

RESEARCH PAPER

# Expression of the ethylene biosynthetic machinery in maize roots is regulated in response to hypoxia

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## Abstract

Ethylene regulates plant growth in response to many adverse environmental conditions, including the induction of aerenchyma, i.e. the formation of air spaces, in flooded roots in an effort to maintain oxygen levels. In this work, quantitative RT-PCR and *in situ* RNA hybridization were used to determine how the expression of the ethylene biosynthetic machinery in maize roots is spatially and temporally regulated following exposure to 4% oxygen (i.e. hypoxia) for up to 24 h, conditions that induced aerenchyma formation in the fully-expanded region of the root and reduced cytoplasmic density throughout the root. Expression of ACC oxidase, the ethylene forming enzyme, was observed in the root cap, protophloem sieve elements, and companion cells associated with metaploem sieve elements. Exposure to 4% oxygen induced ACC oxidase expression in these cell types as well as in the root cortex. ACC synthase, which generates the ethylene precursor, was expressed in the root cap and the cortex and its expression was induced in cortical cells following low oxygen treatment. The induction of expression of the ethylene biosynthetic machinery was accompanied by an induction of ethylene evolution and a reduced rate of root growth. These results suggest that maize roots respond to conditions of hypoxia by inducing the spatially restricted expression of the ethylene biosynthetic machinery, resulting in increased ethylene production.

**Key words:** ACC oxidase, ACC synthase, ethylene, hypoxia, maize, root growth.

## Introduction

The plant hormone ethylene regulates diverse aspects of plant growth and development, including regulating the rate of germination, seedling growth in the absence of light, sex determination, elongation of the stem or other organs in rice, fruit ripening, organ abscission, leaf and flower senescence, and cell death during cereal endosperm development (Ecker and Davis, 1987; Mattoo and Suttle, 1991; Abeles *et al.*, 1992; Grbic and Bleecker, 1995; John *et al.*, 1995; Young *et al.*, 1997). Although not essential for most aspects of plant development, ethylene may be required for growth under certain natural conditions (Harp-ham *et al.*, 1991). Ethylene regulates responses to adverse growth conditions, such as hypoxia, mechanical impedance, and pathogen attack (Drew *et al.*, 1979; Feldman, 1984; Lee *et al.*, 1990; Mattoo and Suttle, 1991; Abeles *et al.*, 1992;

Zacarias and Reid, 1992; Dolan, 1998; Pitts *et al.*, 1998; Clark *et al.*, 1999; Schiefelbein, 2000; Buer *et al.*, 2003). Ethylene also regulates several aspects of root growth and development, including growth rate, adventitious root formation, root hair growth and development, and gravitropism (Feldman, 1984; Lee *et al.*, 1990; Mattoo and Suttle, 1991; Abeles *et al.*, 1992; Zacarias and Reid, 1992; Dolan, 1998; Pitts *et al.*, 1998; Clark *et al.*, 1999; Schiefelbein, 2000; Buer *et al.*, 2003; Hahn *et al.*, 2008).

Roots act as an important sensor of many environmental conditions such as lack of water, soil compaction, or flooding (reviewed in Davies and Zhang, 1991). One means by which roots signal to the aerial parts of the plant is through ethylene-mediated changes in root growth and/or in the synthesis of ACC that is then transported through the

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; ACO, ACC oxidase; CC, companion cells; EtOH, ethanol; MSE, metaploem sieve element; NBT, indoxyl-nitroblue tetrazolium; QC, quiescent centre; QRT-PCR, quantitative RT-PCR; PSE, protophloem sieve element; TE, TRIS-EDTA.  
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plant (Bergner and Teichmann, 1993; Else *et al.*, 1995; Drew, 1997; Beltrano *et al.*, 1999; Hussain *et al.*, 1999; Drew *et al.*, 2000). Ethylene-mediated regulation of root growth is achieved through the inhibition of elongation of cortical cells just distal to the root apex (Whalen and Feldman, 1988). In many species, including maize, the root undergoes remodelling in response to low oxygen that occurs during flooding through the selective death of cortical cells (Drew *et al.*, 1979, 2000; Justin and Armstrong, 1987; Drew, 1997). The hypoxic-induced cell death of cortical cells results in the generation of lysigenous aerenchyma (i.e. air spaces) to promote the diffusion of air into the lower regions experiencing oxygen-limiting conditions (Drew *et al.*, 1979, 2000; Drew, 1997). The generation of aerenchyma increases the survival of the stele, which contains the vascular system, and the root apex, which is highly sensitive to changes in oxygen availability as a consequence of its high rate of respiration. Hypoxia-induced cortical cell death is mediated by ethylene and is accompanied by increases in ACC synthase and ACC oxidase expression in several species (Wang and Arteca, 1992; Zarembinski and Theologis, 1993; He *et al.*, 1994, 1996a; Shiu *et al.*, 1998; Zhou *et al.*, 2001). Aerenchyma formation can be induced by exogenous ethylene and blocked by inhibitors of ethylene synthesis or action (Drew *et al.*, 1979, 1981; Konings, 1982; Jackson *et al.*, 1985; Atwell *et al.*, 1988; He *et al.*, 1994, 1996a, b). The cell death induced by hypoxia is specific to the cortex, suggesting differential expression of the ethylene biosynthetic machinery in the cortex or sensitivity of this cell type to ethylene.

Ethylene is produced from methionine in which the latter is converted initially to *S*-adenosylmethionine (AdoMet) by *S*-adenosylmethionine synthase, which is then converted to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS). The generation of ethylene results from the oxidation of ACC by ACC oxidase (ACO) in a reaction that also produces CO<sub>2</sub> and HCN (Yang and Hoffman, 1984). Both ACS and ACO are encoded by multigene families: for example, the *Arabidopsis* genome contains nine ACS genes which exhibit cell specific and overlapping expression (Liang *et al.*, 1992; Zarembinski and Theologis, 1994; Tsuchisaka and Theologis, 2004). With the exception of two, all members are expressed in the vascular tissue of the maturation zone of the root but only ACS8 is also expressed in the root cap (Tsuchisaka and Theologis, 2004). Expression of *OS-ACS5*, one of the five members of the rice ACS gene family, is restricted largely to vascular tissues of stems and young leaves of air-grown plants and is induced by submergence (Zhou *et al.*, 2002). *OS-ACS5* is also expressed in the elongation zone of lateral roots (Zhou *et al.*, 2002). Because considerably less is known about the spatial or temporal expression of ACC oxidase (Nakatsuka *et al.*, 1998; Sell and Hehl, 2005), it is not known whether those root tissues that express ACS also express ACO.

In maize, the ACS gene family (*ZmACS*) is composed of three members, i.e. *ZmACS2*, *ZmACS6*, and *ZmACS7*, and the ACO gene family (*ZmACO*) is composed of four

members, i.e. *ZmACO15*, *ZmACO20*, *ZmACO31*, and *ZmACO35* (Gallie and Young, 2004). In a knockout approach, *ZmACS6* was identified as being responsible for the bulk of foliar ethylene production, and regulates leaf senescence, photosynthetic function, and drought tolerance (Young *et al.*, 2004). *ZmACS2* knockout mutants exhibited a smaller reduction in foliar ethylene production and an intermediate phenotype related to leaf senescence and photosynthetic function (Young *et al.*, 2004). *ZmACS6*, responsible for the bulk of ethylene produced in roots, regulates root growth in response to mechanical impedance (Gallie *et al.*, 2009).

In this report, it was investigated how the ethylene biosynthetic machinery in maize roots is spatially and temporally regulated under hypoxic conditions. Roots were treated with 4% oxygen for up to 24 h as these were conditions that resulted in the formation of aerenchyma, diagnostic for a hypoxic response (He *et al.*, 1996a), but avoided an anoxic response as oxygen is required for the generation of ethylene and the formation of aerenchyma (Drew *et al.*, 1979). Exposure to 4% oxygen results in an increase in the rate of ethylene production, ACC content, and the activity of ACC synthase and ACC oxidase in maize roots (Drew *et al.*, 1979; Jackson *et al.*, 1985; Atwell *et al.*, 1988; He *et al.*, 1994, 1996a, b). Partial pressures of 3–5 kPa O<sub>2</sub> were the most effective in eliciting aerenchyma formation (Jackson *et al.*, 1985). Treatment with 4% oxygen, therefore, allowed us to examine the changes in the expression of members of the ethylene biosynthetic machinery as part of the optimal response to hypoxia. All three *ZmACS* gene family members were expressed in maize roots: *ZmACS6* was expressed in the root cap and in the root proper whereas *ZmACS2* and *ZmACS7* were expressed in the inner cortex of the elongation zone. Induction of *ZmACS2* and *ZmACS7* in response to hypoxia was observed, particularly in the cortical region distal from the root apex whereas expression of *ZmACS6* was induced proximal to the root apex. Expression of the *ZmACO* subfamily (i.e. *ZmACO15/31*) in roots maintained at a normal oxygen level (i.e. normoxic roots) was restricted to the root cap and the protophloem sieve element (PSE) whereas expression of the *ZmACO20/35* subfamily was confined to the root cap and the companion cells (CC) associated with the protophloem. Transient induction in response to hypoxia was greatest for *ZmACO15*, with induction of the other three gene family members occurring to lower extents. Hypoxic induced expression of *ZmACO* was observed in cortical cells as well as in those tissues expressing these genes under normal growth conditions. As observed with the *ZmACS* gene family members, the level of induction of *ZmACO* gene family members was lower following 24 h of hypoxic treatment than during shorter periods of hypoxia. The induction of the ethylene biosynthetic machinery correlated with an increase in ethylene production in roots subjected to hypoxia, suggesting that maize roots respond to conditions of hypoxia by inducing the spatially restricted expression of the ethylene biosynthetic machinery in order to increase ethylene production.

