Plant Physiol. (1996) 112: 463–472

# Transduction of an Ethylene Signal Is Required for Cell Death and Lysis in the Root Cortex of Maize during Aerenchyma Formation Induced by Hypoxia[1]

**Chuan-Jiu He, Page W. Morgan, and Malcolm C. Drew***

Department of Horticultural Sciences (C.-J.H., M.C.D.) and Department of Soil and Crop Sciences (P.W.M.), Texas A&M University, College Station, Texas 77843

**Ethylene has been implicated in signaling cell death in the lysigenous formation of gas spaces (aerenchyma) in the cortex of adventitious roots of maize (*Zea mays*) subjected to hypoxia. Various antagonists that are known to modify particular steps in signal transduction in other plant systems were applied at low concentrations to normoxic and hypoxic roots of maize, and the effect on cell death (aerenchyma formation) and the increase in cellulase activity that precedes the appearance of cell degeneration were measured. Both cellulase activity and cell death were inhibited in hypoxic roots in the presence of antagonists of inositol phospholipids, $Ca^{2+}$-calmodulin, and protein kinases. By contrast, there was a parallel promotion of cellulase activity and cell death in hypoxic and normoxic roots by contact with reagents that activate G-proteins, increase cytosolic $Ca^{2+}$, or inhibit protein phosphatases. Most of these reagents had no effect on ethylene biosynthesis and did not arrest root extension. These results indicate that the transduction of an ethylene signal leading to an increase in intracellular $Ca^{2+}$ is necessary for cell death and the resulting aerenchyma development in roots of maize subjected to hypoxia.**

Aerenchyma formation in the roots and stems of many non-wetland dicots and monocots, including maize (*Zea mays* L.), takes place in response to an environmental stimulus, $O_2$ deficiency (Drew et al., 1979; Kawase, 1981a, 1981b; Justin and Armstrong, 1991), and accelerates the transfer of $O_2$ from the aerial tissues to the $O_2$-deficient tissues of the stem base and root (Armstrong, 1979). Lysogenic aerenchyma formation involves the death and often complete lysis of cells, with the disappearance of all cell components, including the cytoplasm and cell walls, within about 24 h of the initiation of cell death (Campbell and Drew, 1983). The response in maize nodal roots is highly selective, involving only cortical cells interior to the hypodermis and external to the endodermis (Campbell and Drew, 1983). It is evident that the pattern of cell death during aerenchyma formation has many features in common with programmed cell death or apoptosis in animal cells (Tomei and Cope, 1991).

Aerenchyma formation was long attributed to the direct effects of severe $O_2$ deficiency on the viability of root cortical cells (McPherson, 1939). However, working with stems of sunflower, Kawase (1979, 1981a, 1981b) found aerenchyma formation to be promoted under fully aerobic conditions by exogenous ethylene. Likewise, in nodal roots of maize low concentrations of ethylene (0.1–1.0 $\mu L\ L^{-1}$ air) bubbled through nutrient solution readily induced aerenchyma in the root cortex under conditions in which no $O_2$ deficiency could be involved: the structure of these roots was indistinguishable from the structure induced by hypoxia (Drew et al., 1979; Konings, 1982). Hypoxic root tips contain higher concentrations of ethylene than aerobic ones, suggesting that ethylene biosynthesis might be accelerated under these conditions (Drew et al., 1979). Supporting that notion, higher concentrations of the ethylene precursor ACC (Atwell et al., 1988) and greater activity of ACC synthase (He et al., 1994a) have since been found in hypoxic maize root tips. Furthermore, low, nontoxic concentrations of inhibitors of ethylene biosynthesis (e.g. AVG) or ethylene action ($Ag^+$) effectively block aerenchyma formation in hypoxic roots (Drew et al., 1981; Konings, 1982; Jackson et al., 1985), but the inhibitory effect of AVG (which blocks the activity of ACC synthase) is reversed by simultaneous addition of ACC to the rooting medium (Jackson et al., 1985).

Such results present a strong case for believing that cell death in the maize root cortex is caused not by $O_2$ starvation directly but by an enhanced rate of ethylene production stimulated by hypoxia. The term hypoxia describes the $O_2$ status of cells or tissues in which oxidative phosphorylation is slowed by the external $O_2$ concentration. This term is distinct from anoxia, in which the $O_2$ concentration is so low that oxidative phosphorylation is negligible relative to ATP production in glycolysis and fermentation (Pradet and Bomsel, 1978). No cell lysis or aerenchyma formation takes place in anoxic roots (Drew et al., 1979; Jackson et al., 1985). The fact that ethylene synthesis is halted under anoxia (conversion of ACC to ethylene by ACC oxidase requires molecular $O_2$) is not the full explanation, because treatment of anoxic roots with exogenous

[1] Research was supported by U.S. Department of Agriculture National Research Initiative grants nos. 93–37100–8922 (M.C.D.) and 93–37100–8952 (P.W.M.).

* Corresponding author; e-mail mcdrew@tamu.edu; fax 1–409–845–0627.

Abbreviations: ADH, alcohol dehydrogenase; AVG, aminoethoxyvinylglycine; CaCM, $Ca^{2+}$-calmodulin; GDPβS, guanosine-5′-*O*-(2-thiodiphosphate); GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); G-protein, GTP-binding protein; PR, pathogenesis related.

ethylene also fails to elicit aerenchyma formation (Jackson et al., 1985); anoxic, still-viable cells lack the capacity to respond to the ethylene signal. Additionally, hypoxia increases the activity of ACC synthase, a key enzyme in the biosynthesis of ethylene, in maize root tips, whereas the activity of this enzyme is strongly inhibited under anoxic conditions (He et al., 1994a).

Because of the complete lysis of protoplast and cell wall that can occur following cell death in aerenchyma formation, it is reasonable to assume that a wide array of cell-degrading enzymes are involved. The activity of cellulase increases about 16-fold in hypoxic root tips just prior to aerenchyma formation (He et al., 1994a), and this increase is blocked by AVG (and reversed again by the simultaneous addition of ethylene), indicating the essential role of ethylene in cellulase induction. However, under the electron microscope cell-wall disappearance is observed to take place toward the end of the cell degradation process (Campbell and Drew, 1983); so although cellulase is a useful marker of future cell degradation, it is unlikely to be closely associated with the initiation of cell death.

Apart from aerenchyma formation, ethylene is known to regulate many developmental processes in higher plants, including leaf and flower senescence and abscission, fruit ripening, epinasty, stem elongation, and response to pathogen invasion (Abeles et al., 1992). Several of the components in ethylene perception and signal transduction have recently been defined using various ethylene response mutants isolated in *Arabidopsis thaliana* (Guzman and Ecker, 1990; Hua et al., 1995; reviewed by Zarembinski and Theologis, 1994; Ecker, 1995). Sequence homology between these Arabidopsis genes and prokaryotic and animal genes implicates protein phosphorylation via kinases at several steps in ethylene signal transduction in Arabidopsis.

