A Ca\(^{2+}\) -dependent cysteine protease is associated with anoxia-induced root tip death in maize

Chalivendra C. Subbaiah\(^1,3\), Krishna P. Kollipara\(^1,4\) and Martin M. Sachs\(^1,2\)

\(^1\) Department of Crop Sciences, University of Illinois, Urbana, IL 61801–4730, USA
\(^2\) USDA/ARS, Soybean/Maize Germplasm, Pathology and Genetics Research Unit, Urbana, Illinois 61801, USA

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Abstract

Imposition of anoxia on maize (Zea mays cv. B73) seedlings for 48 h or longer led to the death of the root tip. The necrosis extended into the root axis during post-anoxic treatment, leading to the mortality of 30–50\% of the seedlings. Using zymography, protease profiles in the root tissues of anoxic seedlings were studied. O\(_2\) deprivation for 24 h or longer repressed pre-existing protease activities and induced a novel soluble enzyme in the roots. The anoxia-induced protease (AIP) activity was predominant in the root apex at 24 h of anoxia and, subsequently, became the most abundant soluble activity in the root axis as well. The induction of AIP and its in vitro renaturation were Ca\(^{2+}\)-dependent. Inhibitor sensitivity studies indicated that AIP is a cysteine protease. In SDS-acrylamide gels, the enzyme activity migrated as a 23.5 kDa polypeptide. The anoxic induction of the activity was repressed by cycloheximide treatment, suggesting that new protein synthesis was required for the AIP appearance. Excision of the root tip (de-tipping) before anoxia led to a superior recovery of seedlings from stress injury. De-tipped seedlings showed lesser root damage and an increased production of lateral roots compared to intact seedlings. Furthermore, the superior anoxia tolerance of de-tipped seedlings was associated with a decreased AIP activity. Thus, the appearance of AIP activity in the root tip at 24 h of anoxia was spatially and temporally associated with the root tissue death. These studies further indicate that the root tip elimination early during anoxia may provide an adaptive advantage.

Key words: Anoxia, root tip, cell death, cysteine protease.

Introduction

Oxygen deprivation limits the growth and productivity of submerged plants. The magnitude of tolerance to anaerobiosis not only varies among different plant species (Crawford, 1992; Kennedy \textit{et al}., 1992) or cultivars within a species (Lemke-Keyes and Sachs, 1989), but also within different tissues of an individual plant (Johnson \textit{et al}., 1989; Sachs, 1994; Ellis \textit{et al}., 1999). Maize root tips are very sensitive to anoxia and die within a few hours (Roberts \textit{et al}., 1984; Johnson \textit{et al}., 1989). Root tips (or apices) are composed of tightly packed tissues with few, if any, intercellular spaces and hence suffer from restricted gaseous diffusion. In addition, the young, dividing cells in this region are metabolically-intensive with high O\(_2\) demand and, therefore, experience hypoxia even under normal oxygen partial pressures (Armstrong \textit{et al}., 1991; Crawford, 1992; Andrews \textit{et al}., 1993). Consequently, in submerged seedlings, root tip death may be a natural consequence of oxygen starvation and the attendant repression of substrate transport. A lot of attention has been given to strategies/mechanisms that prolong the anoxia tolerance of the primary root tip in young maize seedlings, as the tip of the primary root is considered to be very important for seedling establishment (for a review, see Drew \textit{et al}., 1994). On the other hand, the hypothesis proposed here is that the death of the primary root tip under severe oxygen deficit can be a tolerance mechanism that eliminates dispensable but metabolically intense tissues, and redirects the limiting resources to prolong the survival of the root axis and the shoot. These two latter organs are essential for the post-anoxic re-growth and autotrophic life of the seedling. Hence, the goal in the present work is to test the proposal that the death of the primary root tip has an adaptive advantage, and to probe...
further the mechanisms that regulate the process. In this report, the time-course of root tip death initiation in the maize inbred, B73, was studied. To identify potential regulators of the cell death process, the changes in protease activities in the root apex and axis were followed. Increasing evidence indicates that proteases play an important regulatory role in the initiation of cell death (Chen and Foolad, 1997, and references therein), although other hydrolases (such as nucleases, lipases and cell wall-acting enzymes, e.g. cellulases, XET) may assume important roles in the later stages of cell lysis. Cysteine and serine proteases have been implicated in the cell death/injury induced by abiotic, biotic or developmental signals in plants (Schaffer and Fischer, 1988; Williams et al., 1994; Jiang et al., 1995; Stroeher et al., 1997; Del Pozo and Lam, 1998; Solomon et al., 1999; for reviews, see Beers, 1997; Hadfield and Bennett, 1997). The results of this study indicate that the root tip self-amputation has an adaptive role in the seedling anoxia tolerance and a Ca\(^{2+}\)-dependent soluble cysteine protease may be involved in the death of the root tip.