## Materials and methods

### Plant growth conditions

The maize inbred line B73 was used throughout the study. The apical 1 cm of roots from 1-week-old seedlings subjected to conditions of hypoxia [i.e., 4% (v/v) oxygen/96% nitrogen] was collected as indicated and fixed overnight with 2% glutaldehyde and paraformaldehyde in 75 mM phosphate buffer, pH 7.2. The roots were dehydrated in an EtOH series, infiltrated and embedded in JB-4 (Polysciences, Inc.). Three  $\mu\text{m}$  longitudinal sections were collected using a Hacker 5030 microtome and stained with 1% Aniline Blue Black (for protein staining) and in some cases counterstained with Periodic Acid Schiff (for carbohydrate staining). Digital images of median sections were collected using a Leica microscope with brightfield optics.

### Semi-quantitative RT-PCR and QRT-PCR

Total nucleic acid was isolated from roots from 1-week-old seedlings subjected to conditions of hypoxia, i.e. 4% (v/v) oxygen/96% nitrogen, as described by Young *et al.* (2004). Following the initial precipitation and resuspension in TE, total RNA was further purified by two rounds of  $\text{LiCl}_2$  precipitation as described by Sambrook *et al.* (1989). 50  $\mu\text{g}$  total RNA was treated with RQ1 DNase (Promega) to ensure that no contaminating DNA was present. Two  $\mu\text{g}$  of total RNA were used for cDNA synthesis using the Omniscript RT kit (Qiagen) with oligo-dT<sub>20</sub> as the primer.

Preliminary analysis of transcript abundance was accomplished using semi-quantitative RT-PCR with the following conditions: 95 °C for 15 min (1 cycle); 95 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min (35–37 cycles); 72 °C for 5 min (1 cycle). Analysis of transcript abundance by quantitative RT-PCR (QRT-PCR) was accomplished using the QuantiTect SYBR Green PCR kit (Qiagen). Reactions contained 1 $\times$  buffer, 0.5  $\mu\text{l}$  of the reverse transcription reaction (equivalent to 50 ng total RNA) and 0.25  $\mu\text{M}$  (final concentration) forward and reverse primers in a total reaction volume of 25  $\mu\text{l}$ . Reactions were carried out using an ABI PRISM 7700 sequence detection system under the following conditions: 95 °C for 15 min (1 cycle); 95 °C for 30 s, 62 °C for 30 s, 72 °C for 2 min (50 cycles); 72 °C for 5 min (1 cycle). Each gene was analysed a minimum of four times and the average and standard deviation reported. All primer combinations (Table 1) were initially run and visualized on an agarose gel to confirm the presence of a single product of the correct size. All amplification products were subcloned into the pGEM-T Easy vector system (Promega) and used for the generation of standard curves to facilitate conversion of the expression data to the number of copies  $\mu\text{g}$  RNA basis.

### RNA in situ localization

RNA *in situ* localization was carried out as described previously by Langdale (1994) with modifications. Roots were placed into FAA (50% EtOH, 10% formalin, 5% acetic acid), vacuum infiltrated,

and stored for 2 d at 4 °C. The fixative was replaced with 70% EtOH and the samples dehydrated through an EtOH series (85%, 95%, and 100%) at 1 d intervals at 4 °C. The EtOH was replaced with Hemo-De through a graded series [2 h 50% EtOH: 50% Hemo-De (Fisher), three treatments in 100% Hemo-De for 2 h]. Samples were then infiltrated in increasing concentrations of Paraplast Plus, embedded in 100% Paraplast Plus, sectioned on a rotary microtome (15  $\mu\text{m}$  thick), and fixed on Probe-On-Plus slides (Fisher). Sections were treated as described by Jackson (1991) with modifications. Sections were deparaffinized in 100% Hemo-De, rehydrated through an EtOH series, equilibrated in PBS, deproteinized with proteinase K, treated with glycine, and washed twice in PBS. Sections were post-fixed with 4% paraformaldehyde, acetylated with acetic anhydride, washed, and finally dehydrated through an EtOH series. For RNA *in situ* hybridization, sense or antisense *ZmACS* or *ZmACO* RNA labelled with DIG-UTP was denatured at 80 °C, added to the hybridization solution (0.3 M NaCl, 10 mM TRIS-HCl, pH 6.8, 10 mM  $\text{NaH}_2\text{PO}_4$ , 5 mM EDTA, 50% formamide, 10% dextran sulphate, 1 $\times$  Denhardtts, 1 mg  $\text{ml}^{-1}$  tRNA), and applied to the slide for overnight hybridization at 55 °C. The sections were washed, treated with RNase, blocked (using 1.0% Boehringer Block), and incubated with anti-DIG antibody. The sections were washed, covered with indoxyl-nitroblue tetrazolium (NBT) substrate solution, and developed in the dark for 1–3 d until the signal was visible.

### Ethylene determination

Ethylene was measured from root sections of 5-d-old seedlings subjected to conditions of hypoxia, i.e. 4% (v/v) oxygen/96% nitrogen, as indicated. Excised roots were allowed to recover for 30 min prior to collecting ethylene. Roots were placed in glass vials with 0.5 ml of water to maintain hydration of the roots and the vials capped with a rubber septum. Following a 2 h incubation, 0.9 ml of headspace was sampled from each vial and the ethylene content measured using a 6850 series gas chromatography system (Hewlett-Packard, Palo Alto, CA) equipped with a HP Plot alumina-based capillary column (Agilent Technologies, Palo Alto, CA), which can detect as little as 10  $\text{nl l}^{-1}$  (10 ppb) ethylene. The ethylene peak was identified as that which had the same retention time as pure ethylene. Tissue fresh weight was measured for each sample. Three replicates were measured and the average and standard deviation reported.

## Results

### Roots undergo a reduction in cytoplasmic density following the imposition of hypoxic conditions

The ethylene biosynthetic machinery exhibits tissue-specific expression within the root cap and within the zones of cell

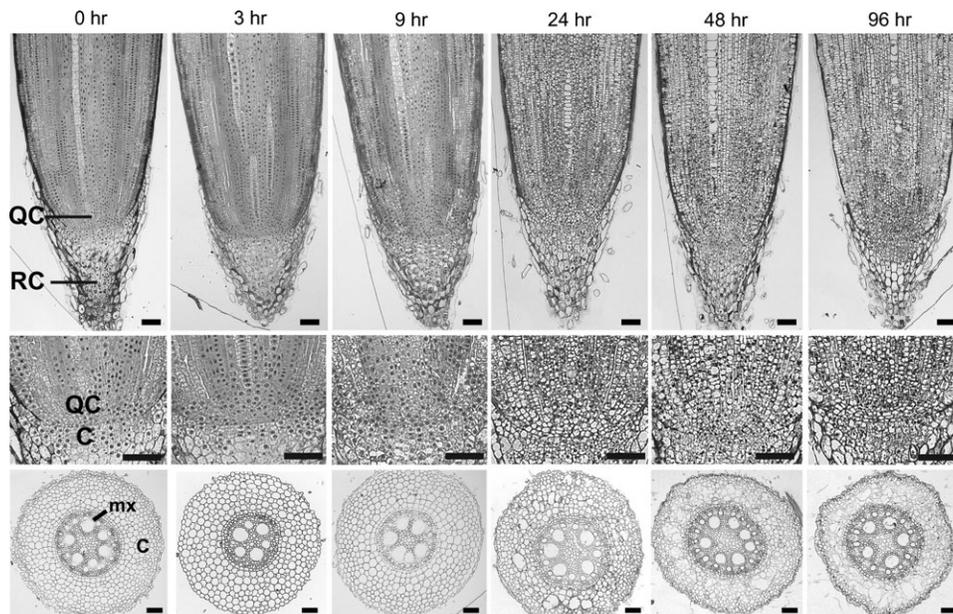
**Table 1.** Forward and reverse primers