Components of an ethylene signal transduction pathway have also been revealed by studying the induction of chitinase and other PR genes in tobacco (Raz and Fluhr, 1992). Both ethylene-dependent and -independent pathways for induction of the PR genes occur in tobacco, whereas in Arabidopsis ethylene does not appear to play a similar role (Zarembinski and Theologis, 1994). In tobacco, reagents that chelate $Ca^{2+}$ block the ethylene-dependent increase in gene expression, whereas drugs that increase endogenous [$Ca^{2+}$] stimulate PR gene expression. Raz and Fluhr (1993) went on to show that ethylene applied to tobacco leaves causes a rapid, transient increase in protein phosphorylation. With the addition of kinase inhibitors, PR gene expression by ethylene was blocked, whereas phosphatase inhibitors enhanced protein phosphorylation and stimulated PR gene expression in the absence of exogenous ethylene. Although these studies offer no clues as to the nature or position in the pathway of the kinases and phosphatases, it is likely that several steps could be affected simultaneously, perhaps close to the site of ethylene perception, as well as downstream, where CaCM might activate a specific protein kinase.

Little is known about the signaling of $O_2$ deprivation in higher plant cells, but it is likely that there is a sensing mechanism for [$O_2$] that activates changes in gene expression (reviewed by Drew, 1990). Recent evidence indicates that anoxia somehow elicits a signal transduction pathway involving $Ca^{2+}$. The anoxic induction of ADH was blocked in maize roots by an inhibitor of organellar $Ca^{2+}$ fluxes (Subbaiah et al., 1994b), whereas a drug that increased cytosolic [$Ca^{2+}$] caused expression of ADH in the absence of anoxia (Subbaiah et al., 1994a). Since ethylene production is known to be blocked by strict anoxia, the pathway is presumably independent of ethylene.

The aim of the present work was to determine whether there is evidence of a signal transduction pathway between (a) hypoxia and ethylene biosynthesis and/or (b) ethylene and the cell death and lysis that occur during aerenchyma formation in maize roots. We applied different classes of antagonists that are known to modify particular steps in signal transduction in other biological systems, and we examined the effect on death and degradation of cortical cells under hypoxic and fully aerobic (normoxic) conditions. The activity of cellulase, which is closely associated with aerenchyma formation (He et al., 1994a), was concurrently measured. Preliminary accounts of this work have appeared previously (He et al., 1993, 1994b).

## MATERIALS AND METHODS

Caryopses of maize (*Zea mays* L. cv TX 5855) were surface-sterilized in a solution of commercial Clorox (final concentration of NaOCl, 0.5%) for 10 min, washed in tap water for 4 h, and allowed to imbibe and germinate on moist germination paper in an incubator at 30°C. Seedlings of uniform root length (about 30 mm) were selected and transferred to a stainless steel mesh over an aerated (0.1×) nutrient solution at 25°C in a laboratory with a PPFD of about 50 μmol photons $m^{-2}$ $s^{-1}$. The roots were protected from light by a layer of 10-mm-diameter black plastic beads placed on the mesh. After 3 d plants were transferred in groups of four to 2-L volumes of 0.5× nutrient solution in a controlled environment (650 μmol photons $m^{-2}$ $s^{-1}$ PPFD, 14-h light period) with day/night conditions of 25/20°C and a RH of 75/65%. After 2 d plants were transferred to 1× nutrient solution containing 1.0 mM $KNO_3$, 4.0 mM $Ca(NO_3)_2$, 1.0 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 0.1 mM Fe as Fe-EDTA, and micronutrients, pH 6.5.

### Experimental Treatments

Experimental treatments began when nodal roots at the first whorl of the stem base were about 50 mm long (11 d from imbibition). To make roots hypoxic, air (20.6% [v/v] $O_2$) and prepurified $N_2$ gas, both from pressurized cylinders and regulated by electronic mass flow controllers, were mixed to give an $O_2$ concentration of 4% (v/v). This was passed into the nutrient solution at a flow rate of 200 mL $min^{-1}$ per container. Antagonists and other reagents (Sigma) were dissolved in small volumes of water, with the exceptions of thapsigargin and okadaic acid, which were dissolved in ethanol, and K-252a, which was dissolved in DMSO. The final concentration of ethanol in the nutrient solution was 5 μL $L^{-1}$, and DMSO was less than 1 μL $L^{-1}$;

these were without effect on roots. The final concentrations of antagonists in the nutrient solution are indicated in Table I, but in preliminary work each was tested over a 100- to 1000-fold concentration range for effectiveness and toxicity.

### Ethylene Production

Ethylene production was measured immediately after excising in air the apical 25 mm from nodal roots at the first whorl. Eight sections were transferred to a 10-mL glass vial containing air and sealed with a rubber serum stopper. To measure ethylene production by hypoxic roots, the glass vial and excised root segments were purged with 4% $O_2$ for 5 min via a hypodermic needle inserted through the septum, with another needle through the septum providing an outflow. After 30 or 60 min of incubation at 25°C, a 1-mL volume of gas was withdrawn from either hypoxic or normoxic vials with a hypodermic needle and syringe. The sample was injected into a gas chromatograph (Varian, Walnut Creek, CA) with a flame ionization detector and $N_2$ as the carrier gas (Drew et al., 1989). The root samples were weighed at the end of the experiment.

### Cellulase Activity

Cellulase activity was measured by a modification of the method described earlier (He et al., 1994a). Nodal root tips (0.5 g fresh weight, 25-mm apical zone) were excised in the cold (about 4°C) and macerated over an ice bath with a pinch of river sand in 3 mL of 30 mM potassium phosphate buffer at pH 6.1 containing 1 M NaCl (final concentration). The extract was centrifuged at 3000*g* for 10 min, and 2.5 mL of the supernatant was passed in the cold through a desalting column and eluted by 3 mL of the same potassium phosphate buffer at pH 6.1 without NaCl. The eluted solution was brought to 25°C, and 2 mL was pipetted into the reaction chamber of a flow viscometer (20–200 centipoise range; Fisher Scientific) containing 4 mL of 1% sodium carboxymethyl cellulose of medium viscosity (Sigma). Cellulase activity was assayed by measuring the viscosity before and after 2 h of incubation at 25°C. A cellulase preparation from *Aspergillus niger* (Sigma c7377) was used as a standard.

### Estimation of Cell Death

Using a fine camel hair brush, nodal roots were marked with a spot of carbon (charcoal slurry) exactly 10 mm behind the tip to provide a reference for subsequent measurements of root extension. All estimations of cell death were done on transverse sections cut at the zone that corresponded to the location of the root tip at the start of the experimental treatment. Camera lucida drawings were made of the transverse sections, identifying the areas that comprised intact cells, cells in the process of disintegration, and clearly delineated, gas-filled spaces (lacunae). These areas were quantified for each section.

## RESULTS

Different classes of antagonists known to modify specific steps in signal transduction in animal and plant cells were tested for their effect on cell death and cellulase activity, as well as on ethylene biosynthesis in maize roots. In some cases we used different antagonists that all act at the same step but by different mechanisms. It was expected that any nonspecific effects of an antagonist would result in an inconsistent result relative to the other antagonists, but no such inconsistency was found. A summary of the main effects of all of the antagonists used in this study is given in Table I and explained below.

