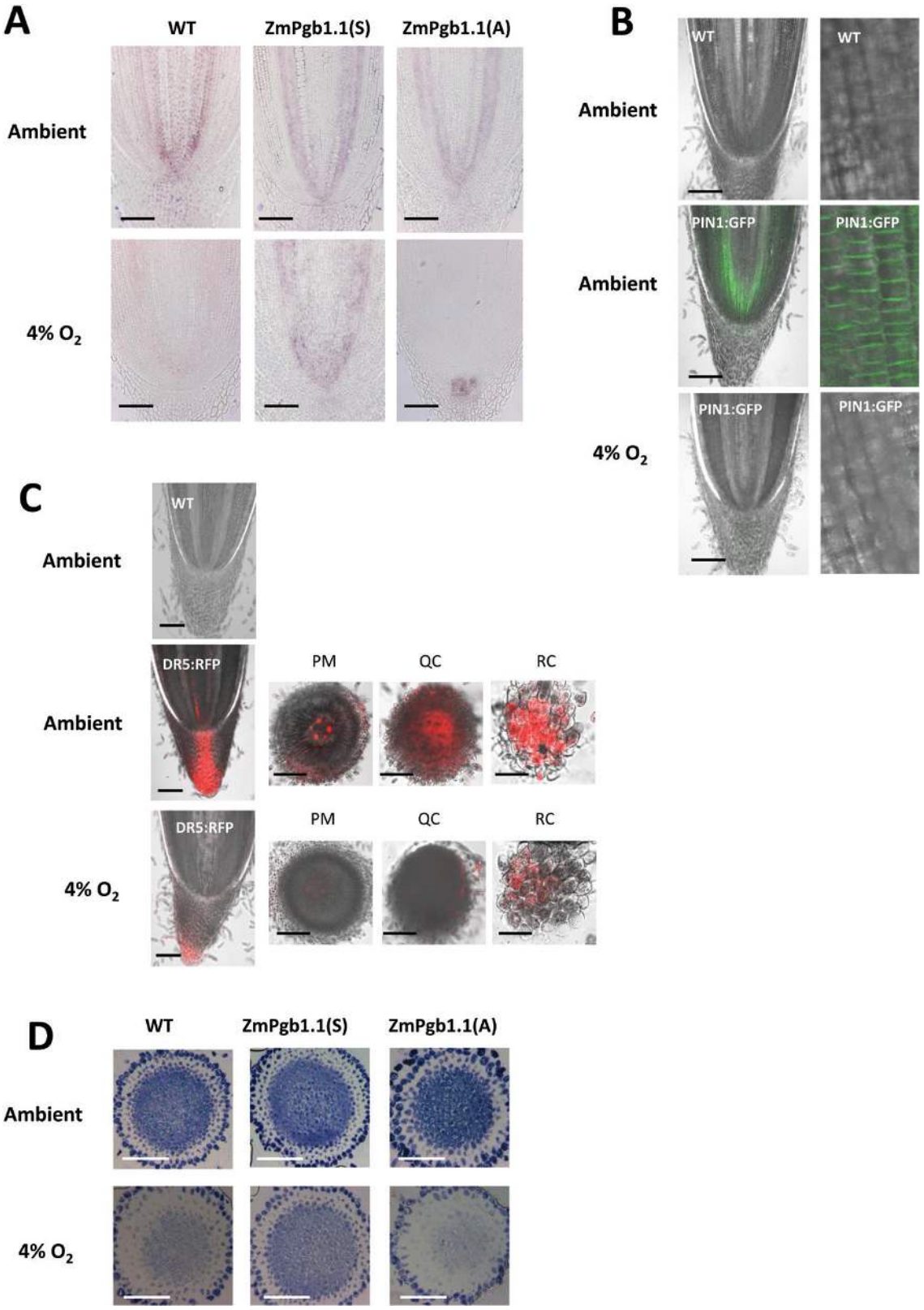

Fig. 2. Localization patterns of *PIN1* and auxin in maize roots. (A) Paraffin section showing the localization of *PIN1* transcripts by RNA *in situ* hybridization in WT roots and roots overexpressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1.* (B) PIN1:GFP signal in WT roots. (C) DR5:RFP signal in whole WT root tips and specific compartments (PM, QC, and RC). (D) Compresstome sections showing the immunolocalization of IAA in QCs dissected from the three different lines. Normoxic (ambient) or hypoxic (4% O_2) treatments were imposed for 12 h. Pictures are representative micrographs from >20 roots. Scale bars=300 μm.

ZmPgb1.1(A) roots [characterized by high levels of NO (Mira *et al.*, 2016*a*)] re-established the ZmPgb1.1(S) phenotype (compare Fig. 2A with Supplementary Fig. S4).

Auxin responses in hypoxic QCs were assayed by measuring the transcript levels of some AUXIN RESPONSIVE FACTORS (ARFs), expressed at the QC niche and correlating to auxin maxima (Jiang *et al.*, 2010). In WT and *ZmPgb1.1*-suppressing QCs, the transcript levels of *ARF2*, *5*, *6*, *7*, *8*, and *10* diminished during the hypoxic treatment; this trend was mitigated in QCs overexpressing *ZmPgb1.1* (Fig. 3).

Collectively these data demonstrate the requirement of *ZmPgb1.1* for the maintenance of the PIN1-auxin accumulation and auxin response in hypoxic QCs.