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
<i>ZmACS2</i>	ATCGCGTACAGCCTCTCCAAGGA	GATAGTCTTTTGTCAACCATCCCATAGA
<i>ZmACS6</i>	AGCTGTGGAAGAAGGTGGTCTTCGAGGT	AGTACGTGACCGTGGTTTCTATGA
<i>ZmACS2</i>	ATCGCGTACAGCCTCTCCAAGGA	GATAGTCTTTTGTCAACCATCCCATAGA
<i>ZmACS7</i>	ATCGCGTACAGCCTCTCCAAGGA	CAACGCTCTGTCACTCTGTGTAATGT
<i>ZmACO15</i>	CTCGTCTTCGATCAATTCCCAAGT	TACATTATCAATTATTCTCCGGCTGT
<i>ZmACO20</i>	CTCATCCTGCTGCTCCAGGACGAC	TCCACGATACACGCATAAACCACCGT
<i>ZmACO31</i>	CTCGTCTTCGATCAATTCCCAAGT	ATAGCAAAGAGGGCAACTAGCTAGT
<i>ZmACO35</i>	CTCATCCTGCTGCTCCAGGACGAC	ACACACATAACTGTGCCACTATAAGCA
$\beta$ -Tubulin	ACCAGATCGGCGCCAAGTTCT	CATCATGTTCTTGGCATCCCACA

division and elongation (Gallie *et al.*, 2009). Consequently, it was examined whether exposure to hypoxia altered cell development in these regions of the root. Roots from 1-week-old seedlings were placed in 0.5% MS solution through which 4% oxygen (i.e. 4% (v/v) oxygen/96% nitrogen) was bubbled while the shoots were maintained in air. The use of an 'open' system provides additional oxygen to the roots through internal diffusion which avoids anaerobic conditions (Raymond *et al.*, 1978; Armstrong, 1979; Erdmann and Wiedenroth, 1988; He *et al.*, 1994). Moreover, treatment with 4% oxygen has been shown to result in increased ethylene production, the formation of aerenchyma, and increased tolerance to anoxia (Andrews *et al.*, 1994; Johnson *et al.*, 1994; Cobb *et al.*, 1995), indicating that this level of oxygen is sufficient to sustain ethylene biosynthesis. The seedlings were immersed to just below the scutellar node while the aerial portions of the seedling remained in air. The roots were maintained under low oxygen conditions for 3, 9, 24, 48, and 96 h and the roots collected, fixed, and embedded in plastic resin for sectioning. Longitudinal sections (3  $\mu$ m) were stained with 1% Aniline Blue Black and counterstained with Periodic Acid Schiff. With the exception of the expanding metaxylem, cells throughout normoxic roots were cytoplasmically dense as were cells of the calyptrogen (root cap initials) and the columella (central column of cells of the root cap) (Fig. 1). Beginning at 9 h of hypoxia, a reduction in the cytoplasmic density was observed throughout the root that became prominent by 24 h of hypoxia and was maintained during further hypoxic treatment. Loss of the cytoplasmic density was observed for virtually all cells,

including those in the quiescent centre (QC), the root initials, and the calyptrogen (Fig. 1). Aerenchyma did not develop in the zones of cell division and elongation but it was observed in the mid-cortex of the zone of the root representing the fully-expanded region (Fig. 1), consistent with previous observations that cell death in the hypoxic root is confined to the zone where cell elongation is complete and formation of aerenchyma becomes increasingly prominent in older zones beginning at about 10 mm behind the root apex (Drew *et al.*, 1979, 2000; Konings, 1982; Campbell and Drew, 1983; Kawai *et al.*, 1998; Schussler and Longstreth, 2000). Under the conditions used, aerenchyma was first observed by 24 h of hypoxic treatment. This is in good agreement with the formation of pre-aerenchyma at 12 h, developing aerenchyma at 18 h, and developed aerenchyma at 26 h in hypoxic roots (Gunawardena *et al.*, 2001). By 4 d of hypoxic treatment, which is equivalent to a prolonged imposition of hypoxic stress, significant portions of the cortex had undergone cell death (Fig. 1). Even following this severe stress, all cell types within the vascular cylinder remained intact, in good agreement with previous observations (He *et al.*, 1996a), which suggests that these cell types are protected from the hypoxic-induced cell death that occurs in the cortex.

Root growth is impeded when exposed to 3% oxygen (Gunawardena *et al.*, 2001). To examine the impact that exposure to 4% oxygen has on root growth, the elongation of roots from 5-d-old seedlings treated with 4% oxygen for up to 27 h was compared to those from normoxic seedlings. The growth rate of hypoxia-treated roots was lower relative to normoxic roots (Table 2). Growth for hypoxic roots



**Fig. 1.** Loss of cytoplasmic density following exposure to hypoxia. B73 roots from 1-week-old seedlings were subjected to hypoxia, i.e. 4% (v/v) oxygen/96% nitrogen, up to 96 h, which were then fixed and embedded prior to sectioning. Three  $\mu$ m sections were stained with 1% Aniline Blue Black and counterstained with Periodic Acid Schiff. The apical 1.5 mm of each root is shown in the longitudinal sections with the QC and calyptrogen shown immediately below each longitudinal section. Cross-sections from the mature portion of the root (i.e. the region following the cell division and elongation zones which represents the fully-expanded region) are shown at bottom.

during the first 3 h of low oxygen treatment was 63% of that of normoxic roots and progressively declined to 52% of that of normoxic roots. These results indicate that treatment of 4% oxygen was sufficient to reduce but not prevent root growth.

*Expression of ZmACS and ZmACO gene family members is induced in maize roots in response to hypoxia*

To measure changes in expression of the ethylene biosynthetic machinery, QRT-PCR was performed on RNA extracted from normoxic roots and roots exposed to 4% oxygen for up to 24 h. To increase the resolution of changes through the root, primary roots were divided into 0–2, 2–4,

and 4–6 mm sections from the root apex as well as 6–10 mm and 10–20 mm from the root apex prior to RNA extraction for QRT-PCR analysis. The analysis of all genes was performed with the same RNAs in order to make direct comparisons between expression levels.

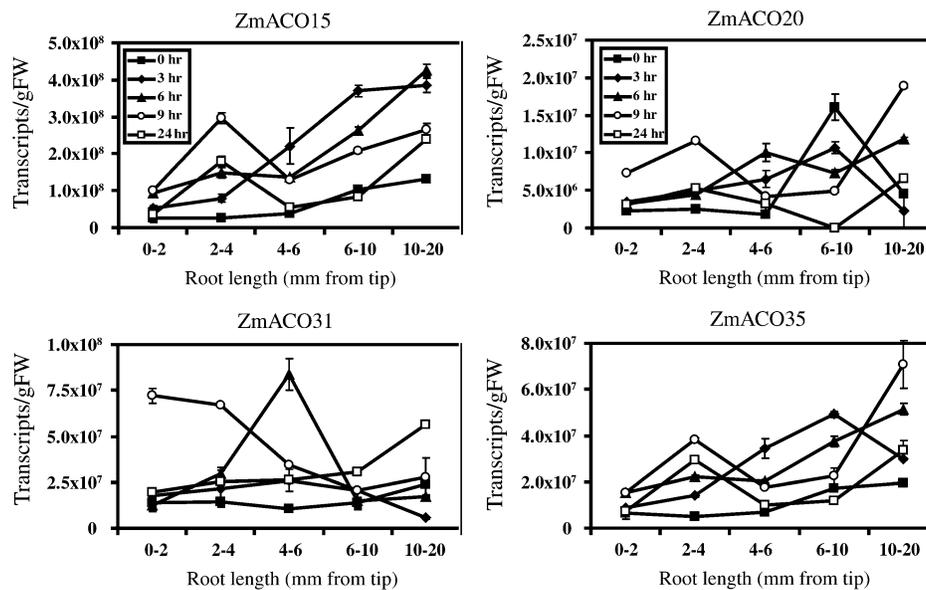
Of the four maize *ZmACO* genes, *ZmACO20* and *ZmACO35* form one subgroup based on sequence similarity and *ZmACO15* and *ZmACO31* form a second (Gallie and Young, 2004). Expression of *ZmACO15* was expressed at a lower level in the first 6 mm from the root apex than in the distal region (Fig. 2). Expression from *ZmACO15* throughout the length of the root examined was induced within 3 h of exposure to 4% oxygen. Even higher levels of induction were observed within the apical 4 mm following 6–9 h of exposure to 4% oxygen. By 24 h of hypoxia, however, expression from *ZmACO15* was reduced relative to shorter periods of hypoxia. Expression from *ZmACO31*, the other member of this subgroup, was also induced following exposure to 4% oxygen but the induction characteristics differed somewhat. Expression of *ZmACO31* was relatively constant throughout normoxic roots (Fig. 2). Exposure to 4% oxygen for 3 h resulted in moderate induction of expression. A 6 h treatment resulted in induction within the region from 2–6 mm whereas following 9 h of treatment, expression from 0–6 mm was substantially higher than in normoxic roots. By 24 h of treatment, the induction observed in the apical region of the root had decreased to near control levels.