**Materials and methods**

**Materials**

Caryopses of inbred maize (Zea mays L.) line B73 were germinated and grown as described previously (Subbaiah et al., 1994b). The root system of seedlings used in the study consisted of only a primary root (5–8 cm) and two small seminal roots (<10 mm). Only the primary root was utilized for this analysis and hence the terms ‘tip’ and ‘axis’ used throughout the text apply to the apical and distal portions of the primary root.

Protease inhibitors were purchased from Calbiochem (La Jolla, CA) or Roche Molecular Biology (Indianapolis, IN). All other chemicals were from Bio-Rad (Hercules, CA) or Sigma (St Louis, MO) and were analytical grade.

**Treatment of seedlings and preparation of root extracts**

Anoxia was induced by submerging the seedlings in gas-tight containers completely filled with flooding buffer (5 mM TRIS-Cl, pH 7.5, 250 mg amoxicillin + 125 mg ampicillin 1\(^{-1}\)) as described earlier (Subbaiah et al., 1994b). In initial experiments, the submerged seedlings were incubated in an anaerobic system to create rapid and complete anoxia (Subbaiah et al., 1994a). The results did not vary between these two methods of imposing oxygen deprivation and, hence, the term ‘anoxia’ was used throughout this report. At selected time points of anoxic treatment, only the primary roots were harvested and used for enzyme extraction or frozen in liquid N\(_2\) for later use. Root tissue was ground in a buffer containing 62 mM TRIS-Cl pH 6.8, 10% glycerol and 2% Triton X-100 for total proteases and the extract was centrifuged at 10,000 g for 10 min. The supernatant was used for protease analysis. Soluble and membrane fractions were prepared from fresh, unfrozen tissue. Triton X-100 was omitted from the extraction buffer. The extract was centrifuged at 10,000 g for 10 min to pellet organelles and cell debris. The clarified extract was further centrifuged at 35,000 rpm for 90 min in a Ti45 rotor (Beckman-Coulter, Fullerton, CA). The supernatant represented the soluble fraction and the pellet was regarded as the membrane fraction. To test the effect of extracellular Ca\(^{2+}\) on protease induction, maize seedlings were submersed in the presence of 2 mM of EGTA or CaCl\(_2\), and used for protease extraction. The effect of cycloheximide (CHI, a translational inhibitor) on protease induction was tested by adding the drug at 5 or 20 \(\mu\)g ml\(^{-1}\) in the submergence buffer. Control seedlings were incubated with methanol (used as solvent for CHI, up to 0.05% at final dilution). In experiments where protease profiles were analysed separately in the tip and axis, the root apex (‘tip’, up to 10–15 mm from the extreme end of the root including the root cap) was excised from the rest of the root (‘axis’), and processed separately.

