Apoptosis-like Programmed Cell Death Occurs in Procambium and Ground Meristem of Pea (Pisum sativum) Root Tips Exposed to Sudden Flooding

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Background and Aims Pea (Pisum sativum) primary roots form long vascular cavities when grown under wet or flooded conditions at 25 °C. It is thought that the cavities are a form of aerenchyma. At 25 °C short roots continue to grow after flooding. After roots reach 10 cm long flooding causes rapid cessation of growth, and root tips often become curled. In longer roots the cavities do not extend into the base of the roots, perhaps rendering them ineffective as aerenchyma. It was hypothesized that the resulting growth arrest was due to programmed cell death (PCD) rather than necrosis.

Methods and Key Results Histological examination by light microscope showed that some cells in the primary meristem (elongation) zone of the primary root tips had morphological abnormalities, including misshapen and fragmented nuclei, and cytoplasmic shrinking and fragmentation. Transmission electron microscopy revealed lobing, invagination and chromatin aggregation in nuclei. The affected cells were positive for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling. Extracted DNA formed a ‘DNA ladder’ during electrophoresis. Cell death usually began in procambium at one or two protoxylem poles and seemed to spread out to nearby tissues, which asymmetrically inhibited growth and resulted in tip curling.

Conclusions The above are symptoms of apoptosis-like PCD. Programmed root tip death may rapidly reduce oxygen demand and sink strength, allowing more rapid diversion of resources to lateral roots growing in more permissive conditions.

Key words: Pisum sativum, root primary meristem, apoptosis, programmed cell death, flooding, TUNEL, DNA ladder, aerenchyma.

INTRODUCTION

Cellular necrosis occurs whenever conditions experienced by a cell become so stressful that it is unable to maintain systems and structures necessary for continued viability. Programmed cell death (PCD), by contrast, is a genetically controlled, systematic process that is a normal part of organismal development or a response to stressful environmental conditions and that usually is adaptive (White, 1996; Gray, 2004; Potten and Wilson, 2004). It is the present authors’ view that recognizing and studying PCD when it occurs offers an opportunity to further our understanding of the role of cell-level processes during development, ecological acclimatization and evolution. Interest in PCD in plants has increased significantly over the past decade or so, and this has resulted in increased numbers of studies examining the role of PCD in plant development and responses to the environment in particular. Some examples are the formation of tracheary elements (Mittler and Lam, 1995; McCann et al., 2000), senescence of peripheral root cap cells (Zhu and Rost, 2000), senescence of leaves (Cao et al., 2003), pollen incompatibility responses (Thomas and Franklin-Tong, 2004), development and germination of seeds (Gallie, 2004), and response to salt stress in root apical meristem cells (Katsuhara and Kawasaki, 1996). Other examples of PCD have been reported as a consequence of an induced mutation (Kossak et al., 1997), as a response to pathogenic infection (Goodman and Novacky, 1994; Mittler et al., 1997; Heath, 2000), in the formation of constitutive aerenchyma in roots of Sagittaria lancifolia (Schussler and Longstreth, 2000) and in the formation of inducible aerenchyma in Zea mays roots (Gunawardena et al., 2001). PCD has also been implicated in the deaths of root tips in maize as a consequence of flooding stress (Subbaiah and Sachs, 2003).

The current project developed as an unexpected outcome of a long-term study that examined the constitutive formation of long, lysigenous gas-filled cavities in the central xylem of legume primary roots grown in moist and warm conditions (Lu et al., 1991; Rost et al., 1991; Niki et al., 1995) and under warm, relatively dry hypoxic conditions (Gladish and Niki, 2000). Vascular cavities will rapidly form in response to sudden flooding following growth under warm, relatively dry conditions (Niki and Gladish, 2001). It was thought that the ability to form long cavities under these conditions is likely to be a PCD response that forms a rare type of aerenchyma: vascular aerenchyma (Gladish and Niki, 2000).