Specification of the QC niche during hypoxia requires ZmPgb1.1

To unequivocally assess if the retention of the auxin flow by *ZmPgb1.1*, through NO, is sufficient for the specification of the QC niche, we followed the re-establishment of a new QC in hypoxic roots in which the original QC was surgically removed. Reformation of a QC, compromised by the polar transport inhibitor NPA [*N*-1-naphthylphthalamic acid (10^{-5} M); Jiang *et al.* (2003)] under normoxic conditions, occurred at higher frequency during hypoxia when NO was diminished genetically [ZmPgb1.1(S) line], or pharmacologically by applications of cPTIO to the ZmPgb1.1(A) line that accumulates

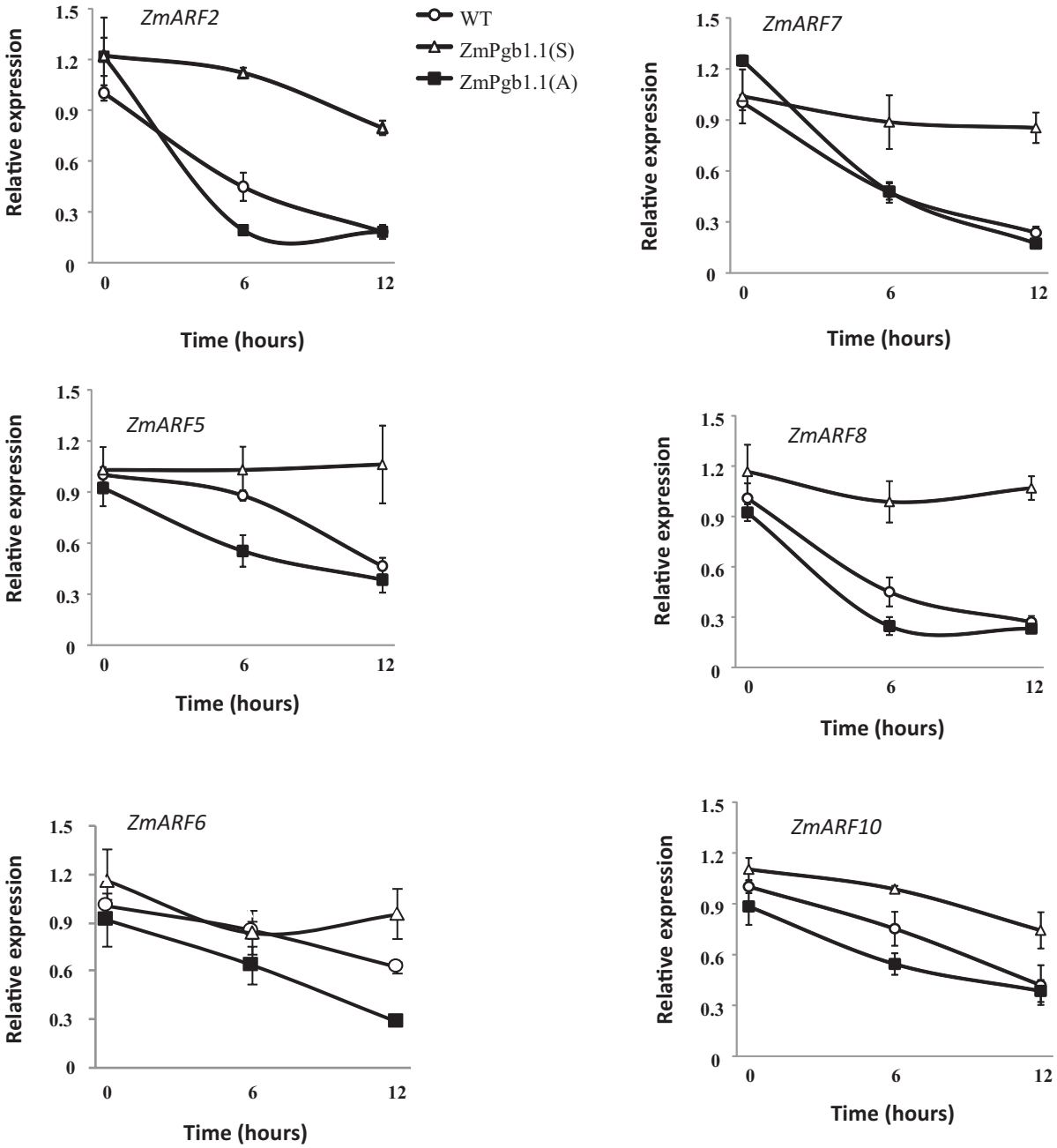

Fig. 3. Auxin response factors in hypoxic QCs. Transcript levels of the *Auxin Responsive Factor* genes (*ZmARF2*, *5*, *6*, *7*, *8* and *10*) in QCs of WT roots and roots overexpressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1*. subjected to hypoxia for 12 h. Values are means ±SE of three biological replicates each consisting of 30 root compartments, and are normalized to the WT (0 h) value set at 1.