**Zymographic analysis of protease activities:** Protein concentration was estimated using DC (detergent-compatible)-Protein Assay kit from Bio-Rad. Protease activities were analysed by native or denaturing PAGE in gelatin-copolymerized acrylamide gels (modified from Fisher et al., 1989). Briefly, proteins (equal amounts in each lane, for all comparisons) were electrophoresed in 6–12% acrylamide gradient gels containing 0.033% gelatin. The gels were washed in 2.5% Triton X-100 and incubated overnight in assay buffer containing 50 mM TRIS-Cl, pH 7.5, 150 mM KCl, 10 mM CaCl\(_2\), 100 \(\mu\)M ZnCl\(_2\), and 0.05% Brij-50. The effect of Ca\(^{2+}\) was tested by omitting CaCl\(_2\) and adding 2 mM EGTA to the buffer. Sensitivity to protease inhibitors was tested by incubating the extracts at indicated concentrations of the inhibitors for 20 min at room temperature, before electrophoresis. Inhibitors were also added in the wash and activity buffers. Water-insoluble inhibitors (calpain inhibitor I, chymostatin and pepstatin) were dissolved in DMSO and used at concentrations indicated in the Results. As a control for these inhibitors, an appropriate concentration of the solvent (up to 5%) was tested on protease activity. Native-SDS two-dimensional gels were run as described previously (Lal et al., 1998).

**In vitro analysis of protease activities:** Protease activity was assayed spectrophotometrically using Azocoll as the substrate (Chavira et al., 1984).

**Flood tolerance tests**

Maize seedlings were submersed and post-anoxic survival as well as growth were measured as described earlier (Subbaiah et al., 1994b). To de-tip seedlings, the apical 15 mm region was excised under flooding buffer and discarded. The de-tipped seedlings were then submersed and tolerance tested.

**Digitization and quantification of zymography results**

Gels were photographed using Polaroid Instant photography (Type 665 or 667). The positives were scanned using a flat-bed scanner and stored as digitized images. The images were analysed using an NIH Image program (developed at the United States National Institute of Health and available on the internet at http://rsb.info.nih.gov/nih-image). The band intensities were measured, subtracted from background, integrated with areas and averaged.

**Results**

**Imposition of anoxia on maize seedlings for 48 h or beyond leads to the death of the root tip and the proximal axis**

Three-day-old dark-grown maize (cv. B73) seedlings were submersed for different periods and were planted in the sand bench to recover. Seedlings were gently uprooted after 2 d and observed for root and shoot injury/
re-growth. The seedlings when submerged had only primary roots and rudimentary seminal roots. Anoxia (i.e. complete submergence in sealed containers) rapidly suppressed all growth and developmental processes in maize seedlings. No new meristems were produced until after reoxygenation. Therefore, the terms root ‘tip’ and ‘axis’ refer to those of the primary root, throughout this report. Post-anoxic recovery of root and shoot growth was inversely proportional to the duration of anoxia, but the stress injury was particularly severe at 48 h of O$_2$ deprivation, when roots showed necrosis of the tip and the elongation zone after reoxygenation treatment (Fig. 1). The seedling recovery in this genotype was close to 100% until 24 h of anoxia and thereafter it declined (Table 1; Lemke-Keyes and Sachs, 1989). Furthermore, the tip growth resumed after re-aeration in seedlings submerged for 24 h or less (Fig. 1), although cell death indicators such as callose deposits and associated biochemical changes appeared in the tip within 24 h or shorter duration of anoxia (CC Subbaiah and MM Sachs, unpublished data). This indicated that the death process, although initiated before 24 h, became irreversible at 48 h of inundation. Even in 48 h submerged seedlings, tissue browning did not occur as long as seedlings were submerged, but was seen only after an exposure to air for at least 12 h, indicating that oxygen is required for the appearance of visual symptoms of the death process. In this report, the term root tip ‘death’ signifies the post-anoxic appearance of necrotic symptoms and growth cessation, while ‘survival’ is the lack of such symptoms and resumption of growth.

**Anoxia-induced changes in root protease activities of maize seedlings**

Total collagenase (azocoll-hydrolysing) activities in root extracts from aerobic and submerged seedlings are shown in Fig. 2. Protease activity declined in the root under anoxia, particularly after 12 h and corresponded with the anoxia-induced growth-suppression. This was further supported by the recovery of protease activity within 24 h of re-oxygenation (see below). In seedlings submerged for 48 h, the activity was only 50% of the aerobic levels. Azocoll activity declined by only 18–20% in seedlings that remained under aerobic incubation for 48 h (Fig. 2).