### Cellulase Activity and Cell Death

K-252a, a staurosporine analog, is an inhibitor of protein kinases with a degree of selectivity for protein kinase C (Kase et al., 1986). It is an effective inhibitor in tomato cells in culture (Grosskopf et al., 1990) and in tobacco cells (Raz and Fluhr, 1993). Okadaic acid, a potent tumor promotor, is a specific inhibitor of protein Ser/Thr (type 1 and type 2A) phosphatases (Cohen, 1989; Cohen et al., 1990), and in leaves of tobacco it enhances protein phosphorylation (Raz and Fluhr, 1993). Neomycin forms complexes with phosphatidylinositol-4-monophosphate and phosphatidylinositol-4,5-bisphosphate (Gabev et al., 1989) and interferes in the binding of these inositol phospholipids in plasma membrane preparations from wild carrot (Chen and Boss, 1991). When these antagonists were supplied to maize roots at the start of hypoxia, K-252a and neomycin effectively blocked cell death

**Table I.** *Effect of signal transduction antagonists on aerenchyma formation, cellulase activity, and ethylene production in nodal roots of maize under $O_2$ stress*

| Antagonists | | Aerenchyma | Cellulase | Ethylene |
|---|---|---|---|---|
| G-protein modifier | GTPγS (25 μM) | Promote[a] | Promote[a] | NS[b] |
| | GDPβS (25 μM) | NS | NS | NS |
| Inhibitor of inositol phospholipids | Neomycin (50 μM) | I[c] | I | NS |
| Modifiers of intracellular free $Ca^{2+}$ | EGTA (50 μM) | I | I | NS |
| | Thapsigargin (1 μM) | Promote[a] | Promote[a] | NS |
| | Caffeine (500 μM) | Promote[a] | Promote[a] | I |
| | Ruthenium red (25 μM) | I | I | NS |
| CM antagonist | W-7 (10 μM) | I | I | NS |
| Protein kinase inhibitor | K-252a (1 μM) | I | I | NS |
| Protein phosphatase inhibitor | Okadaic acid (50 nM) | Promote[a] | Promote[a] | NS |

[a] Promotion also occurred in normoxic roots. [b] NS, No significant effect. [c] I, Inhibited.

in the zone behind the apical meristem (Figs. 1 and 2, E and F). By contrast, okadaic acid caused a distinct promotion of cell death at 36 to 72 h (Figs. 1 and 2D). It is interesting that okadaic acid also promoted death in normoxic (control) roots (Figs. 1A and 2C), although not to the same extent that hypoxia did. Promotion of cell death in normoxic roots was about the same when we doubled the concentration of okadaic acid to 100 nM (data not presented). Normoxic roots in nutrient solution sparged with 20.6% $O_2$ (air) extended at about 3 cm $d^{-1}$, and each of the three antagonists slowed their extension to about 60% of that value. During hypoxia roots continued to extend at about 6 mm $d^{-1}$, and the above antagonists did not significantly affect the extension rate.

Cellulase activity in the 25-mm apical zone showed a pattern similar to that of cell death, with marked inhibition of induction in hypoxic roots in the presence of K-252a and neomycin (Fig. 3), and with appreciable promotion in the presence of okadaic acid. In normoxic roots, okadaic acid promoted cellulase activity, but the other antagonists had no effect.

A number of compounds that modify the intracellular free [$Ca^{2+}$] by different mechanisms were tested. Thapsigargin blocks the activity of the ER $Ca^{2+}$-ATPase in animal cells, thereby increasing intracellular [$Ca^{2+}$] (Thastrup et al., 1990; Ziegelstein et al., 1994), and is effective in tobacco cells (Raz and Fluhr, 1992). Caffeine gates $Ca^{2+}$ channels in animal cells and thus acts to increase intracellular [$Ca^{2+}$] (Vites and Pappano, 1992); it is effective in maize cells in suspension culture (Subbaiah et al., 1994a). EGTA strongly chelates $Ca^{2+}$ at physiological pH and can be used to reduce free [$Ca^{2+}$] in the external medium or to immediately lower cytosolic [$Ca^{2+}$] by intracellular injection (Hepler and Wayne, 1985; Gilroy et al., 1986). The presence of a low concentration of EGTA in the external solution for 4 d totally suppressed cell death during hypoxia (Figs. 2G and 4), presumably because of permeation of the plasma membrane by EGTA during prolonged contact. By contrast, caffeine and thapsigargin, which tend to increase cytosolic [$Ca^{2+}$], stimulated cell death in normoxic roots at 72 and 96 h (Fig. 4); doubling the concentration of either of these reagents did not enhance the effect (data not shown). Hypoxic roots also responded to these reagents, with enhanced areas of dead cortical cells at 48 and 72 h; however, by 96 h an aerenchyma was fully developed in hypoxic roots receiving no additional treatment. Aerenchyma in young maize roots usually involves a maximum of 30 to 40% of the total cortical area, so the presence of the cell-death-promoting drugs does not modify the final pattern of cell death across the root but accelerates it. Caffeine and thapsigargin had no effect on the extension of hypoxic or normoxic roots, whereas EGTA inhibited extension by 10 and 30%, respectively. Similar results were obtained with 100 μM EGTA, but at 500 μM and above there was toxicity to roots and shoots (data not presented).

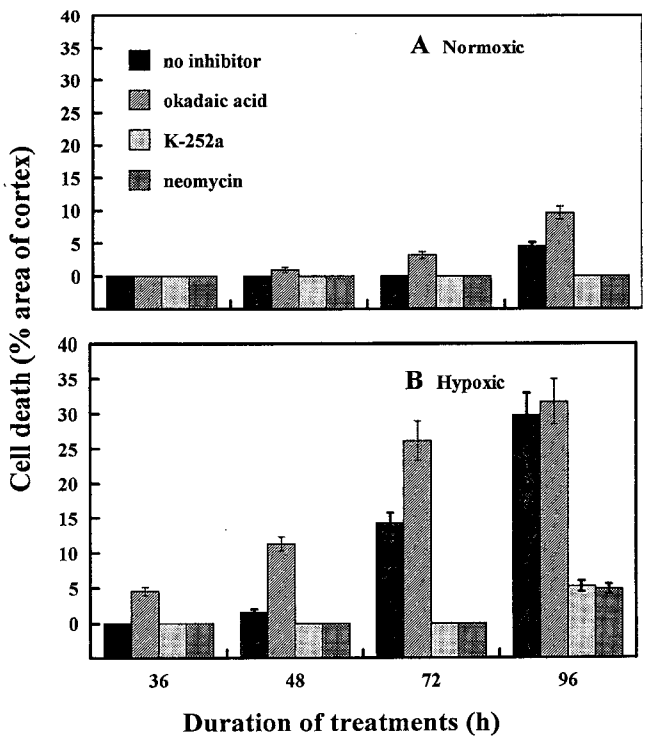


**Figure 1.** Effect of antagonists on cell death in the root cortex of maize. A, Normoxic control root treated with air (20.6% $O_2$). B, Hypoxic (4% $O_2$) root. Results are means ± SE ($n$ = 6 roots).

Cellulase activity (Fig. 5) again very closely paralleled the relative extent of cell death. EGTA completely suppressed cellulase activity in hypoxic roots, whereas both thapsigargin and caffeine stimulated it in controls at 72 and 96 h and in hypoxic roots at 48 and 72 h.