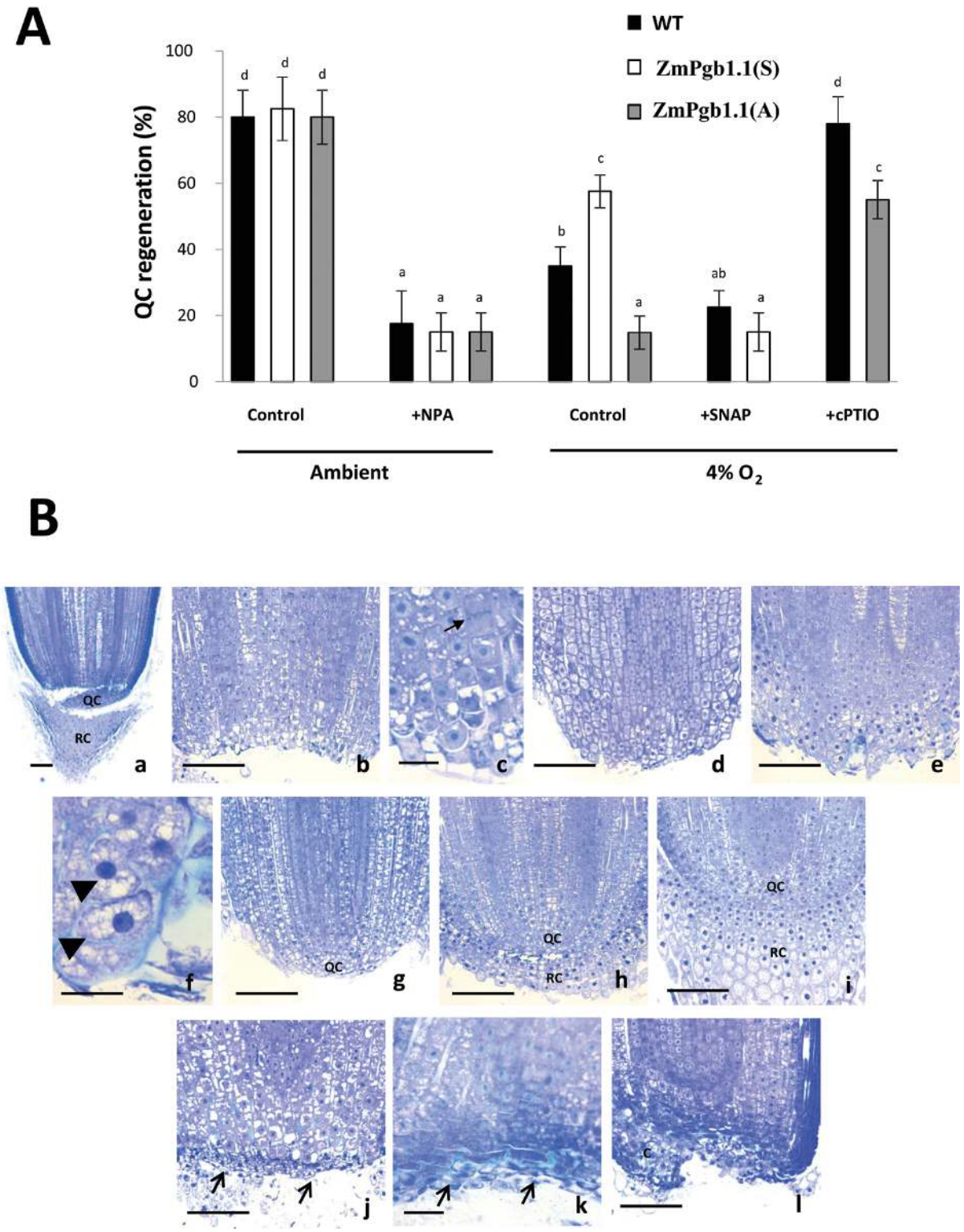

Fig. 4. *De novo* regeneration of QCs in normoxic (ambient) or hypoxic (4% O_2) maize roots. (A) The frequency of roots able to regenerate a new QC was measured 24 h after the removal of the original QC. Roots were also treated with the auxin flow inhibitor NPA. Roots with low levels of NO [(ZmPgb1.1(S)] were treated with the NO donor SNAP, while roots with high levels of NO [ZmPgb1.1(A)] were treated with the NO scavenger cPTIO. Both compounds were also used in WT roots. Values are means ±SE of four biological replicates each consisting of >10 roots. Letters indicate statistically significant differences (P<0.05). (B) Epoxy section showing the structural events during the *de novo* regeneration of the QCs. The RC and the QC can be removed from hypoxic maize roots (a). After 3 h (b), mitotic figures (arrow) become visible in cells above the removed QC (c). Cellular proliferation contributes to the regeneration of new apical tissue (d) and, by 6 h, cells in the apical region become vacuolated (e, and arrowheads in f), a characteristic feature of the QC niche. After 12 h, a newly formed QC is apparent (g) and this is followed by the reformation of a RC by 24 h (h and i). This developmental pattern was observed in all the roots able to reform a new QC, regardless of the genotype. In roots unable to regenerate a new QC, mainly from WT and ZmPgb1.1(A) lines, accumulation of heavily stained compounds, possibly phenols (arrows), preceded cell death and growth arrest (j and k). In some instances growth cessation was observed after the incomplete regeneration of the apical tissue (l). Scale bars=200 μm (a, b, d, e, g, h, i, j, and l) and 20 μm (c, f, and k).

high levels of NO (Mira *et al.*, 2016*a*) (Fig. 4). Low QC regeneration frequency was observed in the ZmPgb1.1(A) line, accumulating high levels of NO (Mira *et al.*, 2016*a*), or by applications of the NO donor SNAP to the ZmPgb1.1(S) line (Fig. 4) that is known to have low NO levels (Mira *et al.*, 2016*a*).