Of the members of the *ZmACO* gene family, expression from *ZmACO20* was lowest (Fig. 2). Expression from *ZmACO20* was induced in most of the distal region of the

**Table 2.** Growth of roots under ambient and 4% oxygen

Time (h)	Root elongation <sup>a</sup>		Relative rate (hypoxic/control)
	Ambient air (mm)	4% Oxygen (mm)	
3	1.58±0.74	1.00±0.68	0.63
5.5	2.77±0.78	1.66±0.78	0.60
9	4.41±1.25	2.46±1.05	0.56
27	15.14±2.73	7.89±2.97	0.52

<sup>a</sup> Measurements were taken from 5-d-old normoxic seedlings or those subjected to 4% oxygen. Data represent the total amount of growth relative to the root length at 0 h. Normoxic roots had an average rate of elongation of 0.57 mm h<sup>-1</sup> whereas roots grown in 4% oxygen had an average rate of elongation of 0.29 mm h<sup>-1</sup>, 50.9% of that observed for normoxic roots.

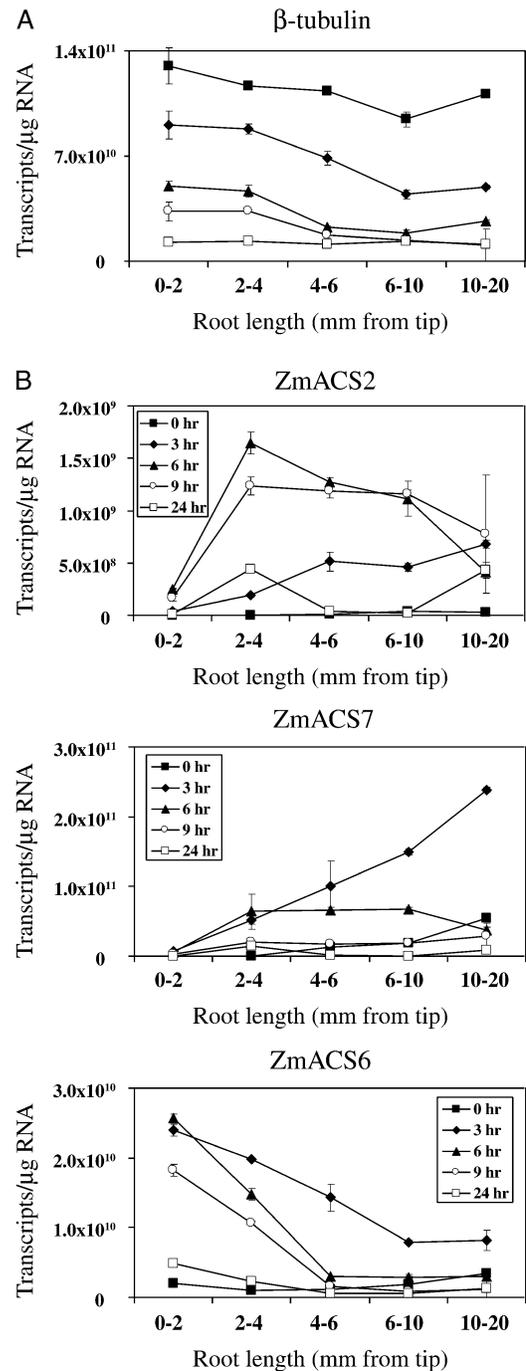


**Fig. 2.** Quantitative RT-PCR analysis of *ZmACO* gene family members in normoxic and hypoxic maize roots. Real-time QRT-PCR analysis of expression of *ZmACO15*, *ZmACO20*, *ZmACO31*, and *ZmACO35* was performed on RNA isolated from 2 mm sections for the first 6 mm of the apical region of the root as well as 6–10 mm and 10–20 mm from the root apex. Transcript amounts during development are expressed in exponential form and plotted on a RNA basis. Each gene was analysed a minimum of four times and the average and standard deviation are reported. Filled squares, normoxic roots; filled diamonds, 3 h hypoxic roots; filled triangles, 6 h hypoxic roots; open circles, 9 h hypoxic roots; open squares, 24 h hypoxic roots.

root within 3–6 h of exposure to 4% oxygen, but its expression in the apical region was induced by 9 h of the treatment. Expression was substantially reduced throughout the length of the root examined at 24 h of hypoxia relative to shorter hypoxic treatments, suggesting that induction of *ZmACO20* by hypoxia was transient. As with the other gene family members, expression from *ZmACO35* was induced following exposure to hypoxia (Fig. 2). Like *ZmACO20*, expression from *ZmACO35* was induced in the distal region of the root within 3–6 h of exposure to 4% oxygen but its expression in the apical region was also induced by 6–9 h of the treatment. Expression of *ZmACO35* was substantially reduced throughout the length of the root examined at 24 h of hypoxia relative to shorter hypoxic treatments.

Of the three members in the *ZmACS* gene family, *ZmACS2* and *ZmACS7* are closely related (95% amino acid identity) whereas the third gene (i.e. *ZmACS6*) is considerably more divergent (54% and 53% amino acid identity with *ZmACS2* and *ZmACS7*, respectively) (Gallie and Young, 2004). In the apical region of the root, expression from *ZmACS6* was highest followed by *ZmACS7*, with expression from *ZmACS2* the lowest. Expression from *ZmACS6* was induced substantially in the apical region of the root within 3–9 h of exposure to 4% oxygen which declined towards the distal portion of the root (Fig. 3). By 24 h of hypoxic treatment, however, induction of expression from *ZmACS6* had largely returned to control levels. The induction of expression from *ZmACS2* and *ZmACS7* following exposure to 4% oxygen differed from that of *ZmACS6* in that expression was induced as a function of the distance from the root apex, although some induction of expression from *ZmACS2* was observed within the apical 2 mm (Fig. 3). Expression from *ZmACS2* was induced moderately at 3 h of exposure to 4% oxygen, increased further by 6–9 h and declined again by 24 h of hypoxic treatment. Expression from *ZmACS7* was induced strongly by 3 h of exposure to 4% oxygen and declined progressively with longer hypoxic treatment to the point that expression following 9–24 h of exposure to 4% oxygen was lower than in normoxic roots. In contrast to the induction of expression from *ZmACS* and *ZmACO* genes, expression of  $\beta$ -tubulin mRNA was expressed at a relatively constant level from the root apex to at least 2 cm from the apex (Fig. 3).  $\beta$ -tubulin expression was highest in normoxic roots and declined as a function of the length of the hypoxic treatment. These results demonstrate that much of the induction of *ZmACS* and *ZmACO* gene expression is temporally restricted to the first 24 h of exposure to conditions of low oxygen.

To examine whether the same hypoxic treatment employed for the QRT-PCR analysis that resulted in the induction of *ZmACS* and *ZmACO* gene expression also resulted in an increase in ethylene production, ethylene was measured from roots treated to conditions of low oxygen. Ethylene evolution increased 4.2-fold in roots exposed to 4% oxygen for 3 h relative to normoxic roots (Table 3). A 5.8-fold increase in ethylene evolution was observed



**Fig. 3.** Quantitative RT-PCR analysis of *ZmACS* gene family members in maize roots. (A) Real-time QRT-PCR analysis of  $\beta$ -tubulin expression was performed on RNA isolated from 2 mm sections for the first 6 mm of the apical region of the root as well as 6–10 mm and 10–20 mm from the root apex. (B) Real-time QRT-PCR analysis of *ZmACS2*, *ZmACS7*, and *ZmACS6* expression performed as described for  $\beta$ -tubulin. Transcript amounts are expressed in exponential form and plotted on a RNA basis. Each gene was analysed a minimum of four times and the average and standard deviation are reported. Filled squares, normoxic roots; filled diamonds, 3 h hypoxic roots; filled triangles, 6 h hypoxic roots; open circles, 9 h hypoxic roots; open squares, 24 h hypoxic roots.