Ruthenium red is an endomembrane $Ca^{2+}$-channel blocker that strongly inhibits $Ca^{2+}$ release to the cytosol in animal cells and in yeast (Lee and Tsien, 1983; Calvert and Sanders, 1995). In tobacco seedlings, ruthenium red prevents any increase in cytosolic [$Ca^{2+}$] otherwise caused by wind-induced motion (Knight et al., 1992). Additionally, in animal cells ruthenium red competitively binds to $Ca^{2+}$-binding proteins (Charuk et al., 1990), including CM, with inhibition of CaCM-dependent enzyme activity (Sasaki et al., 1992). In roots of maize seedlings and in maize suspension-cultured cells, ruthenium red blocked the increase in cytosolic [$Ca^{2+}$] that would have occurred when cells were made anoxic (Subbaiah et al., 1994a, 1994b). The CM antagonist W-7 (*N*-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide) inhibits CaCM-modulated ion channels in barley aleurone cells (Bethke and Jones, 1994). In the present study we found that in hypoxic maize roots both ruthenium red and W-7 inhibited cellulase activity and aerenchyma formation (Table I). The extent of inhibition for each was similar to that shown in Figures 1 and 3 for the kinase inhibitor K-252a. Root extension was insensitive to W-7, whereas ruthenium red inhibited normoxic roots by about 30%, without significant effect on hypoxic ones.

Involvement of G-proteins in a signal transduction pathway is indicated by the response to reagents that modify G-protein function. GTPγS is an analog that locks G-proteins in an active state, whereas GDPβS locks the proteins in an inactive state. These analogs have been used in guard cells of *Vicia faba* to provide evidence of G-protein involvement in stomatal regulation (Fairley-Grenot and Assmann,

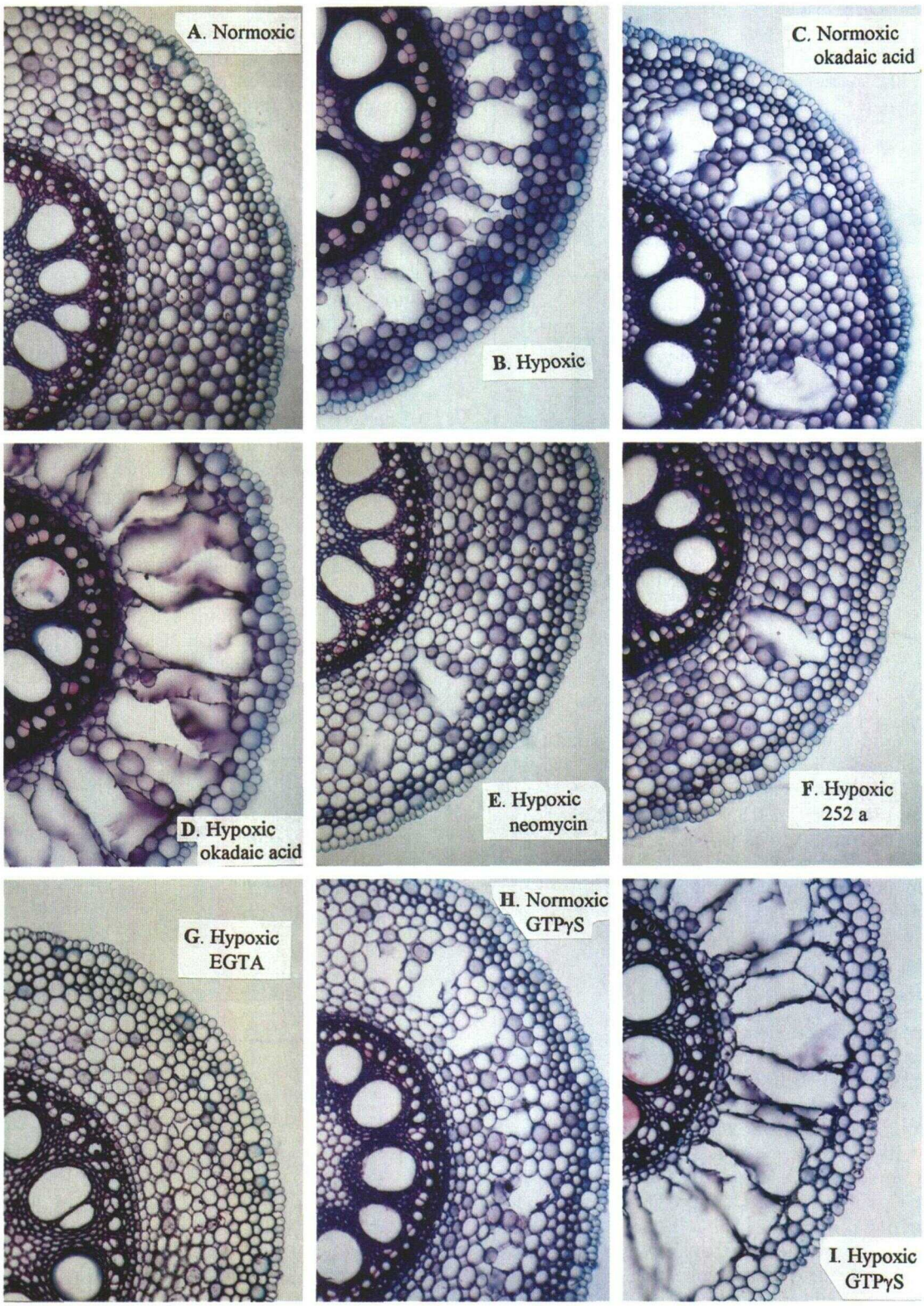


**Figure 2.** Transverse sections of maize nodal roots following treatment for 96 h under the stated conditions (see "Materials and Methods"). Magnification is ×56. The concentrations of antagonists were as given in Table I. Sections were cut from 96-h-old root zones and stained in toluidine blue. B, Note the disappearance of many cells in the mid-cortex with formation of gas (air)-filled spaces and the persistence of some radial cell wall residues and some radial files of intact cells. C–I, Treatments are as noted in each panel.

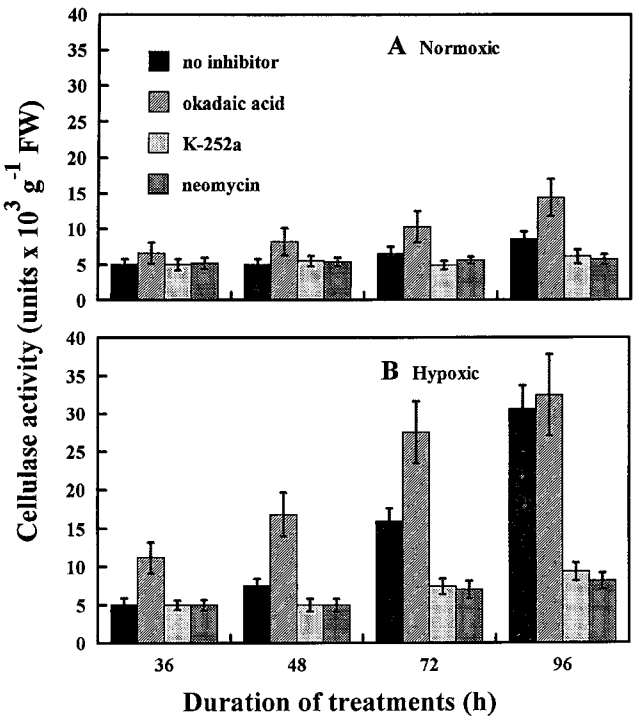


**Figure 3.** Effect of antagonists on cellulase activity in the root tip of maize. A, Normoxic control root treated with air (20.6% $O_2$). B, Hypoxic (4% $O_2$) root. Results are means ± SE ($n$ = 6). FW, Fresh weight.