The activity profile as resolved by native-PAGE shows that a prominent gelatinase activity seen in the root axis of aerobic seedlings (marked by an arrowhead)
Table 1. Effect of anoxia up to 48 h on survival and fresh weight accumulation in intact and de-tipped maize seedlings (cv. B73)

Results are means ± SE of three independent experiments.

<table>
<thead>
<tr>
<th>Hours of anoxia</th>
<th>% Survival</th>
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<tr>
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<td>Intact</td>
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<td>48</td>
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AIP is a soluble protease and migrates as a 23.5 kDa polypeptide in denaturing gels

When the extract was separated into soluble and microsomal fractions, AIP was recoverable from the soluble part of the extract with no detectable activity in the membrane fraction (Fig. 4). In this report, it has not been determined if the enzyme is cytoplasmic or localized to any organelle. Zymography of anoxic and aerobic extracts in SDS-denaturing gels showed a greater diversity of activities than was found in native gels. This indicated that many protease species existing inactive in the root extracts were activated during renaturation in vitro (in addition to the superior resolving power of SDS-PAGE). There were activities that corresponded with the bands in native gels. However, in initial attempts, no activity in SDS-(or native/SDS 2-dimensional) gels could be indicated. Duration (0–48 h) were assayed for protease activity using Azocoll assay. Protease activity in seedlings that were incubated aerobically for 48 h (A₄₈) is also shown for comparison. Results are means ± SE of four independent experiments.

Fig. 2. Total protease activity in root tissue of maize seedlings subjected to anoxia. Root extracts from seedlings anoxically-stressed for the indicated duration (0–48 h) were assayed for protease activity using Azocoll assay. Protease activity in seedlings that were incubated aerobically for 48 h (A₄₈) is also shown for comparison. Results are means ± SE of four independent experiments.

rapidly declined during anoxia (Fig. 3A). O₂ deprivation increased a high molecular weight band in the root axis (indicated by the asterisk) and induced a faster migrating gelatinase species in the tip as well as the axis (indicated by an arrow). By 48 h of anoxia, the high molecular activity of the axis (marked by the asterisk) declined to a barely detectable level, while the fastest migrating species became the most predominant activity both in the tip and the axis. No activity in the range was detectable in aerobic extracts, even after 48 h of aerobic incubation (Fig. 3B), indicating that this may be a novel protease induced by anoxia. This activity, hereafter referred to as AIP (anoxia-induced protease), is the object of the present investigation.

In root extracts of seedlings left under aerobic incubation for 48 h, a slow-migrating activity became intense (arrowhead, Fig. 3B) indicating that total gelatinase activity increased substantially during aerobic growth. Apparently this species cannot utilize azocoll as the substrate, as total azocoll activity showed a small but consistent decline by 48 h from ‘zero’-time aerobic control (Fig. 2).
Maize cysteine protease in root tip death

Fig. 3. (A) Gelatinase profiles of root tip and axis in aerobic and anoxic maize seedlings. Maize seedlings were treated anoxically for the indicated duration in hours. Root tissue was collected and divided into tip (up to 1.5 cm from the apex, 'T') and axis (rest of the root, 'Ax'). Extracts were made separately and assayed for gelatinase activities by native PAGE (6–12%), as described in Materials and methods. The inset shows an analysis of the extracts in a 10% gel, which gave a superior resolution of the slow migrating activities. Arrowhead indicates the major aerobic activity. The asterisk indicates the protease activity that increased up to 24 h of anoxia. (B) Comparison of total root gelatinase activities in '0-time' aerobic (A₀), 48 h aerobic (A₄₈) and 48 anoxic (S₄₈) seedlings. AIP, the anoxia-induced protease, is shown by an arrow in all the figures.

Fig. 4. AIP is a soluble enzyme. Maize seedlings were anoxically-stressed for 48 h and soluble and membrane fractions were prepared from the whole root as detailed in Materials and methods. The extracts were analysed by native gelatin zymography.

under anoxia, was used. In seedlings submerged in the presence of CHI (at 20 μg ml⁻¹), AIP was totally repressed (Fig. 6A) and at a lower concentration of the inhibitor (5 μg ml⁻¹), the activity was decreased by 75% (Fig. 6B). These results indicated that the AIP synthesis was induced by O₂ deprivation. The effect was fairly specific to AIP as all the major protease activities present in untreated seedlings, including a few novel ones, were detectable in extracts from CHI incubated seedlings.