**Table 3.** Ethylene evolution from maize roots during hypoxia

Length of hypoxic treatment <sup>a</sup> (h)	Ethylene evolution (nl g <sup>-1</sup> FW h <sup>-1</sup> )
0 h	0.63±0.19
3 h	2.63±0.41
6 h	3.68±0.74
12 h	6.28±0.28
24 h	4.54±0.92
48 h	2.98±0.20

<sup>a</sup> Measurements were taken from 5-d-old seedlings treated with 4% oxygen.

following 6 h of hypoxic treatment and a 10-fold increase was observed by 12 h of hypoxic treatment. However, ethylene evolution increased 7.2-fold following 24 h of hypoxic treatment and only 4.7-fold following 48 h of hypoxic treatment. The induction of ethylene synthesis as measured with excised root tips is consistent with the induction measured by a continuous flow system of roots exposed to 3 kPa O<sub>2</sub> (Brailsford *et al.*, 1993). Our results suggest that, as with the induction of *ZmACS* and *ZmACO* gene expression, ethylene evolution is also induced under these hypoxic conditions. As hypoxia stimulates the formation of aerenchyma in maize roots by promoting ethylene synthesis, the appearance of aerenchyma in roots exposed to 4% oxygen supports the conclusion that oxygen sufficient to support a level of ethylene biosynthesis needed to signal aerenchyma formation was present in the roots during the hypoxic treatment. Moreover, the continued formation of aerenchyma for at least 4 d suggests that ethylene synthesis was ongoing during this period.

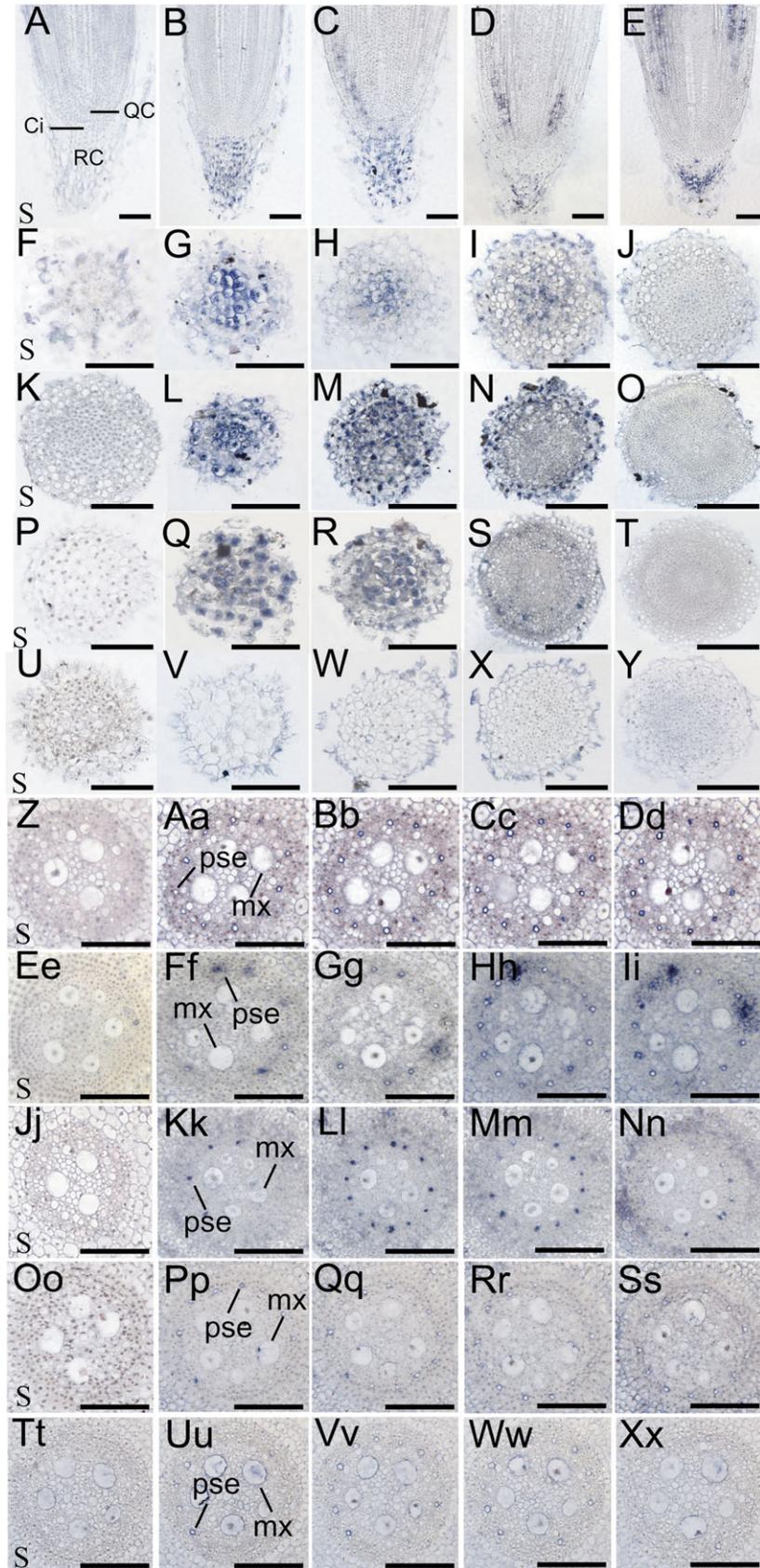
#### Induction of *ZmACS* and *ZmACO* gene expression during hypoxia is restricted to specific cell types

To determine whether the induction of *ZmACS* and *ZmACO* gene expression following exposure to conditions of hypoxia is spatially restricted, *in situ* RNA localization was performed on primary roots of 1-week-old seedlings exposed to hypoxic conditions. Because of the high degree of similarity within each of the two subgroups of the *ZmACO* gene family (i.e. the *ZmACO15/ZmACO31* and the *ZmACO20/ZmACO35* subgroups) (Gallie and Young, 2004), the *in situ* RNA localization analysis represents the combined expression of *ZmACO15* and *ZmACO31* and the combined expression of *ZmACO20* and *ZmACO35*. The *ZmACO15/31* subgroup is expressed in the root cap of normoxic roots but was limited to the columella with no detectable expression in the calyptrogen (Fig. 4B). No signal was detected when *ZmACO15/31* sense RNA was used as the probe (Fig. 4A). Expression was detected in the cortex of roots exposed to 4% oxygen for 3 h (Fig. 4C) and was also observed following 6 h of hypoxic treatment (Fig. 4D). Expression was concentrated in the mid-cortex close to the root initials. Following exposure to 4% oxygen for 9 h, expression in the mid-cortex was further from the root apex

than observed during shorter hypoxic treatments (Fig. 4E). Analysis of cross-sections at different positions within the root cap confirmed that *ZmACO15/31* expression was concentrated in the central columella (Fig. 4G–I). Hypoxic treatment increased expression to include a greater number of cells of the columella (Fig. 4L–N and Q–S), but no expression was observed in the calyptrogen (Fig. 4J, O, T). Little or no expression was detected in the root cap of roots exposed to 4% oxygen for 24 h (Fig. 4V–Y).

In the root proper of normoxic roots, *ZmACO15/31* expression was detected in the PSE but not in the metaphloem sieve element (MSE) or the metaxylem (Fig. 4Aa–Dd). No signal was detected in the PSE in sections probed with *ZmACO15/31* sense RNA (Fig. 4Z). No expression of *ZmACO15/31* was detected in the PSE in the mature region of the root (data not shown). In 3 h hypoxic roots, expression of *ZmACO15/31* was substantially induced (Fig. 4Ff–Ii). Expression of *ZmACO15/31* was not induced simultaneously in all PSE cells, but expression was observed in most PSE within 1–2 cells of the cell file, consistent with the observation that PSE do not to initiate differentiation at exactly the same stage (Eleftheriou, 1996). Expression of *ZmACO15/31* also occurred prior to the appearance of the MSE or the elongation and enucleation of the metaxylem. Expression of *ZmACO15/31* was also observed in most PSE of roots exposed to 4% oxygen for 6 h although the signal was less intense than observed in PSE in 3 h hypoxic roots, but remained stronger than that observed in normoxic roots (Fig. 4Kk–Nn). Expression of *ZmACO15/31* observed in cortical cells proximal to the endodermis in longitudinal sections of roots exposed to 4% oxygen for 6 h (Fig. 4E) can also be seen in cortical cells proximal to the endodermis in cross-sections of roots exposed to 4% oxygen for 6 h (Fig. 4Nn). The signal intensity in PSE of 9 h hypoxic roots was reduced further (Fig. 4Pp–Ss) and in roots exposed to 4% oxygen for 24 h, little expression was detected (Fig. 4Uu–Xx). These results suggest that the expression of *ZmACO15/31* in the root cap and in the PSE is induced following hypoxic treatment and that expression of *ZmACO15/31* is also induced in the mid-cortex.