1991). We found that in hypoxic roots of maize GTPγS strongly promoted cell death and cellulase activity (Table I; Fig. 2I), but GDPβS was without affect, so that aerenchyma formation and the associated increase in cellulase activity continued at the same rate as with hypoxia alone. GTPγS also promoted cell death (Fig. 2H) and cellulase activity in normoxic roots, to a similar extent as shown for okadaic acid (Figs. 1 and 3). Neither GTP analog affected extension of hypoxic or normoxic roots.

### Ethylene Production

The extent to which ethylene biosynthesis is modified by the different antagonists was tested to look for evidence of a signal transduction pathway between hypoxia and ethylene biosynthesis and because inhibition or promotion of ethylene production might account for changes in aerenchyma formation. Measurements were made on ethylene collected from root apical segments freshly excised from intact plants so that the production of wound ethylene would be minimal during 1 h of collection. In a separate experiment, a pattern similar to that shown in Figure 6 was obtained at 30 min of ethylene collection, before wound ethylene was detectable (data not presented). Ethylene production was strongly stimulated by exposure to an atmosphere of 4% $O_2$ (Fig. 6) but was not affected by the inclusion of EGTA or K-252a. Caffeine approximately halved the rate of ethylene production under hypoxia, but this does not explain the observed promotion of aerenchyma formation. To avoid congestion, Figure 6 shows the response to only a few of the modifiers of signal transduction that were tested. With the exception of caffeine, none of the modifiers affected the rate of ethylene production during hypoxia or normoxia, as summarized in Table I.

## DISCUSSION

### Components of the Ethylene Signal Transduction Pathway

It is well established that hypoxia strongly stimulates the rate of ethylene production in maize roots and that enhanced ethylene signals cell death and lysis during aerenchyma formation (see the introduction). We have shown that the death of cells in the cortex of maize roots during hypoxia is blocked by the reagents EGTA and ruthenium red, which tend to decrease the cytosolic free [$Ca^{2+}$] (Table I). The $Ca^{2+}$ chelator EGTA was effective at concentrations much lower than that of $Ca^{2+}$ in the external solution, suggesting that EGTA acted intracellularly to decrease cytosolic $Ca^{2+}$. Ruthenium red decreases cytosolic [$Ca^{2+}$] by blocking endomembrane $Ca^{2+}$ channels. Fluorescence-probe imaging of maize suspension-culture cells in vivo provided direct evidence that ruthenium red prevents any increase in cytosolic free [$Ca^{2+}$] in response to anoxia (Subbaiah et al., 1994a). Other evidence of the critical role of $Ca^{2+}$ in the induction of cell death comes from experiments with the CM antagonist W-7, which also blocked cell death during hypoxia.

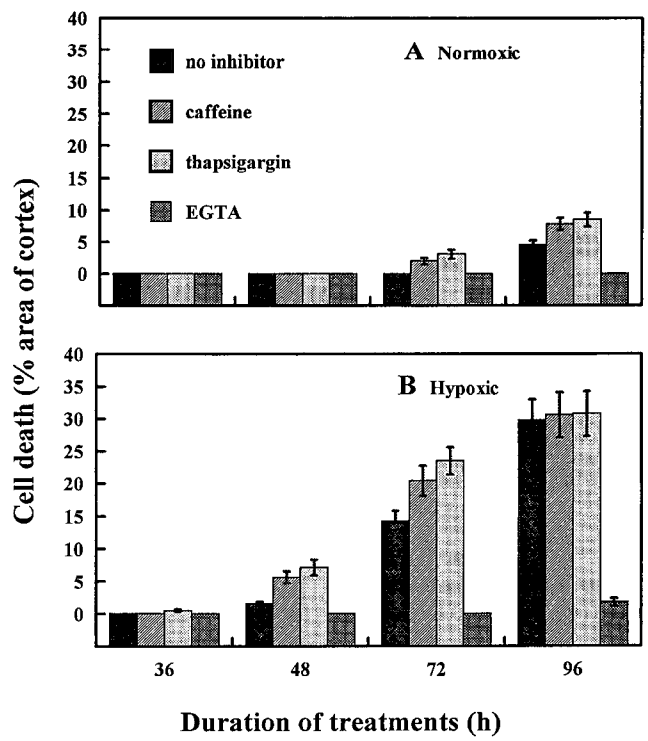


**Figure 4.** Effect of modifiers of cytosolic $Ca^{2+}$ on cell death in the root cortex of maize. A, Normoxic control root treated with air (20.6% $O_2$). B, Hypoxic (4% $O_2$) root. Results are means ± SE ($n$ = 6 roots).

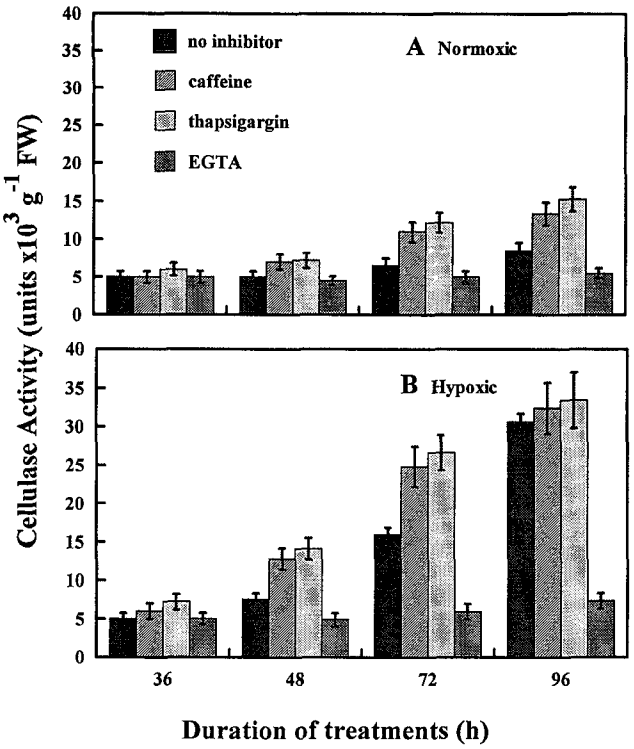


**Figure 5.** Effect of modifiers of cytosolic $Ca^{2+}$ on cellulase activity in the root tip of maize. A, Normoxic control root treated with air (20.6% $O_2$). B, Hypoxic (4% $O_2$) root. Results are means ± SE ($n$ = 6). FW, Fresh weight.

We also found that reagents that increase cytosolic free [$Ca^{2+}$] also promote cell death, under both hypoxic and anoxic conditions (Table I). Thapsigargin and caffeine achieve this by contrasting mechanisms (see "Results"), but the outcome at the level of cell death is the same. There is direct evidence that caffeine increases cytosolic free [$Ca^{2+}$] in maize cells in suspension culture (Subbaiah et al., 1994a), so that its mode of action is apparently the same as in animal cells.