In vitro renaturation of AIP and its in vivo induction are Ca²⁺ dependent

It was investigated whether Ca²⁺ is really required for AIP activity, as the cation was routinely added to the assay buffer. In native gels, removal of Ca²⁺ by EGTA addition decreased the enzyme activity by ~90% (data not shown). In denaturing gels, AIP activity was totally abolished by EGTA and Ca²⁺ was necessary to activate
Fig. 5. (A) AIP migrates as 23.5 kDa polypeptide in SDS-acrylamide gels. Maize seedlings were submerged for the indicated duration. Root tissue was collected and divided into tip (up to 1.5 cm from the apex, ‘T’) and axis (rest of the root, ‘Ax’). Renaturable gelatinase activities were profiled in root tip and axis extracts by SDS-PAGE. (B) Root extracts from ‘aerobic’ and 48-h stressed (‘anoxic’) seedlings were first resolved in a non-denaturing 6–12% gradient acrylamide gel. Lanes from the native gel were excised and resolved in the second-dimension in denaturing 6–12% gradient acrylamide gels. Left lanes indicate single dimension separation by SDS-PAGE. Numerals on the left side of panels in all the figures indicate molecular mass in kDa.

The active site specificity of AIP was probed by testing the effects of class-specific inhibitors. E-64 (1.64 μM), a potent cysteine protease inhibitor completely blocked AIP activity (Fig. 8). Chymostatin (100 μM), an inhibitor of most of the cysteine proteases and the Calpain Inhibitor I (6 μM), a peptide inhibitor of animal calpain-type neutral cysteine proteases, decreased the activity by 66%
Maize cysteine protease in root tip death

Fig. 7. AIP is a Ca$^{2+}$-dependent protease (A). Whole root extracts made from aerobic (lane A) or 48 h-anoxically stressed (lane S) seedlings were tested for gelatinase activities in the presence of 2 mM EGTA (−Ca$^{2+}$) or 2 mM EGTA + 3 mM CaCl$_2$ (+Ca$^{2+}$). Zymographic analysis was carried out in SDS-denaturing gels. AIP induction may be regulated by cytosolic Ca$^{2+}$ (B). Maize seedlings were subjected to anoxia for 24 or 48 h in the presence of either 2 mM EGTA or CaCl$_2$. Extracts made from tip (T$_{24}$ and T$_{48}$) and axis tissues (Ax$_{24}$ and Ax$_{48}$) were tested for native gelatinase activities.

Fig. 8. AIP is a cysteine protease. Effects of cysteine-specific (E-64 at 1.4 µM; Cl: calpain inhibitor at 6 µM; CS: chymostatin at 100 µM), serine-specific (PB: pefablock at 0.4 mM) or aspartate-specific (PS: pepstatin at 1 µM) protease-inhibitors were tested on the AIP activity as described in Materials and methods. Gelatinase assays were done in non-denaturing gels. A, aerobic; S, 48 h-anoxic seedlings.

and 83%, respectively. Pepstatin (1 µM), an inhibitor of aspartic proteases did not cause a decrease in AIP activity. Pefablock (0.4 and 2 mM), used as an inhibitor of serine proteases, but also known to act against certain type of cysteine proteases (Alonso et al., 1996), showed a mild inhibitory effect on AIP activity. These results suggest that AIP is a cysteine protease, functionally similar to the calpain type of Ca$^{2+}$-dependent thiol proteases. Calpains have been reported to occur in a wide variety of organisms such as vertebrate and invertebrate animals, fungi and algae (for reviews, see Mykles and Skinner, 1990; Sorimachi et al., 1996; Johnson and Guttman, 1997). Results of many studies strongly suggest that cell death under ischemia and hypoxia in vertebrates is a result of calpain activation (Johnson and Guttman, 1997).

