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 metaphloem 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 (Harpham 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, metaphloem 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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The ACOS three members, i.e., 1992; Zarembinski and Theologis, 1993; He accompaned by increases in ACC synthase and ACC a consequence of its high rate of respiration. Hypoxia- highly sensitive to changes in oxygen availability as contains the vascular system, and the root apex, which is undergoing 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; Shi 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 CO2 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 axenic 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 O2 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% glutaraldehyde 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 μm 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 LiCl precipitation as described by Sambrook et al. (1989). 50 μg total RNA was treated with RQ1 DNase (Promega) to ensure that no contaminating DNA was present. Two μg of total RNA were used for cDNA synthesis using the Omniscript RT kit (Qiagen) with oligo-dT20 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 2 μl × buffer, 0.5 μl of the reverse transcription reaction (equivalent to 50 ng total RNA) and 0.25 μM (final concentration) forward and reverse primers in a total reaction volume of 25 μl. 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 μg 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 μm 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 NaHPO4, 5 mM EDTA, 50% formamide, 10% dextran sulphate, 1× Denhardts, 1 mg 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 nl⁻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')                  |
|---------|---------------------------------------------|----------------------------------------|
| ZmACS2  | ATCGGTGTAACGCTTCTCCAAGGA                   | GATAGCTTCTTTGTCACCACCATCCATAGA        |
| ZmACS6  | ACGTTGGAAGAGGTGTCCTCCGAGGT                | AGTACGTTCTTTGTCACCACCATCCATAGA        |
| ZmACS2  | ATCGGTGTAACGCTTCTCCAAGGA                   | GATACGTTCTTTGTCACCACCATCCATAGA        |
| ZmACS7  | ATCGGTGTAACGCTTCTCCAAGGA                   | CAACGCTTCTGTCACCTGTCATAGT            |
| ZmACO15 | CTGTCCTGCATTCAACCTCCAAGT                  | TACCATTACATTTTCCTCCGCTGT             |
| ZmACO20 | CTGACCTCTGTGCTCCAACGAGC                   | TCCAGCCTAGACGCTAAACACCAGT            |
| ZmACO31 | CTGTCCTGCATTCAACCTCCAAGT                  | ATAGAAAGAGGAGGCAACTGACGAGT           |
| ZmACO35 | CTGTCCTGCATTCAACCTCCAAGT                  | ACAACACATACCTGTCGACCTATAAGCA         |
| β-Tubulin | ACCAGATCGGCGCGAAGTTCT                  | CATCAGTCTCTGTCGACCTCGA             |
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 μm) 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 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 μm 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.

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

| Time (h) | Root elongationa | Relative rate |
|---------|-----------------|--------------|
|         | Ambient air (mm) | 4% Oxygen (mm) | (hypoxic/control) |
| 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              |

a 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⁻¹ whereas roots grown in 4% oxygen had an average rate of elongation of 0.29 mm h⁻¹, 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 β-tubulin mRNA was expressed at a relatively constant level from the root apex to at least 2 cm from the apex (Fig. 3). β-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...
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 O₂ (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.

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

| Length of hypoxic treatment (h) | Ethylene evolution (nl g⁻¹ FW h⁻¹) |
|---------------------------------|-----------------------------------|
| 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                         |

*Measurements were taken from 5-d-old seedlings treated with 4% oxygen.*

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 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. 4Un–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-old normoxic root.
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. 5Fi–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 calyptrogen 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 calyptrogen 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

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 calyptrogen (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, calyptrogen; 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-Ii) with the exception that digoxigenin-labelled riboprobe representing sense ZmACO20/35 RNA was used to probe a median longitudinal...
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 hypoxic roots 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 μm thick) in a progressive series. CC, companion cells; Ci, calyptrogen; Mse, metaphloem sieve element; mx, metaxylem; pse, protophloem sieve element; QC, quiescent centre; RC, root cap. The bar is equivalent to 100 μM except in U–II where it is 50 μM.
ethylene production in the roots while subjected to hypoxic conditions? Continuous flow measurements of ethylene production from roots exposed to 3 kPa O$_2$ 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 ZmACS genes in phloem tissues of normoxic and hypoxic roots appears to be spatially separate from ZmACO expression where expression of ZmACS genes 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 ZmACO1531 or ZmACO2035 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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