Confirmation of the oxidized environment in the QC and variations in redox state during hypoxia

Mitotic activation patterns in the root tip are governed by the auxin flow. The establishment of an auxin maximum at the QC favors a highly oxidized environment of lower AA/DHA and GSH/GSSG ratios, conferring a quiescent state (Jiang *et al.*, 2003). A more reduced environment with elevated AA/DHA and GSH/GSSG ratios within the PM is conducive to proliferation (Jiang *et al.*, 2003). This model, which held true prior to hypoxic treatments (Supplementary Tables S4–S6), with variations in absolute values from those reported previously (Jiang *et al.*, 2003) ascribed to genotypic differences, was perturbed by the imposition of hypoxia.

In QCs from WT and ZmPgb1.1(A) roots, hypoxia diminished the levels of DHA and GSSG while elevating those of AA and GSH (Supplementary Fig. S5). These changes, generating a more reduced state with higher AA/DHA and GSH/GSSG ratios, were minimized in QCs overexpressing *ZmPgb1.1* which retained a highly oxidized environment (Fig. 5). The different redox environments among hypoxic QCs of the different lines were verified by visualizing AA, which accumulated in WT and, especially in ZmPgb1.1(A) QCs; and the signal of the redox sensitive dye H2DCFDA, fluorescing preferentially in oxidized environments, which was retained in hypoxic QCs overexpressing *ZmPgb1.1* and almost lost in those of WT and ZmPgb1.1(A) roots (Fig. S6).

The observed redox patterns in hypoxic QCs were further examined by analyzing the activity of ascorbate and glutathione redox enzymes. Relative to QCs from WT and *ZmPgb1.1* suppressing roots, the activity levels of AAO and APX were higher in hypoxic QCs overexpressing *ZmPgb1.1* (Fig. 6). An opposite trend was observed for DHAR, MDHAR, and, to a lesser extent, GR, which were more highly active in hypoxic QCs of WT roots and roots suppressing *ZmPgb1.1* (Fig. 6). A similar pattern was observed at the transcriptional level (Supplementary Fig. S7, S8).

The energy status is altered by ZmPgb1.1 *expression in hypoxic QCs*

Mitochondrial function, sensitive to oxidative stress (Fujie *et al.*, 1993), can be estimated using MitoTraker Orange which stains mitochondria able to maintain a membrane potential. In normoxic QCs, staining was confined to the peripheral domains of the QCs (Supplementary Fig. S9), confirming the need for impaired mitochondrial function in the central domain of the QC to maintain the quiescent state (Jiang *et al.*, 2006). This characteristic pattern was retained in hypoxic QCs overexpressing *ZmPgb1.1*, but altered in QCs from WT and ZmPgb1.1(A) roots where fluorescence often extended to the central domains of the QC without a precise pattern (Supplementary Fig. S9)

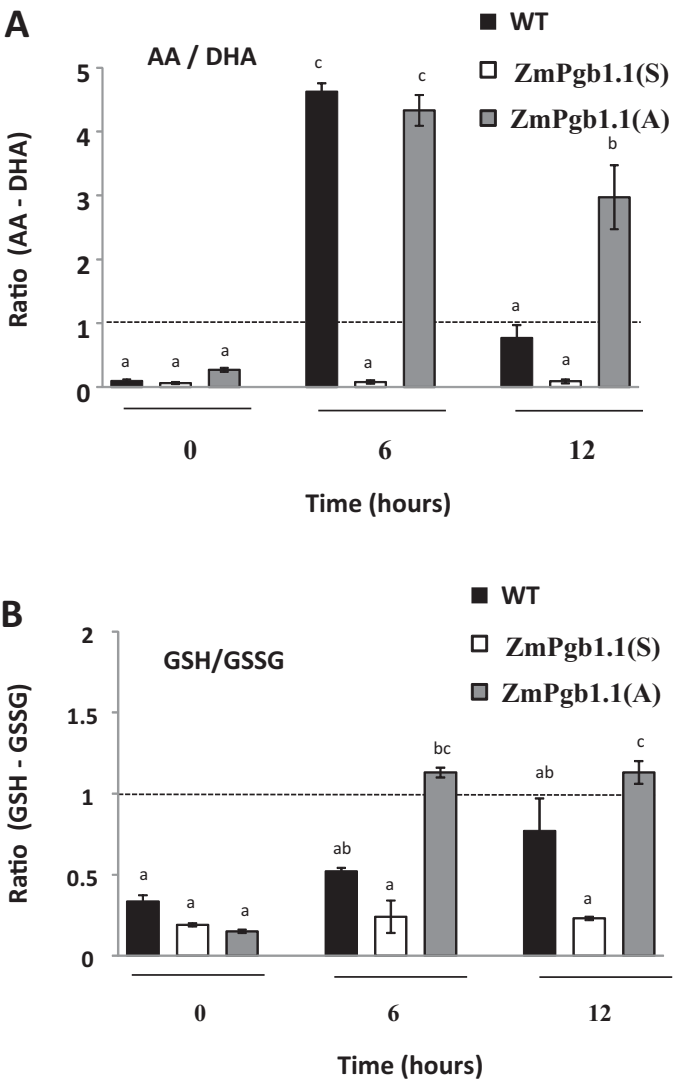

Fig. 5. Ascorbate and glutathione redox state in hypoxic QCs. Measurements of the AA/DHA and GSH/GSSG ratios in QCs dissected from WT roots and roots overexpressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1*. Measurements were conducted during the 12 h of hypoxic treatment. Values are means ±SE of two biological replicates each consisting of 80 QCs. Letters indicate statistically significant differences ($P<0.05$).