The above conclusions are consistent with the hypothesis that the increase in ethylene induced by hypoxia signals an increase in the cytosolic [$Ca^{2+}$] in maize roots and that this is an important second messenger in the initiation of cell death. The direct involvement of ethylene in this signaling is supported by evidence (C.J. He, P.W. Morgan, and M.C. Drew, unpublished data) that induction of cell death by exogenous ethylene in normoxic roots is enhanced or completely blocked, respectively, by caffeine or EGTA and follows a pattern identical with that found with hypoxia.

The results of the present study have some features in common with the response of maize roots and suspension culture cells to anoxia, but there are also some important differences. In maize seminal roots (Subbaiah et al., 1994b) inhibition by ruthenium red of the release of intracellular $Ca^{2+}$ into the cytosol repressed the anoxic induction of ADH mRNA and enzyme activity and greatly shortened the period that seedlings could remain viable during submergence. Maize suspension-culture cells showed a perceptible increase in cytosolic free [$Ca^{2+}$] within 1 to 2 min of the onset of anoxia (Subbaiah et al., 1994a), a response so rapid that it seems unlikely that ethylene is involved. Additionally, responses to the presumed increase in cytosolic [$Ca^{2+}$] must be different when roots are anoxic compared with when they are hypoxic. Anoxia does not induce selective cell death of the root cortex, and anoxic root cells are not able to respond to exogenous ethylene (Drew et al., 1979; Jackson et al., 1985), so cell lysis and aerenchyma formation do not occur.

The specific steps involved in the ethylene signal transduction pathway in maize roots cannot be identified unambiguously based on the present evidence, but it is instructive to consider the possibilities and thus construct a working hypothesis. What steps might precede the putative increase in cytosolic [$Ca^{2+}$]? The actions of the GTP/GDP analogs and of neomycin (Table I) suggest that earlier stages in signal transduction could involve G-proteins (reviewed by Ma, 1994) and phosphoinositides, perhaps leading to inositol-1,4,5-triphosphate and its release of $Ca^{2+}$ from intracellular organelles (Drobak, 1993; Bush, 1993; Clapham, 1995). Activation of protein kinases by cytosolic $Ca^{2+}$ is well documented (Roberts and Harmon, 1992). In hypoxic maize roots, phosphorylation of one or more proteins is indicated by the clear contrast between the effects of the protein kinase inhibitor that blocked cell death (K-252a) and the phosphatase inhibitor that promoted it (okadaic acid) (Table I). Raz and Fluhr (1993) likewise concluded that the ethylene signal transduction pathway in tobacco leaves, which leads to the induction of PR proteins, involved protein phosphorylation events. Involvement of G-proteins, increased cytosolic [$Ca^{2+}$], and protein phosphorylation have also been implicated in the increased ethylene sensitivity of

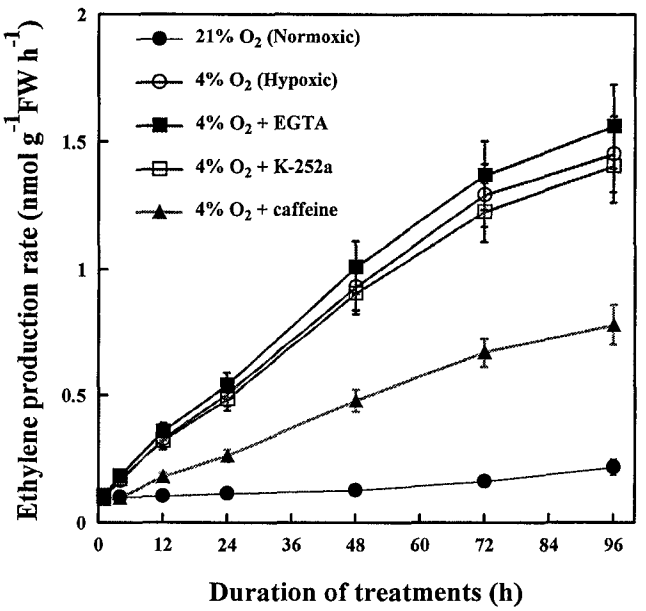


**Figure 6.** Effect of antagonists on the rate of ethylene production by freshly excised maize root tips. Treatments with intact plants began at the start of the time course. Results are means ± SE ($n$ = 4). FW, Fresh weight.

*Phalaenopsis* petals, which are induced to senesce following pollination (Porat et al., 1994).

At this stage it is a matter of conjecture as to the location of the protein kinases in the ethylene signal transduction pathway in maize roots. Several of the components in ethylene perception and signal transduction have recently been defined by various ethylene response mutants isolated in *A. thaliana* (Guzman and Ecker, 1990; reviewed by Zarembinski and Theologis, 1994; Ecker, 1995). The *ETR1* gene represents an early step in the signal transduction pathway and has sequence homology with both components of the prokaryotic "two-component" system (Chang et al., 1993). This implies that an early step (by analogy with the prokaryotic sensor-response regulator system) involves phosphorylation of a His kinase. The *CTR1* gene is downstream of *ETR1* in the signal transduction pathway, encodes a Ser/Thr protein kinase with homology with the Raf protein kinase family, and is a potential target for okadaic acid. Genes hybridizing to *CTR1* have been found in many plant species, including maize, suggesting that this pathway or parts of it may be conserved in plant species and have features in common with animal cells. Clearly, as in Arabidopsis, protein phosphorylation via kinases might occur at several steps in the ethylene signal transduction in maize.

### Cellulase Activity and Cell Death

All reagents that enhanced cell death caused a parallel increase in cellulase activity and vice versa. Although it could be argued that this merely reflected the extent of postmortem degradation of cells, this is unlikely to be so. The enzyme has to be synthesized by living cells, and this presumably occurs specifically in those cortical cells that are programmed to die in response to ethylene, as is the case in the leaf abscission zone (del Campillo et al., 1990). Also, increases in cellulase activity occur close to the root tip (He et al., 1994a) and precede or possibly are coincident with the first signs of cell abnormality (Campbell and Drew, 1983). It is therefore of interest to note that enhanced cellulase activity and cell death were not uncoupled by any of the antagonists we tested. This suggests that increased cellulase activity, and presumably also increased activity of other cell-degrading enzymes, is part of a coordinated response associated with cell death.

### Ethylene Production

The rate of ethylene production was not affected by any of the antagonists we tested, with the exception of caffeine, which was inhibitory. This means that none of the responses we found at the level of cell death or cellulase activity could be attributed to greater ethylene production. Increased ethylene synthesis in maize roots under hypoxia is associated with greater activity of ACC synthase (He et al., 1994a, 1996), higher levels of ACC (Atwell et al., 1988), and increased activity of ACC oxidase (C.-J. He, S.A. Finlayson, M.C. Drew, W.R. Jordan, and P.W. Morgan, unpublished data). Apparently, neither of these enzymes' activity is affected by reagents that modify the ethylene signal transduction pathway. The pathway is unknown by which hypoxia is sensed, causing the activity of ACC synthase and ACC oxidase to increase, but that pathway is clearly very different from the ethylene signal transduction pathway that we have tested.

The lack of effect on ethylene production by inhibitors of protein kinases and protein phosphatases contrasts markedly with the behavior of suspension-cultured cells of tomato (Spanu et al., 1994). Induction of ACC synthase in response to fungal elicitor molecules was blocked by a protein kinase inhibitor (K-252a), whereas an inhibitor of protein phosphatase (calyculin A) enhanced enzyme activity both in the presence and in the absence of fungal elicitors. The authors concluded that such rapid regulation of enzyme activity (within minutes) was posttranslational and explicable in terms of inactivation/degradation of the dephosphorylated enzyme.