De-tipping decreases AIP activity and alleviates anoxic stress injury

To test if the apical part of the root is the origin and major source of AIP, the root tip (10–15 mm of the apex) was removed before seedlings were submerged. The AIP activity in de-tipped roots was compared with the activity in the root axes of intact seedlings (Fig. 9A). De-tipping reduced the enzyme activity by ~50% in the root axis of seedlings submerged for 24 h and about 30% at 48 h of anoxia. This indicated that the tip or tip-associated processes may play a role in the induction of AIP even in the axis region. The dynamics of AIP activity were also tested soon after reoxygenation. Re-aeration decreased AIP activity in the root axis (the tip, being necrotic, was discarded) by greater than 50% within 24 h (Fig. 9B).

The aim here is also to determine if the root tip removal before submergence would lead to superior anoxic tolerance. The post-anoxic survival of de-tipped and intact maize seedlings was compared after they had been submerged for different periods. Removal of the root tip did not affect the survival or growth of aerobic seedlings except that there was an increased partitioning of biomass into the roots in comparison to intact seedlings (Table 1), most likely due to a loss of root apical dominance. This trend continued until 12 h of anoxia. However, better
Fig. 9. De-tipping or reaeration diminishes AIP activity. Root extracts were made from de-tipped seedlings treated anoxically for 1 or 2 d (dT24 and dT48) and tested for native gelatinase activities. For comparison, AIP activity in the root axis of seedlings stressed for 24 or 48 h (Ax24 or Ax48) is also shown (A). Seedlings subjected to 48 h of anoxia were transplanted in vermiculite to recover for 24 h in the dark (R). For comparison, AIP activity in the root axis of seedlings submerged continuously for 72 h (Ax72) is also shown (B). In de-tipped and reaerated seedlings, only the root axis was available (root tip being excised or dead) for protease extraction and hence, comparison was made with the extract from the root axis of intact seedlings.

Fig. 10. De-tipped maize seedlings show improved post-anoxic survival and growth. Seedlings were either submerged with the root tip ‘Intact’ or removed (‘de-tipped’). After the indicated duration, they were planted in the greenhouse to score for re-growth. Seedling survival (A) and root as well as shoot growth (B) were recorded after 2 weeks. Results are means ± SE of three separate experiments. Intact or de-tipped seedlings were anoxically-stressed for 48 h and transplanted on the sand-bench. They were gently uprooted after 3 d to observe the re-growth (C).

Discussion

The primary root tip is a dispensable energy drain in anoxic maize seedlings

The seedling primary root plays a brief but important role in the life cycle of maize, as in the case of most herbaceous monocotyledons. In most maize genotypes, the seminal roots grow and contribute to nutrient and water absorption, within the first few weeks after germination. After a month of seedling emergence, the seminal root system (the primary and seminal roots) assumes less importance and frequently decays following the
development of the nodal or permanent root system (Feldman, 1994). The primary root tip is needed for even a shorter duration, i.e. only during the first few days of germ growth, to establish seedling orientation in space and to produce initially tissues involved in water/nutrient uptake. The root apex is metabolically very intense and thus has greater O$_2$ demand than the rest of the primary root (Armstrong et al., 1991; Lin and Lin, 1992). In young maize seedlings subjected to prolonged anoxia, the primary root tip appears to serve no perceptible function essential for seedling survival. Instead, it may be a non-essential sink which, if eliminated, can conserve limiting resources. The conserved substrate and energy can usefully be redirected to prolong the maintenance of organs essential for growth after re-aeration. These studies show that excision of the primary root tip was not detrimental to either the aerobic or anoxic growth of young maize seedlings (Table 1). On the contrary, removal of the root tip before imposing anoxia alleviated the stress injury and prolonged the tolerance of maize seedlings to oxygen deprivation. Thus, the death of the metabolically intensive root tip, induced by prolonged anoxia, served as a tolerance mechanism against the severe energy starvation known to occur under O$_2$ deprivation (Ricard et al., 1994). Recently, Zeng et al. (Zeng et al., 1999) compared the anoxic tolerance of excised root tips with that of intact seedlings, in three different maize genotypes. Maize variety Oh43 which has the least anoxic tolerance in excised root tips shows greater whole seedling tolerance to anoxia than NK508 and W22, indicating that the early death of the primary root tip confers superior flooding tolerance in young maize seedlings. The natural ‘detipping’ of the primary root may be a genetically controlled flooding tolerance mechanism in plants, although the importance of the root tip during normal growth and development is indisputable.