The activity of the fermentation enzyme ADH increased during hypoxia, especially in QCs of WT and ZmPgb1.1(A) lines, and to a lesser extent in QCs from the ZmPgb1.1(S) line (Fig. 7A). The *ZmPgb1.1* effect on ADH activity was specific to the QC and not observed in other root tip compartments (i.e. RC and PM; Supplementary Fig. S10).

The higher increase of ADH activity observed in hypoxic QCs of WT and *ZmPgb1.1* suppressing lines was accompanied by a substantial decrease in the ATP/ADP ratio. This decrease was attenuated in QCs overexpressing *ZmPgb1.1* (Fig. 7B)

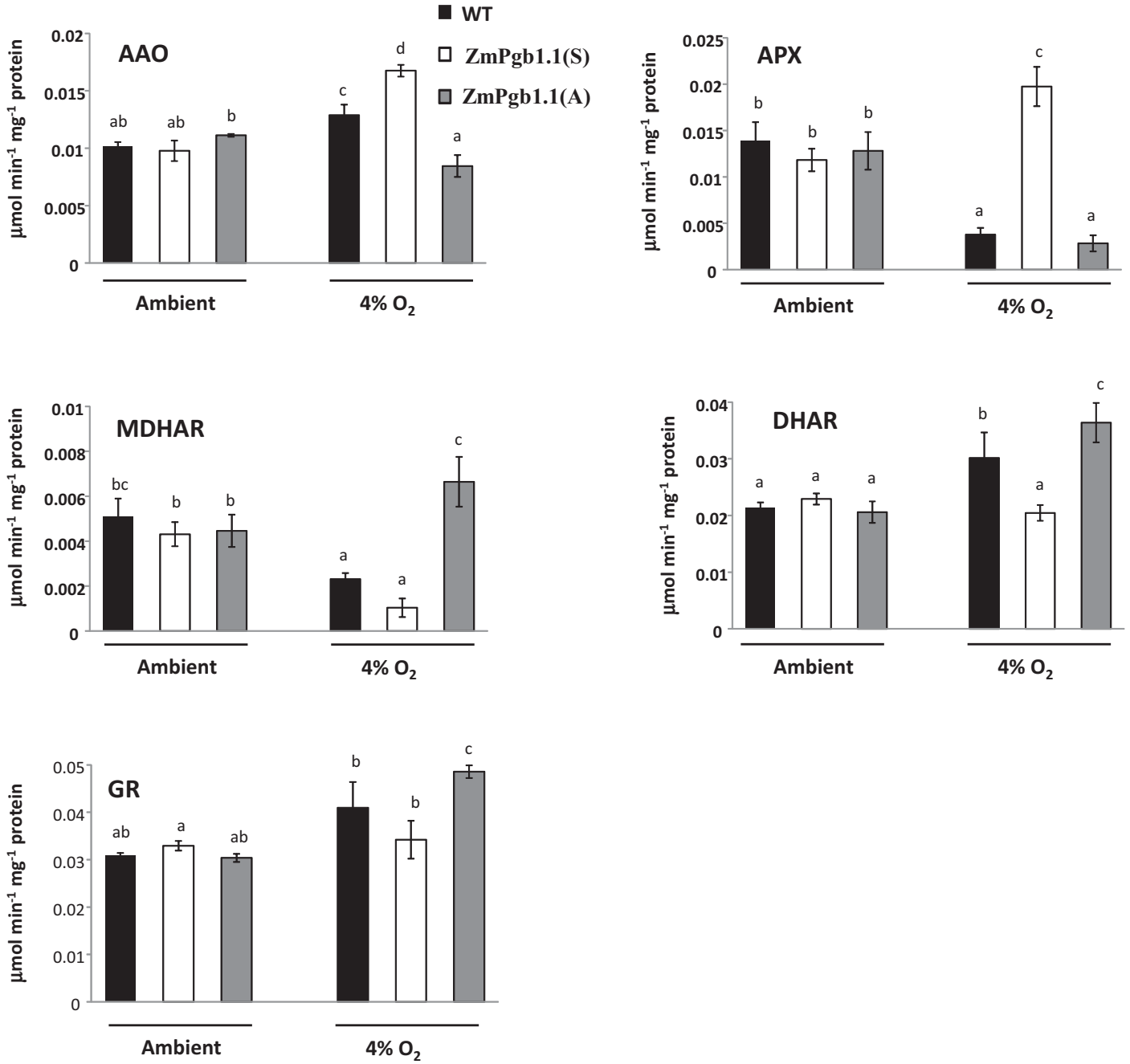

Fig. 6. Activities of ascorbate oxidase (AAO), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) in QCs dissected from WT roots and roots overexpressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1* . Measurements were conducted after 12 h of normoxic (ambient) or hypoxic (4% O_2) treatments. Values are means ±SE of two biological replicates each consisting of 80 QCs. Letters indicate statistically significant differences (P<0.05).