### Mechanism of Cell Death

The highly selective death of cells in the root cortex in response to ethylene is reminiscent of programmed cell death or apoptosis in animal cells (Kerr and Harmon, 1991). Apoptosis is a type of programmed cell death that characteristically involves activation by $Ca^{2+}$ of endonucleases that degrade nDNA as part of a gene-regulated process in which the dying cell actively participates (Kerr, 1993; Arends and Harrison, 1994; McConkey and Orrenius, 1995). The program leading to cell death is initiated in response to either an environmental or a developmental signal and is distinct from necrosis, which characteristically involves early damage to cell membranes followed by cell lysis (Lockshin and Zakeri, 1991; Kerr, 1993). Processes akin to apoptosis have recently been identified in higher plants during the hypersensitive reaction to pathogens (Greenberg et al., 1994; Mittler and Lam, 1995a), during death of cultured plant cells induced by exposure to host-selective fungal toxins (Wang et al., 1996), and during maturation and death of xylem cells (Mittler and Lam, 1995b). DNA fragmentation and formation of typical apoptotic bodies have also been found in sloughing (dying) root cap cells (Wang et al., 1996), indicating that elements of apoptosis are conserved in plant cells as well as in animal cells. Alternatively, the enhanced activity of cellulase that is associated with cell death in the root cortex may represent but one of an entire suite of cell-degrading enzymes that are coordinately activated or released from intracellular compartments early in autolysis. Proteases, for example, are implicated in the timed senescence and death of plant organs such as flowers and leaves (Carrasco and Carbonell, 1988; Cercos and Carbonell, 1993; reviewed by Viestra, 1993), and it may be that cytosolic $[Ca^{2+}]$ somehow initiates the autolytic process without further interaction with the nucleus. Increased cytosolic $[Ca^{2+}]$ has been implicated in the activation of proteases and phospholipases in programmed cell death of neuronal cells (Server and Mobley, 1991). It remains to be seen which model of programmed cell death, or necrosis, resembles most closely the phenomenon of ethylene-promoted cell death and aerenchyma formation in the root cortex of maize.

Received February 2, 1996; accepted May 28, 1996.
Copyright Clearance Center: 0032–0889/96/112/0463/10.

## LITERATURE CITED

**Abeles FB, Morgan PW, Saltveit ME** (1992) Ethylene in Plant Biology, Ed 2. Academic Press, New York

**Arends MJ, Harrison DJ** (1994) Apoptosis: molecular aspects and pathological perspective. *In* C Crocker, ed, Molecular Biology in Histopathology. Wiley, Chichester, UK, pp 151–170

**Armstrong W** (1979) Aeration in higher plants. Adv Bot Res **7:** 225–331

**Atwell BJ, Drew MC, Jackson MB** (1988) The influence of oxygen deficiency on ethylene synthesis, 1-aminocyclopropane-1-carboxylic acid levels and aerenchyma formation in roots of *Zea mays* L. Physiol Plant **72:** 15–22

**Bethke PC, Jones RL** (1994) $Ca^{2+}$-calmodulin modulates ion channel activity in storage protein vacuoles of barley aleurone cells. Plant Cell **6:** 277–285

**Bush DS** (1993) Regulation of cytosolic calcium in plants. Plant Physiol **103:** 7–13

**Calvert CM, Sanders D** (1995) Inositol triphosphate-dependent and -independent $Ca^{2+}$ mobilization pathways at the vacuolar membrane of *Candida albican*. J Biol Chem **270:** 7272–7280

**Campbell R, Drew MC** (1983) Electron microscopy of gas space (aerenchyma) formation in adventitious roots of *Zea mays* L. subjected to oxygen shortage. Planta **157:** 350–357

**Carrasco P, Carbonell J** (1988) Involvement of a neutral proteolytic activity in the senescence of unpollinated ovaries of *Pisum sativum*. Physiol Plant **72:** 610–616

**Cercos M, Carbonell J** (1993) Purification and characterization of a thiol-protease induced during senescence of unpollinated ovaries of *Pisum sativum*. Physiol Plant **88:** 267–274

**Chang C, Kwok SF, Bleecker AB, Meyerowitz EM** (1993) *Arabidopsis* ethylene-response gene ETR1: similarity of product to two-component regulators. Science **262:** 539–544

**Charuk JHM, Pirraglia CA, Reithmeier RAF** (1990) Interaction of ruthenium red with $Ca^{2+}$-binding proteins. Anal Biochem **188:** 123–131

**Chen Q, Boss WF** (1991) Neomycin inhibits the phosphatidylinositol monophosphate and phosphatidylinositol bisphosphate stimulation of plasma membrane ATPase activity. Plant Physiol **96:** 340–343

**Clapham DE** (1995) Calcium signaling. Cell **80:** 259–268

**Cohen P** (1989) The structure and regulation of protein phosphatases. Annu Rev Biochem **58:** 453–508

**Cohen P, Holmes CFB, Tsukitani Y** (1990) Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem Sci **15:** 98–102

**del Campillo E, Reid PD, Sexton R, Lewis LN** (1990) Occurrence and localization of 9.5 cellulase in abscising and nonabscising tissues. Plant Cell **2:** 245–254

**Drew MC** (1990) Sensing soil oxygen. Plant Cell Environ **13:** 681–693

**Drew MC, He CJ, Morgan PW** (1989) Decreased ethylene biosynthesis, and induction of aerenchyma, by nitrogen- or phosphate-starvation in adventitious roots of *Zea mays* L. Plant Physiol **91** 266–271

**Drew MC, Jackson MB, Giffard S** (1979) Ethylene-promoted adventitious rooting and development of cortical air spaces (aerenchyma) in roots may be adaptive response to flooding in *Zea mays* L. Planta **147:** 83–88

**Drew MC, Jackson MB, Giffard SC, Campbell R** (1981) Inhibition by silver ions of gas space (aerenchyma) formation in adventitious roots of *Zea mays* L. subjected to exogenous ethylene or to oxygen deficiency. Planta **153:** 217–224

**Drobak BK** (1993) Plant phosphoinositides and intracellular signaling. Plant Physiol **102:** 705–709

**Ecker JR** (1995) The ethylene signal transduction pathway in plants. Science **268:** 667–675

**Fairley-Grenot K, Assmann SM** (1991) Evidence for G-protein regulation of inward $K^+$ channel current in guard cells of fava bean. Plant Cell **3:** 1037–1044

**Gabev E, Kasianowicz J, Abbott T, McLaughlan S** (1989) Binding of neomycin to phosphatidylinositol 4,5-bisphosphate ($PIP_2$). Biochim Biophys Acta **979:** 105–112

**Gilroy S, Hughes WA, Trewavas AJ** (1986) The measurement of intracellular calcium levels in protoplasts from higher plant cells. FEBS Lett **199:** 217–221

**Greenberg JT, Guo A, Klessig DF, Ausubel FM** (1994) Programmed cell death in plants: a pathogen-triggered response activated coordinately with multiple defense functions. Cell **77:** 551–563