Is AIP an initiator or executor of the root tip death?

AIP is a Ca$^{2+}$-dependent soluble cysteine protease that was inducible in the root apical tissue within 24 h of anoxia. Its temporal precedence to the root necrosis and spatial distribution within the root indicate that AIP may play an important role in the initiation of root tip death. Furthermore, a decrease in AIP activity as well as a greatly reduced root necrosis in de-tipped seedlings suggests that the enzyme may serve as a molecular switch that turns on the cell death process. However, its continued presence, albeit at reduced levels, after re-oxygenation does not rule out that it may serve as an executor of cell degradative processes. As root necrosis in anoxically-treated maize seedlings continued even after reoxygenation, the continued presence of AIP activity in re-aerated seedlings may not be totally surprising. AIP was the only detectable gelatinase, which showed sensitivity to cysteine protease inhibitors (including calpain inhibitor 1) and also Ca$^{2+}$ dependence, suggesting that the enzyme may be functionally analogous to animal calpains. Calpains are regulated by Ca$^{2+}$ as well as cellular redox state (by virtue of having cysteine in the active site, they are inhibited by oxidants) and initiate hypoxic cell death in vertebrates (Johnson and Guttmann, 1997). It is necessary to identify the molecular targets of AIP and events downstream to its induction in order to understand whether the enzyme has a role in cell death. Furthermore, other proteases (particularly those, which cannot degrade gelatin) cannot be ruled out for their role in the root tip death.

With the present data, it is not possible to compare AIP with other proteases that have previously been identified from maize or other species. AIP is similar in its size and active site to the cysteine protease implicated in the programmed cell death during tracheary element differentiation in *Zinnia elegans* (Ye and Varner, 1996). However, it is not known if the zinnia enzyme requires Ca$^{2+}$ for its activity. At the same time, AIP is different from ACDP, a Ca$^{2+}$-dependent cysteine protease partially purified from *Arabidopsis* root tissue, in its size and sensitivity to calpain inhibitors (Safadi et al., 1997).

**Cytosolic Ca$^{2+}$ changes may be involved in the initiation of cell death in anoxic root tips**

Earlier work showed that an elevation of cytosolic Ca$^{2+}$ triggers gene activation and tolerance to O$_2$ deprivation (Subbaiah et al., 1994a, b). Ca$^{2+}$-release from intracellular stores is critical for anoxic tolerance during the first few hours of anoxia (Subbaiah et al., 1994b) and seedling survival is compromised in the absence of extracellular Ca$^{2+}$, beyond 12 h of anoxia (CC Subbaiah, unpublished data). Subsequently, it has been shown that hypoxia-induced cell death during aerenchyma formation is also a Ca$^{2+}$-dependent process (He et al., 1996). Thus, cytosolic Ca$^{2+}$-rise appears to switch on the co-ordinate gene expression induced under oxygen deprivation in maize roots. The current work shows that the AIP induction is inhibited by EGTA when added to the submergence buffer (Fig. 7C) and thus its synthesis and cell death initiation may depend on elevated cytosolic Ca$^{2+}$ levels in the root tip cells. Furthermore, the inhibition of AIP induction by the translational inhibitor, cycloheximide, gives a preliminary indication that this protease may be one of the gene products whose synthesis is co-ordinately activated under anoxia (Sachs et al., 1980).

In summary, these studies show that the death of the primary root tip in young maize seedlings between 24–48 h of anoxia may be regulated by AIP, a Ca$^{2+}$-dependent cysteine protease. Further, this tissue-suicide, if initiated earlier, appears to provide greater anoxia tolerance to submerged seedlings.
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