Overexpression of ZmPgb1.1 *retains the functionality of QCs during hypoxia*

The physiological alterations observed at the QC suggest that hypoxia compromises QC functionality, and that overexpression of *ZmPgb1.1* mitigates these effects. To unequivocally demonstrate this concept, functionality of QCs was evaluated by monitoring the ability of QCs dissected from normoxic and hypoxic roots to regenerate roots *in vitro* (Feldman, 1976) (Supplementary Fig. S11), and measuring the transcript levels of *WUSCHEL RELATED HOMEOBOX* (*WOX5*) and *PLETHORA* (*PLT*), both markers of QC specification (Aida *et al.*, 2004; Forzani *et al.*, 2014).

About 50–60% of QCs dissected from roots grown in ambient air regenerated roots, a trend observed for all genotypes (Fig. 8A). This percentage diminished markedly in ZmPgb1.1 (A) QCs and, to a lesser extent, in WT QCs, dissected from hypoxic roots. The decline in root regeneration from hypoxic QCs was attenuated by overexpressing *ZmPgb1.1*. These effects were dependent upon the level of DHA [added to the ZmPgb1.1(A) line characterized by a high AA/DHA ratio, Fig. 5A], and AA [added to the ZmPgb1.1(S) line characterized by a low AA/DHA ratio, Fig. 5A] (Fig. 8A).

While remaining unchanged or declining marginally in hypoxic QCs overexpressing *ZmPgb1.1*, the expression of *ZmPLT*

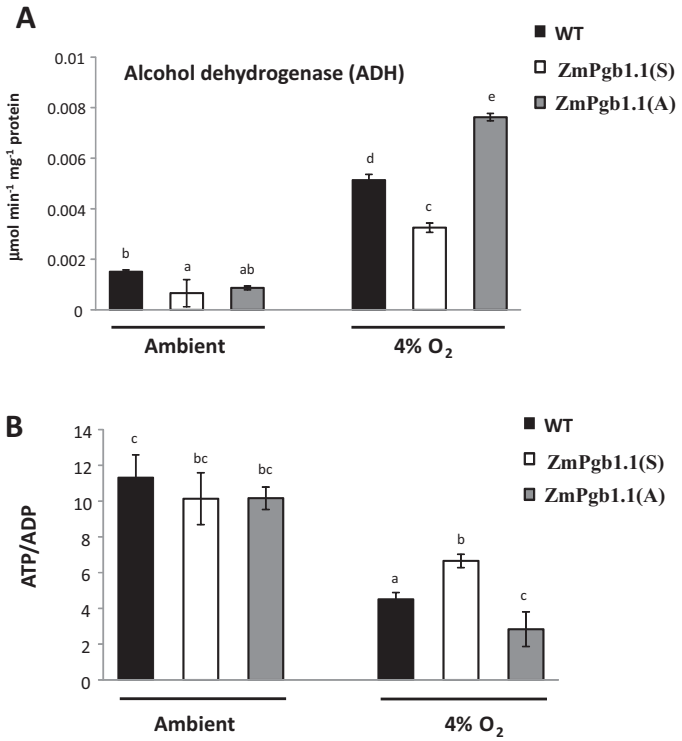

Fig. 7. Activity of the fermentation enzyme alcohol dehydrogenase (ADH) (A) and energy status (ATP/ADP) (B) in QCs of WT roots and roots overexpressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1*. Measurements were conducted after 12 h of normoxic (ambient) or hypoxic (4% O_2) treatments. Values are means ±SE of two biological replicates each consisting of 80 QCs. Letters indicate statistically significant differences (P<0.05).

and *ZmWOX5* diminished markedly in WT and *ZmPgb1.1* suppressing QCs. (Fig. 8B).

Discussion

Located in the center of the RAM, the QC acts as an 'organizing center' conferring a meristematic undifferentiated state to the surrounding initials, which through iterative divisions provide the diverse cell types for the root system (Dolan *et al.*, 1993). This conserved function is paramount for the regulation of cell division and differentiation within the meristematic region (Hong *et al.*, 2015). Factors damaging or activating the QC perturb the dynamic homeostasis and lead to root tip abnormalities (Jiang *et al.*, 2003), as was also observed under stress conditions (Mira *et al.*, 2016*a*, 2017). Thus, maintaining the viability of the QC is a determinant factor for root survival. Here, we demonstrate that changing the expression level of the maize Pgb gene *ZmPgb1.1* causes alterations in the state of the QC cells when exposed to hypoxic stress. Suppressing *ZmPgb1.1*, whose expression increases during hypoxia in the QC (Fig. 1A), induced the mitotic activation and eventually the degradation of the QC after 24 h of hypoxic treatment (Fig. 1B; Supplementary Fig. S3). Overexpression of *ZmPgb1.1* mitigated these effects and retained the state of quiescence in the QC. The requirement for high levels of *ZmPgb1.1* for QC niche specification and functionality during conditions of hypoxia was unequivocally demonstrated by several pieces of evidence. First, the expression level of *ZmPLT* and *ZmWOX5*, genes associated with QC specification (Aida *et al.*, 2004; Forzani *et al.*, 2014), remained high in hypoxic ZmPgb1.1(S) QCs relative to QCs from WT and *ZmPgb1.1* suppressing roots (Fig. 8B). In addition, the capacity of hypoxic roots to regenerate new QC cells following excision of the QC (Fig. 4) and of QCs dissected from hypoxic roots to regenerate viable roots *in vitro* (Fig. 8A) was enhanced under conditions in which the expression of *ZmPgb1.1* was elevated. Finally, several normoxic-like physiological conditions were retained in hypoxic QCs overexpressing *ZmPgb1.1*, relative to their WT and *ZmPgb1.1* suppressing counterparts. These include the typical pattern of mitochondria membrane depolarization (Supplementary Fig. S9), linked to the quiescence of the QC (Jiang *et al.*, 2006), as well as a lower fermentation activity (Fig. 7A) with a concomitant higher energy status (Fig. 7B). The significant differences in ADH activity and ATP/ADP ratios among the different lines are indicative of the role of *ZmPgb1.1* in reducing the need for substrate level phosphorylation in hypoxic QCs.