Expression of *ZmACO20/35* in normoxic roots was also detected predominantly in the root cap, and like expression of *ZmACO15/31*, it was concentrated largely in the columella (Fig. 5B). No signal was detected in the root cap when sections were probed with *ZmACO20/35* sense RNA (Fig. 5A). Expression of *ZmACO20/35* was also detected in the root cap following several hours of hypoxic treatment (Fig. 5C, D). Analysis of cross-sections at different positions within the root cap confirmed its expression in the columella (Fig. 5L–N) but not in the calyptrogen (Fig. 5O). Expression of *ZmACO20/35* continued in the root cap of 9 h hypoxic roots (Fig. 5Q–T) but it was not detected following 24 h of hypoxic treatment (Fig. 5E). Although little expression of *ZmACO20/35* was detected in the cortex of normoxic roots (Fig. 5B, F), it was detected in 3 h hypoxic roots (Fig. 5C). This expression was also observed in the mid-cortex of the expansion zone of 6 h hypoxic roots (Fig. 5H–J). In addition, expression of *ZmACO20/35* was



**Fig. 4.** *In situ* localization of *ZmACO15/31* mRNA in maize roots. Median longitudinal sections from a 1-week-old normoxic root (B) and from hypoxic roots treated for 3 h (C), 6 h (D), or 9 h (E) with 4% (v/v) oxygen were hybridized with digoxigenin-labelled riboprobe representing antisense *ZmACO15/31* RNA. Similar hybridizations were performed on cross-sections (F-Xx) with the exception that digoxigenin-labelled riboprobe representing sense *ZmACO15/31* RNA was used to probe a median longitudinal section from a 1-week-

detected in the companion cells (CC) adjacent to the MSE in normoxic roots (Fig. 5F) and in roots subjected to hypoxic treatment (Fig. 5G–J). Expression of *ZmACO20/35* was observed following enucleation of the PSE, but prior to the enucleation of the MSE (Fig. 5V–Y) or metaxylem (Fig. 5F–J). By 6 h of hypoxic treatment, expression continued to be observed in the CC and the signal was more readily detected (Fig. 5Aa–Dd). Following 9 h of hypoxic treatment, expression of *ZmACO20/35* in the CC increased substantially (Fig. 5Ff–Ii). These results suggest that expression of *ZmACO20/35* in the root cap is maintained for a few hours after exposure to hypoxic treatment but decreases within 24 h of the treatment. Concurrent with this is an increase in expression of *ZmACO20/35* in the CC and an induction of its expression in the mid-cortex.

Expression of *ZmACS6* was detected in the peripheral cell layer of the root cap of normoxic roots but not in the columella or the calyptragen of the root cap (Fig. 6A). Low expression of *ZmACS6* was also detected in the outer cortex of normoxic roots (Fig. 6A). Following 3 h of hypoxic treatment, expression of *ZmACS6* was induced throughout the cortex, largely in a stochastic manner but somewhat concentrated in the mid to outer cortex (Fig. 6B–D). Analysis of cross-sections at different positions in the root cap confirmed its induction in the cortex following 3 h (Fig. 6M–O) or 9 h (Fig. 6R–T) of hypoxic treatment.

Because of the high degree of homology between *ZmACS2* and *ZmACS7*, the *in situ* RNA localization analysis could not discriminate between these two members and therefore the analysis represents the combined expression of *ZmACS2* and *ZmACS7* (i.e. *ZmACS2/7*). Expression of *ZmACS2/7* in normoxic roots was detected at the apex of the root cap (Fig. 6U, Aa–Bb) but not in the columella or the calyptragen of the root cap or the QC (Fig. 6U) and this expression was little changed following the onset of hypoxia (Fig. 6V). *ZmACS2/7* expression was also detected in the cortical cells proximal to the vascular cylinder in the elongation zone of normoxic roots (Fig. 6U). An increase in the expression of *ZmACS2/7* was observed in the inner cortex by 3 h of hypoxic treatment (Fig. 6V) with further increases in expression observed by 9 h of hypoxic treatment (Fig. 6W–X). By 24 h of hypoxic treatment, expression of *ZmACS2/7* had declined and was largely limited once again to cortical cells proximal to the endodermis (Fig. 6Y).

## Discussion

In this study, it is shown that the cell-specific expression of *ZmACS* and *ZmACO* gene family members in maize roots

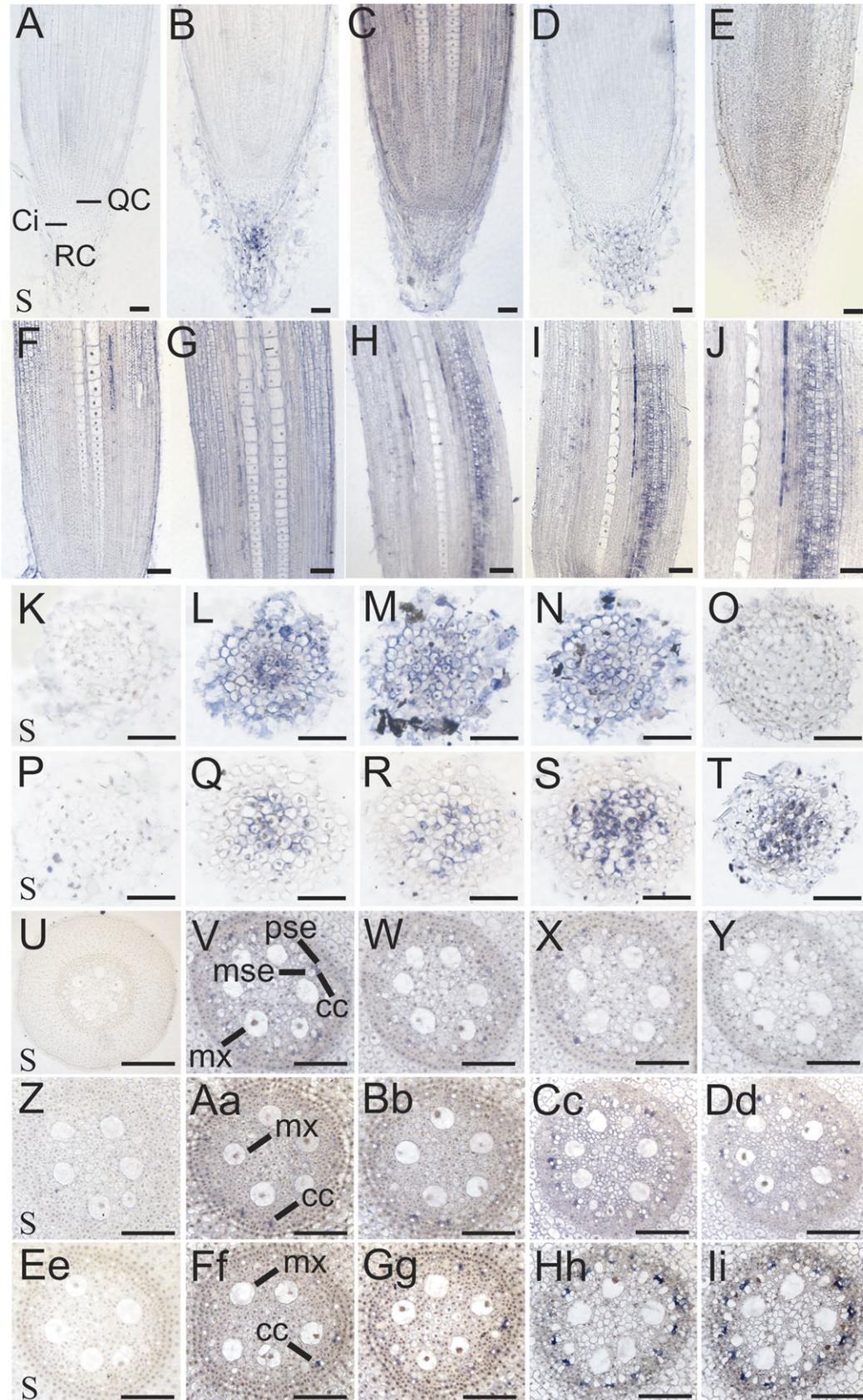
is transiently induced in a cell-specific manner following exposure to conditions of hypoxia as determined by QRT-PCR, *in situ* hybridization, and direct measurement of ethylene evolution. *ZmACS6* was expressed to a substantially higher level and was subject to greater induction in the first 2 mm of the root tip than either *ZmACS2* or *ZmACS7*. Expression of *ZmACS7*, however, was induced to increasingly higher levels as a function of the age of root tissue, becoming the most abundantly expressed member in the fully expanded region of the root by 3 h of hypoxic treatment. *ZmACS2* remained the lowest expressed member throughout the portion of the root analysed. Of the *ZmACO* gene family members, *ZmACO15* was expressed to a higher level and induced by hypoxia to a greater extent than the other members. Both its expression and its induction increased with distance from the root apex. The induction of *ZmACO20* and *ZmACO35* expression followed a similar pattern but to a lower level. The induction of *ZmACS* and *ZmACO* expression in response to treatment with 4% oxygen was transient. A similar induction was observed for ACC synthase activity in maize roots exposed to 4% oxygen (He *et al.*, 1994). The activity of ACC synthase increased for the first 12 hours of hypoxic treatment after which it declined (He *et al.*, 1994), a pattern similar to our observations of *ZmACS* expression. The induction of *ZmACS* and *ZmACO* gene expression during hypoxia was developmentally restricted to the root cap, the cortex, and protophloem sieve elements and companion cells associated with metaphloem sieve elements as summarized briefly below.