In experiments using hydroponic growth containers, 10 °C pea (Pisum sativum ‘Alaska’) roots were tolerant of flooding. The level of aeration had no significant effect on primary root growth rate, and the roots did not form vascular cavities. At 25 °C, however, vascular cavities formed at very high frequencies in primary roots of the flooded seedlings. Aeration did affect growth rate in that case (Gladish and...
Niki, 2000). These results indicate the importance of temperature in the relative sensitivity of pea roots to oxygen status and the complexity of the response.

In a study using large beakers containing vermiculite, it was observed that at 25 °C suddenly flooded pea primary roots would continue to grow effectively at certain times, whereas at other times the roots would quickly stop growing, a response often accompanied by curving or coiling of the root tip (Niki and Gladish, 2001). The former occurred when the roots were relatively young and therefore short, typically less than 10 cm long. Vascular aerenchyma in young, suddenly flooded pea roots could form in less than 6 h, and typically extended nearly the entire length of the root. Older, longer roots formed aerenchyma just as rapidly, but these cavities did not extend into the older, basal part of the root, presumably because cells of the central xylem were becoming irreversibly differentiated and thus incapable of water transport.

When flooding began, a response often accompanied by curving or coiling of the root tip (Niki and Gladish, 2001). Microscopic examination of the tips of roots with arrested primary growth revealed that groups of cells in the cortices of their elongation zones, the location of the primary meristem tissues, showed signs of cytological and nuclear abnormalities. When flooding was sustained for 24 h or more the abnormalities were also sometimes found in the promeristem (Niki and Gladish, 2001). In roots with tips that had curled the cell clusters were located on one flank of the root tip. In others the affected cells were distributed across the root symmetrically. These were roots that stopped growing without curving. It was suspected that this, too, was a PCD response and, by virtue of the nature of the nuclear abnormalities, that this response was apoptotic (see further below; Niki and Gladish, 2001). Niki and Gladish (2001) concluded that this was further evidence of the role of lysigenous vascular cavities in legume roots as functional aerenchyma.

Necrotic cells in animals develop abnormalities in their mitochondria that lead to loss of membrane integrity and eventually to mitochondrial disintegration (Kerr et al., 1972; Subbaiah and Sachs, 2003; Potten and Wilson, 2004). Apoptotic PCD, such as has been observed in animals, is characterized by rapid chromatin condensation and fragmentation, blebbing and/or invagination of the nuclear envelope followed by its condensation and breakup, shrinking of the cell, fragmentation of the cell into ‘apoptotic bodies’ and ultimately phagocytic absorption of those bodies by surrounding cells (Cohen, 1993; White, 1996; Hengartner and Bryant, 2000; Cook, 2001; Potten and Wilson, 2004). For the present study it was hypothesized that the root cell clusters described above were undergoing apoptotic PCD. Four different types of analysis were undertaken to try to determine if the observed cell abnormalities were due to apoptotic PCD or if they were due to necrosis. The morphology of tissues, cells and nuclei was studied using light microscopy. Cellular and nuclear ultrastructure was studied using transmission electron microscopy (TEM). DNA was examined for systematic fragmentation by in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL; Gavrieli et al., 1992) and by agarose gel electrophoresis (Gunawardena et al., 2001; Potten and Wilson, 2004).

Materials and Methods

Plant material preparation

Seedlings were grown as per Niki and Gladish (2001). Tall 1-L beakers (20 cm) were lined with chromatographic filter paper, filled with vermiculite and moistened with 375 mL of distilled water (DW). After they were covered with aluminium foil, the beakers were autoclaved for 80 min. Under axenic conditions, seeds of pea (Pisum sativum L. ‘Alaska’) were surface-sterilized for 5 min in a solution of 1 % sodium hypochlorite with 6 drops L–1 Tween 20 (Kanto Chemical Co., Tokyo, Japan). After rinsing in sterile DW, 25–27 seeds were planted in each beaker 3 cm from the top between the paper and glass. The beakers were recovered and placed in a dark growth chamber at 25 °C for four or more days.