**Grosskopf DG, Felix G, Boller T** (1990) K-252a inhibits the response of tomato cells to fungal elicitors in vivo and their microsomal protein kinase in vitro. FEBS Lett **275:** 177–180

**Guzman P, Ecker JR** (1990) Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell **2:** 513–523

**He CJ, Drew MC, Morgan PW** (1994a) Induction of enzymes associated with lysigenous aerenchyma formation in roots of *Zea mays* during hypoxia or nitrogen starvation. Plant Physiol **105:** 861–865

**He CJ, Jordan WR, Drew MC, Morgan PW** (1994b) Transduction of an ethylene signal is required for aerenchyma development in roots of maize (abstract no. 503). Plant Physiol **105:** S-96

**He CJ, Morgan PW, Jordan WR, Drew MC** (1993) The role of calcium in aerenchyma development in roots of maize (abstract no. 910). Plant Physiol **102:** S-159

**Hepler PK, Wayne RO** (1985) Calcium and plant development. Annu Rev Plant Physiol **36:** 397–439

**Hua J, Chang C, Sun Q, Meyerowitz EM** (1995) Ethylene insensitivity conferred by *Arabidopsis* ERS gene. Science **269:** 1712–1714

**Jackson MB, Fenning TM, Drew MC, Saker LR** (1985) Stimulation of ethylene production and gas-space (aerenchyma) formation in adventitious roots of *Zea mays* L. by small partial pressures of oxygen. Planta **165:** 486–492

**Justin SHF, Armstrong W** (1991) The anatomical characteristics of roots and plant response to soil flooding. New Phytol **106:** 465–495

**Kase H, Iwahashi K, Matsuda Y** (1986) K-252a, a potent inhibitor of protein kinase C from microbial origin. J Antibiot **39:** 1059–1065

**Kawase M** (1979) Role of cellulase in aerenchyma development in sunflower. Am J Bot **66:** 183–190

**Kawase M** (1981a) Anatomical and morphological adaptations of plants to waterlogging. Hortscience **16:** 30–34

**Kawase M** (1981b) Effect of ethylene on aerenchyma development. Am J Bot **68:** 651–658

**Kerr JFR** (1993) Introduction: definition of apoptosis and overview of its incidence. *In* M Lavia, D Watters, eds, Programmed Cell Death: The Cellular and Molecular Biology of Apoptosis. Harwood Academic Publishers, Chur, Switzerland, pp 1–15

**Kerr JFR, Harmon BV** (1991) Definition and incidence of apotosis: an historical perspective. *In* LD Tomei, FO Lope, eds, Apoptosis: The Molecular Basis of Cell Death. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 5–29

**Knight MR, Smith SM, Trewavas AJ** (1992) Wind-induced plant motion immediately increases cytosolic calcium. Proc Natl Acad Sci USA **89:** 4967–4971

**Konings H** (1982) Ethylene-promoted formation of aerenchyma in seedling roots of *Zea mays* L. under aerated and non-aerated conditions. Physiol Plant **54:** 119–124

**Lee KS, Tsien RW** (1983) Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. Nature **302:** 790–794

**Lockshin RA, Zakeri Z** (1991) Programmed cell death and apoptosis. *In* LD Tomei, FO Lope, eds, Apoptosis: The Molecular Basis of Cell Death. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 47–60

**Ma H** (1994) GTP-binding proteins in plants: new members of an old family. Plant Mol Biol **26:** 1611–1636

**McConkey DJ, Orrenius S** (1995) Calcium and cyclosporin in the regulation of apoptosis. *In* G Kroemer, C Martinez, eds, Apoptosis in Immunology. Springer-Verlag, Berlin, pp 95–105

**McPherson MC** (1939) Cortical air spaces in the roots of *Zea mays* L. New Phytol **38:** 190–202

**Mittler R, Lam E** (1995a) Identification, characterization and purification of a tobacco endonuclease activity induced upon hypersensitive response cell death. Plant Cell **7:** 1951–1962

**Mittler R, Lam E** (1995b) *In situ* detection of nDNA fragmentation during the differentiation of tracheary elements in higher plants. Plant Physiol **108:** 489–493

**Porat R, Borochov A, Halevy AH** (1994) Pollination-induced senescence in phalaenopsis petals. Relationship of ethylene sensitivity to activity of GTP-binding proteins and protein phosphorylation. Physiol Plant **90:** 679–684

**Pradet A, Bomsel JL** (1978) Energy metabolism in plants under hypoxia and anoxia. *In* DD Hook, RMM Crawford, eds, Plant Life in Anaerobic Environments. Ann Arbor Science Publishers, Ann Arbor, MI, pp 89–118

**Raz V, Fluhr R** (1992) Calcium requirement for ethylene-dependent responses. Plant Cell **4:** 1123–1130

**Raz V, Fluhr R** (1993) Ethylene signal is transduced via protein phosphorylation events in plants. Plant Cell **5:** 523–530

**Roberts DM, Harmon AC** (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu Rev Plant Physiol Plant Mol Biol **43:** 375–414

**Sasaki T, Naka M, Nakamura F, Tanaka T** (1992) Ruthenium red inhibits the binding of calcium to calmodulin required for enzyme activation. J Biol Chem **267:** 21518–21523

**Server AC, Mobley WC** (1991) Neuronal cell death and the role of apoptosis. *In* LD Tomei, FO Lope, eds, Apoptosis: The Molecular Basis of Cell Death. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 263–278

**Spanu P, Grosskopf DG, Felix G, Boller T** (1994) The apparent turnover of 1-aminocyclopropane-1-carboxylate synthase in tomato cells is regulated by protein phosphorylation and dephosphorylation. Plant Physiol **106:** 529–535

**Subbaiah CC, Bush DS, Sachs MM** (1994a) Elevation of cytosolic calcium precedes anoxic gene expression in maize suspension-culture cells. Plant Cell **6:** 1747–1762

**Subbaiah CC, Zhang J, Sachs MM** (1994b) Involvement of intracellular calcium in anaerobic gene expression and survival of maize seedlings. Plant Physiol **105:** 369–376

**Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP** (1990) Thapsigargin, a tumor promoter, discharges intracellular $Ca^{2+}$ stores by specific inhibition of the endoplasmic reticulum $Ca^{2+}$-ATPase. Proc Natl Acad Sci USA **87:** 2466–2470

**Tomei DL, Cope FO (eds)** (1991) Apoptosis: The Molecular Basis of Cell Death. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

**Viestra RD** (1993) Protein degradation in plants. Annu Rev Plant Physiol Plant Mol Biol **44:** 385–410

**Vites AM, Pappano AJ** (1992) Ruthenium red selectively prevents Ins (1,4,5)P3- but not caffeine-gated calcium release in avian atrium. Am J Physiol **262:** H268–H277

**Wang H, Li J, Bostock RM, Gilchrist DG** (1996) Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. Plant Cell **8:** 375–391

**Zarembinski TI, Theologis A** (1994) Ethylene biosynthesis and action: a case for conservation. Plant Mol Biol **26:** 1579–1597

**Ziegelstein RC, Cheng L, Aversano T, Ouyang P, Lakatta EG, Silverman HS** (1994) Increase in rat aortic endothelial free calcium mediated by metabolically sensitive calcium release from endoplasmic reticulum. Cardiovasc Res **28:** 1433–1439