Within the root cortex of the zones of cell division and elongation, *ZmACS6* expression was induced in a stochastic pattern, principally in the mid- to outer cortex following exposure to 4% oxygen, whereas expression of *ZmACS2/7* was induced in the inner to mid-cortex. Expression of *ZmACO* genes was induced in the mid-cortex in the zones of cell division and elongation in response to hypoxia. Uniform expression of *ZmACS* and *ZmACO* also occurred throughout the cortex of the fully expanded region of the root (data not shown), correlating with the QRT-PCR analysis which detected *ZmACS* gene family expression in this region (i.e. 10–20 mm behind the root apex).

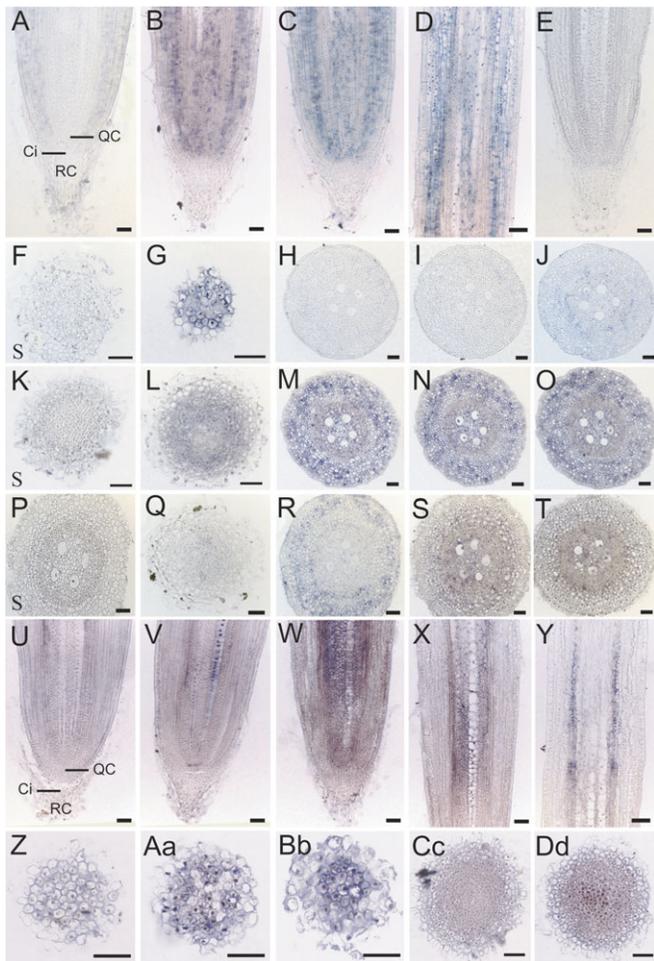
No expression of *ZmACS* gene family members was detected in the stele but *ZmACO15/31* expression was detected in the PSE and *ZmACO20/35* expression was observed in the CC associated with the MSE of normoxic roots as reported previously (Gallie *et al.*, 2009). *ZmACO20/35* expression was observed following enucleation of the PSE but before enucleation and elongation of the MSE in

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old normoxic root (A) or other sections as indicated with an S in the lower left hand corner of the panel. The root cap from normoxic roots (F–J) and from 3 h (K–O), 9 h (P–T), or 24 h (U–Y) hypoxic roots were analysed from the root cap apex (G H; L M; Q R; V W) progressively up to the calyptragen (I J; N O; S T; X, Y). The developing vasculature (within the elongation zone) from normoxic roots (Z–Dd) and from 3 h (Ee–Ii), 6 h (Jj–Nn), 9 h (Oo–Ss), or 24 h (Tt–Xx) hypoxic roots was also analysed. Cross-sections of the developing vasculature within a given row represent every other section (15 µm thick) in a progressive series. Hybridization was detected as a blue precipitate. Ci, calyptragen; mx, metaxylem; pse, protophloem sieve element; QC, quiescent centre; RC, root cap. The bar is equivalent to 100 µM except in Z–Xx where it is 50 µM.



**Fig. 5.** *In situ* localization of *ZmACO20/35* mRNA in maize roots. Median longitudinal sections from a 1-week-old normoxic root (B, F) and from hypoxic roots treated for 3 h (C, G), 6 h (D, H, I, J), or 24 h (E) with 4% (v/v) oxygen were hybridized with a digoxigenin-labelled riboprobe representing antisense *ZmACO20/35* RNA. Similar hybridizations were performed on cross-sections (K-I) with the exception that digoxigenin-labelled riboprobe representing sense *ZmACO20/35* RNA was used to probe a median longitudinal



**Fig. 6.** *In situ* localization of *ZmACS6* and *ZmACS2/7* mRNAs in maize roots. Median longitudinal sections from a 1-week-old normoxic root (A) and from hypoxic roots treated for 3 h (B–D) or 9 h (E) with 4% (*v/v*) oxygen were hybridized with a digoxigenin-labelled riboprobe representing antisense *ZmACS6* RNA. *ZmACS6* expression in cross-sections within the elongation zone from normoxic roots (F–J) and from 3 h (K–O) or 9 h (P–T) hypoxic roots was similarly analysed with the exception that the digoxigenin-labelled riboprobe representing sense *ZmACS6* RNA was used to probe cross-sections as indicated with an S in the lower left hand corner of the panel. Similar median longitudinal (U–Y) and cross (Z–Dd) sections were hybridized with digoxigenin-labelled riboprobes representing antisense *ZmACS2/7* RNA. *ZmACS2/7* expression in the root cap from a normoxic root (Z) and from 3 h (Aa) or 9 h (Bb) hypoxic roots was similarly analysed, as was the calyptrigen from a normoxic root (Cc) and from a 3 h hypoxic root (Dd). S, sections probed with sense RNA; Ci, calyptrigen; QC, quiescent centre; RC, root cap. The bar is equivalent to 100  $\mu$ M.

hypoxic and normoxic roots. Both *ZmACO15/31* and *ZmACO20/35* subgroups were induced in their respective cell types in response to hypoxia, but the induction was transient, consistent with the QRT-PCR results.

*ZmACS6* and both *ZmACO15/31* and *ZmACO20/35* subgroups were expressed in the root cap of normoxic roots and *ZmACO* expression was induced in response to hypoxia, results consistent with the ability of the root cap to generate ethylene. As ethylene regulates gravitropism (Feldman, 1984; Lee *et al.*, 1990; Chang *et al.*, 2004; Buer *et al.*, 2006; Hahn *et al.*, 2008) and serves as a sensor of its environment, for example, soil density (Feldman, 1984), the expression of the ethylene biosynthetic machinery in the root cap is consistent with the role that the root cap plays in these response programs (Hahn *et al.*, 2008).