The changes in QC functionality under hypoxic conditions would be expected to result in changes in metabolism that reflect the state of cells. Under hypoxia the environment in the QC cells shifts from one that is highly oxidized, conferring the state of quiescence (Jiang *et al.*, 2003) as typically observed in QCs of normoxic roots, to one that is highly reduced as evidenced by the high AA/DHA and GSH/GSSG ratios (Fig. 5). The establishment or the imposition of a reduced environment is linked to the induction of cell division and the disorganization of the QC (Liso *et al.*, 1984; Innocenti *et al.*, 1990; Jiang *et al.*, 2003), an observation confirmed here (Fig. 1B; Supplementary Fig. S3). The localization of AA and the use of the redox-sensitive dye H2DCFDA further support the changes in the redox state of hypoxic QCs (Supplementary Fig. S5). Overexpression of *ZmPgb1.1* is sufficient to retain an oxidized environment in the QCs. Fluctuations in the AA/DHA and GSH/GSSG ratios are probably determined by reactions within the ascorbate–glutathione cycle. In a more oxidized environment, an increase in activity in AAO and APX would be anticipated, while in a reduced environment enzymes such as MDHAR, DHAR, and GR would predominate. This holds true in the QC for these enzymes under hypoxic conditions when comparing the ZmPgb1.1(S) versus ZmPgb1.1(A) line (Fig. 6). A similar regulation of Pgb by the redox enzymes was also observed in alfalfa roots (Igamberdiev *et al.*, 2006).

Changes related to auxin play a fundamental role in QC function. The formation of an auxin maximum at the QC is essential for the establishment of a highly oxidized environment and the state of quiescence (Jiang *et al.*, 2003). Physiological or genetic perturbations in auxin distribution cause abnormalities at the QC site (Sabatini *et al.*, 1999; Jiang *et al.*, 2003). Here we show that by modulating NO homeostasis, expression of *ZmPgb1.1* influences PIN1 localization and ultimately auxin distribution (Fig. 2; Supplementary Fig. S4). The auxin maximum, which dissipates in the central domains of QCs of hypoxic WT and *ZmPgb1.1* suppressing lines, is unaltered in QCs of roots overexpressing *ZmPgb1.1* (Fig. 2C, D). These latter QCs also better retain the ability to respond to