The cell-specific expression of the ethylene biosynthetic machinery in the root raises the question of what purpose its induction serves during conditions of hypoxia and does the hypoxic conditions used (i.e. 4% oxygen) provide sufficient oxygen to support ethylene biosynthesis? Ethylene production during hypoxia signals the formation of aerenchyma (Drew *et al.*, 1979; Jackson *et al.*, 1985; He *et al.*, 1996b) and regulates root growth, at least in normoxic roots (Whalen and Feldman, 1988; Lee *et al.*, 1990; Sarquis *et al.*, 1991; Gallie *et al.*, 2009). In the present study, an increase in ethylene production during hypoxic treatment correlated with the induction of *ZmACS* and *ZmACO* expression: an increase of 4.7-fold in ethylene evolution was observed as early as 3 h following the onset of hypoxia which increased to 10-fold by 12 h before decreasing upon longer exposure to hypoxia. The level of ethylene production remained elevated for longer than the increase in transcript levels for most members of the *ZmACS* and *ZmACO* gene families. Our analysis of the effect of hypoxia focused on transcript levels for the ethylene biosynthetic machinery and on ethylene production. It is possible that any increase in protein level resulting from a hypoxia-induced increase in transcript level persists for longer than the increase in RNA level. Whether the increase in ethylene evolution and its subsequent decline correlates with changes at the protein level will require antiserum specific for each member of the ethylene biosynthetic machinery.

For the determination of ethylene evolution, measurements were made from normoxic and hypoxic roots kept in ambient air during the 2 h collection period. This would have provided sufficient oxygen to support a level of ethylene biosynthesis that reflects the full potential of the ethylene biosynthetic machinery expressed for each hypoxic treatment. Was 4% oxygen sufficient, however, to support

section from a 1-week-old normoxic root (A) or other sections as indicated with an S in the lower left hand corner of the panel. The root cap from normoxic roots (K–O) and from 9 h (P–T) hypoxic roots were analysed from the root cap apex (L M; Q R) progressively up the columella (N O; S T). The developing vasculature (within the elongation zone) from normoxic roots (U–Y) and from 6 h (Z, Dd) or 9 h (Ee–Ii) hypoxic roots was also analysed. Cross-sections of the developing vasculature within a given row represent every other section (15  $\mu$ m thick) in a progressive series. CC, companion cells; Ci, calyptrigen; Mse, metaphloem sieve element; mx, metaxylem; pse, protophloem sieve element; QC, quiescent centre; RC, root cap. The bar is equivalent to 100  $\mu$ M except in U–Ii where it is 50  $\mu$ M.

ethylene production in the roots while subjected to hypoxic conditions? Continuous flow measurements of ethylene production from roots exposed to 3 kPa O<sub>2</sub> revealed that this level of oxygen was sufficient to support ethylene synthesis and elicited an increase in ethylene evolution to physiologically active levels (Brailsford *et al.*, 1993). Micro-electrode measurements within maize roots have indicated that oxygen is present as a gradient across the root and the gradient is determined by several factors including the internal diffusion of oxygen from the shoot, the distance from the shoot, and oxygen diffusion from the external medium (Armstrong *et al.*, 1994). Because most of the oxygen in the root in that study was supplied from the shoot while the root was embedded in agar, conditions that differ substantially from those used in our study, it is not possible to assume the internal levels of oxygen measured would be precisely applicable to our study. However, a general conclusion from Armstrong *et al.* (1994) relevant to the present work is that the outer stele, containing the phloem tissues had similar levels of oxygen as the cortex, while the inner stele had a considerably lower level of oxygen (Armstrong *et al.*, 1994). Induction of *ZmACO* expression was confined to the phloem tissues of the outer stele (as well as in the cortex) during hypoxia whereas no *ZmACO* or *ZmACS* expression was detected in the inner stele.

The  $K_m$  for oxygen of ACC oxidase has been reported to range from 0.2% in apple to 2.2% in banana (Burg, 1973; Bank, 1985). Previous work has shown that hypoxic maize roots contain higher levels of ACC synthase and ACC oxidase activity within 4 h of exposure to 4% oxygen (He *et al.*, 1994, 1996a), resulting in higher levels of ACC and ethylene as well as the subsequent formation of aerenchyma (Drew *et al.*, 1979; Jackson *et al.*, 1985; Atwell *et al.*, 1988; He *et al.*, 1996a, b). The formation of aerenchyma requires ethylene production during hypoxia and inhibitors of ethylene production or signalling applied during hypoxia prevent the formation of aerenchyma (Drew, 1997; He *et al.*, 1996b; Drew *et al.*, 2000). By contrast, anoxia results in the repression of ACC synthase activity and ethylene biosynthesis as oxygen is required by ACC oxidase to convert ACC into ethylene (Drew *et al.*, 1979). As ethylene synthesis requires oxygen and ethylene is required to signal aerenchyma formation, the observation that 4% oxygen elicited aerenchyma formation in the roots used in this and in previous studies demonstrates that treatment with 4% oxygen provides sufficient oxygen to support hypoxic-induced ethylene biosynthesis in the roots. That the amount of aerenchyma generated increased during the 4 d hypoxic treatment suggests that ethylene production was ongoing during the treatment. While it is not possible to conclude that sufficient oxygen penetrated throughout the root to permit every cell expressing ACC oxidase to produce ethylene, the formation of aerenchyma is strong evidence that ethylene biosynthesis did occur in the hypoxic roots.

Because ACC oxidase catalyses the production of ethylene, its expression serves as the best indicator of where ethylene is actually produced if ACC is also present. Induction of *ZmACO* and *ZmACS* expression was observed

in the cortex of hypoxic roots suggesting that this tissue is competent to produce ethylene and may contribute to the increased evolution of ethylene in response to exposure to hypoxia. By contrast, the expression of *ZmACO* genes in phloem tissues of normoxic and hypoxic roots appears to be spatially separate from *ZmACS* expression where expression of *ZmACS7* in the inner cortex is at least one cell layer away from the phloem-specific expression of the *ZmACO* genes. This would suggest that the expression of *ZmACO* genes in phloem tissues does not contribute to the ethylene produced during hypoxia or that ACC is transported to these cells if they are to produce ethylene. The latter possibility would also require that sufficient oxygen be able to penetrate to the stele. Transport of ACC generated under conditions of low oxygen has been observed, for example, in tomato roots where it was generated and transported through the xylem to the shoot where it was converted to ethylene (Bradford and Yang, 1980). Such transport of ACC in flooded roots has been suggested to be important in root–shoot signalling (Jackson, 1997). ACC transport from leaves to roots was observed in tomato, suggesting that ACC can also be transported through the phloem (Amrhein *et al.*, 1982). Although induced by hypoxia, the increase in expression from *ZmACO15/31* or *ZmACO20/35* did not alter PSE or MSE development in that, following induction of *ZmACO* genes, both cell types underwent normal clearing with the subsequent appearance of mature sieve elements.

Although the induction of the ethylene biosynthetic machinery in response to hypoxia is necessary to promote aerenchyma formation (Drew *et al.*, 1979; Jackson *et al.*, 1985; He *et al.*, 1996b), what purpose does the observed induction of the ethylene biosynthetic machinery serve in those regions of the root, for example, the zones of cell division and elongation, that are not yet competent to form aerenchyma? In maize, as in other species, ethylene serves to regulate root elongation (Whalen and Feldman, 1988; Lee *et al.*, 1990; Sarquis *et al.*, 1991; Ghassemian *et al.*, 2000; Ruzicka *et al.*, 2007; Swanup *et al.*, 2007; Gallie *et al.*, 2009). The inhibitory effect of ethylene on root growth is rapid, occurring within 20 min and largely inhibited cortical cell elongation in the region just distal to the root apex (Whalen and Feldman, 1988). A reduction in the endogenous evolution of ethylene resulted in enhanced growth under conditions of unimpeded growth but impaired growth in soil, whereas increased production of ethylene inhibited root elongation under conditions of unimpeded growth (Gallie *et al.*, 2009). Although any increase in ethylene production in the fully-expanded region of a root would be unable to affect the growth of fully-expanded cells, the induction of ethylene biosynthesis in the zones of cell division and elongation may serve to inhibit root elongation during hypoxia as it does in normoxic roots, as this is where ethylene can function directly to affect root elongation. This is consistent with our observation that the rate of root growth was reduced by approximately 50% following exposure to 4% oxygen. The lower level of oxygen available under conditions of hypoxia may also contribute to slower root growth. A reduction in root growth may

serve to slow further growth into oxygen-deprived soils, to reduce the problem of oxygen availability. A second possibility that has been proposed is that low oxygen levels in the stele of hypoxic roots may signal ACC synthesis where it is then transported to other tissues (Jackson, 1994). However, no expression of ACC synthase was detected in the stele in normoxic or hypoxic root tips. Instead, the induction of expression of *ZmACS* in the cortex in the zones of cell division and elongation may generate ACC for transport to other tissues or organs, for example, the aerial regions of the plant, as observed in tomato (Bradford and Yang, 1980). A third, non-mutually exclusive, possible role for the generation of ethylene in the developing cortex during hypoxia may be to alter the sensitivity to ethylene which may function to alter the rate of aerenchyma formation during prolonged exposure to hypoxia.

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