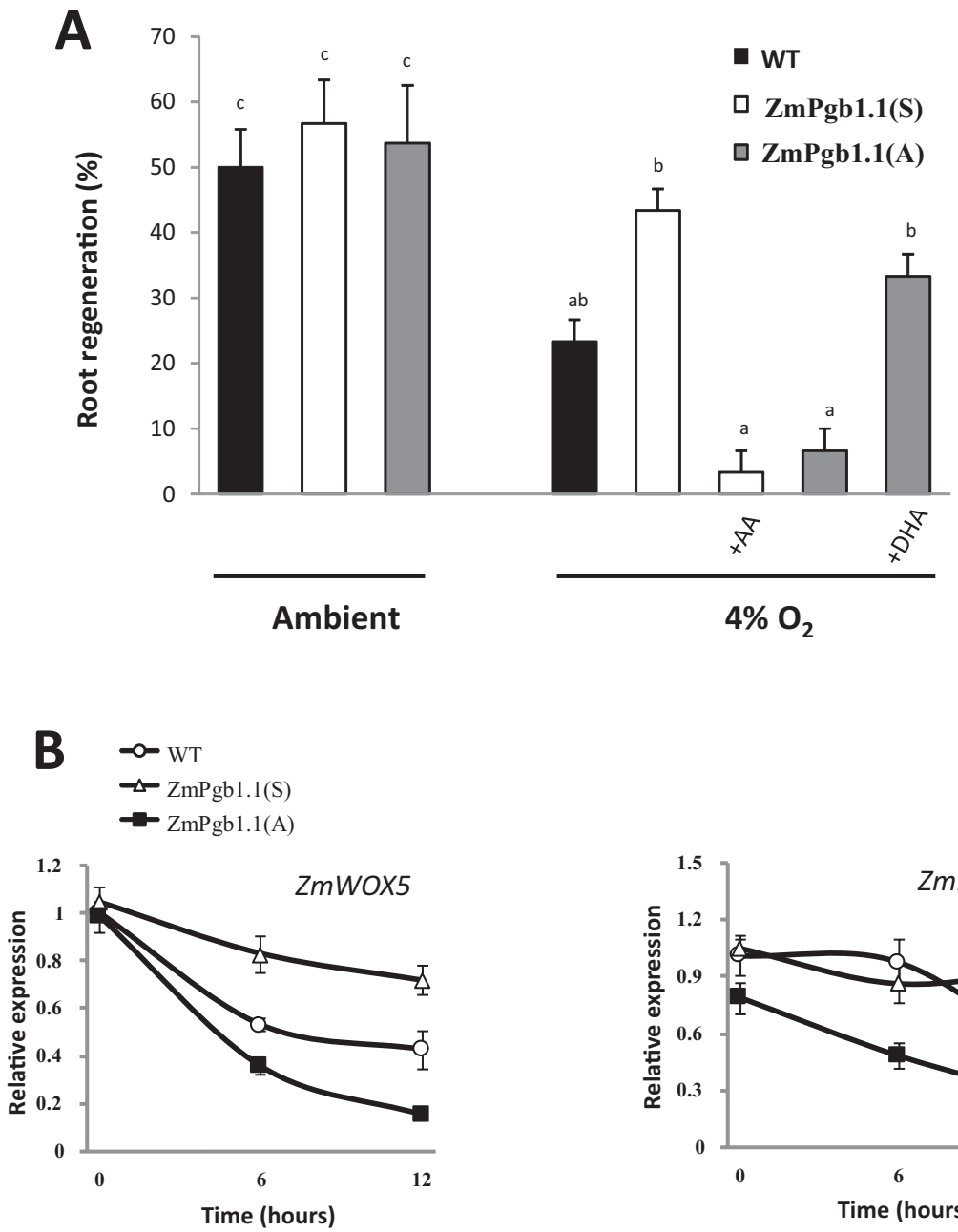

Fig. 8. Viability of maize QCs. (A) Percentage of *in vitro* root regeneration from isolated QCs dissected from WT roots and roots overexpressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1*. QCs were excised after 12 h of normoxic (ambient) or hypoxic (4% O_2) treatments. Excised QCs from the ZmPgb1.1(S) line characterized by a low AA/DHA ratio (Fig. 5A) were treated with exogenous AA, while QCs of the ZmPgb1.1(A) line, characterized by a high AA/DHA ratio (Fig. 5A), were treated with DHA. Values are means ±SE of three biological replicates each consisting of 20 QCs. Letters indicate statistically significant differences (P<0.05). (B) Relative transcript levels of the QC marker genes *ZmWOX5* and *ZmPLT* in QCs isolated from the three lines during the 12 h of hypoxic treatment. Values are means ±SE of three biological replicates each consisting of 30 QCs and are normalized to the WT (0 h) value set at 1.

auxin (Fig. 3). The observed differences in auxin response and distribution among lines are attributable to the influence of *ZmPgb1.1* level on PIN1 localization, which is compromised by hypoxia in WT and *ZmPgb1.1* suppressing roots, but not in those in which *ZmPgb1.1* is elevated (Fig. 2A). We have previously established that suppression of an Arabidopsis *Pgb* increases auxin production and alters PIN protein polarization during Arabidopsis somatic embryogenesis (Elhiti *et al.*, 2013), and that the effects on auxin production require the presence of Pgb in the nucleus (Godee *et al.*, 2017). Thus, if a similar situation exists in hypoxic maize roots, the effect of *ZmPgb1.1* expression may occur in the cell cytoplasm to achieve the observed results.

Hypoxic stress limits root functionality by perturbing the auxin flow and distribution required for the establishment of an oxidized redox environment in the QC, a prerogative for the maintenance of a state of quiescence and functionality in the QC. As a consequence, mitotic proliferation in the QC is activated, resulting in structural abnormalities at the root tip. These effects are aggravated by the suppression of *ZmPgb1.1*

and are alleviated when *ZmPgb1.1* is overexpressed. In light of the previously described participation of Pgbs in stress responses (Hill, 2012), their link with auxin movement (Elhiti *et al.*, 2013), and their requirement for root tip functionality during hypoxia (Mira *et al.*, 2016*a*) and drought (Mira *et al.*, 2017), it is suggested that Pgbs influence plant response to diverse conditions of stress.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Dissection of root compartments from maize roots.

Fig. S2. Structure of QCs and proximal initials in normoxic roots.

Fig. S3. Detection of S-phase cell division in hypoxic QCs.

Fig. S4. Effects of NO manipulations on PIN1 expression.

Fig. S5. Measurements of ascorbate and glutathione in QCs.

Fig. S6. Localization of ascorbic acid and measurement of the redox environment in QCs.

Fig. S7. Relative abundance of transcript levels of ascorbate enzymes.

Fig. S8. Relative abundance of transcript levels of glutathione reductase.

Fig. S9. Mitochondrial membrane potential in maize QCs.

Fig. S10. Activity of alcohol dehydrogenase in root compartments.

Fig. S11. Developmental sequence of *de novo* root formation from cultured QCs.

Table S1. List of primers utilized in the experiments.

Table S2. Efficiency of primers used for q(RT)–PCR.

Table S3. Expression level of *ZmPgb1.1* in QCs of the transgenic lines.

Table S4. Levels of AA and DHA in QCs and PMs before the imposition of hypoxia.

Table S5. Levels of GSH and GSSG in QCs and PMs before the imposition of hypoxia.

Table S6. AA/DHA and GSH/GSSG ratios in QCs and PMs before the imposition of hypoxia.

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