Except for control beakers, beakers containing 4-d-old (4 d post-inhibition) and 5-d-old seedlings were filled to just below the planting level of the seeds with DW, and the position of each root tip was marked on the outside of the beakers. The beakers were then returned to the growth chamber for 3, 6, 12, 24 or 48 h. After the prescribed period, the position of each root tip was again marked to allow for the measurement of growth, and the seedlings were removed from the beakers for further processing as described below.

Light microscopy (LM)

Tissue sections were prepared as per Nikki et al. (1995). Tip segments 4–5 mm long (n = 18–24 for each treatment) were excised and treated overnight in an osmotically balanced fixative solution: 2.4 % glutaraldehyde and 0.3 % paraformaldehyde in 0.03 mol L–1 phosphate buffer (pH 7.2). After fixation, the segments were rinsed in two changes of 0.2 mol L–1 phosphate buffer (pH 7.2; 30 min each) and one rinse in DW, then dehydrated through a graded series of ethanol (30, 40, 50, 65, 80, 90, 100, 100 %, 20 min for each step). Dehydrated root tip segments were embedded in Historesin (Leica Instruments GmbH, Heidelberg, Germany) via a graded series (Historesin: ethanol – 1:3, 1:1, 3:1, 1:0, 2 h each step). Embedded root segments were sectioned at 2 μm with a Leica RM2065 ultramicrotome (Leica Microscopy and Systems GmbH, Wetzlar, Germany) and mounted on glass slides. Four or five embedded root segments were selected at random and sectioned for each treatment. For general observations, sections were stained with 0.025 % toluidine blue ‘O’ dissolved in 0.02 M phosphate buffer (pH 7.2) and observed using bright-field LM. To assay for nuclear changes, sections were stained with 1 mg L–1 DAPI (4′,6-diamidino-2-phenylindol dihydrochloride) in 10 mmol L–1 Tris/HCl buffer (pH 7.4) for 10 min. Sections were observed after excitation at 340–380 nm with a Leica DML epifluorescence microscope (Leica Microscopy and Systems GmbH). Sections were photographed with Kodak Gold 400 ASA film.

To assay for chromatin fragmentation (TUNEL method; after Gavrieli et al., 1992), fixed and dehydrated sections...
were embedded in Paraplast+ (Oxford Labware, St. Louis MO, USA) by using two sequential series: (1) ethanol:2-methyl-2-propanol (3:1, 1:1, 1:3, 100 %, 20 min each step); and (2) 2-methyl-2-propanol:Paraplast (3:1, 1:1, 1:3, 20 min each step), followed by 100 % Paraplast overnight. Embedded segments were sectioned at 8 μm on a rotary microtome (Uchida Yoko Co., Ltd, Tokyo, Japan) and mounted on glass slides. Four or five embedded root segments were selected at random and sectioned for each treatment. The sections were dewaxed by washing twice with xylene for 2 min and rehydrated through a graded ethanol series (100, 95, 90, 80, 50 %), followed by 0.1 mol L⁻¹ phosphate buffer adjusted to pH 7.4. The sections were then incubated in protease K (20 mg L⁻¹ in 10 mmol L⁻¹ Tris/HCl buffer, pH 7.4) for 30 min at 37 °C, and rinsed twice with phosphate-buffered saline. For fluorescence microscopy, sections were stained using the ‘fluorescein in situ cell death detection kit’ (Boehringer Mannheim GmbH, Mannheim, Germany) according to manufacturer’s instructions. TUNEL incubation was for 60–180 min at 37 °C. Nuclei were observed after excitation at 460–500 nm with a Leica DML epifluorescence microscope (Leica Microscopy and Systems GmbH) and photographed with Kodak Gold 400 ASA and Fujicolor Super HG 1600 ASA film.

**DNA extraction and gel electrophoresis**

Tips of pea primary roots, grown as described above (n = 60 roots for each treatment; repeated three times), were ground with a mortar and pestle. The homogenate was bathed in 3 mL of lysis buffer (10 mmol L⁻¹ Tris/HCl, pH 7.4, 10 mmol L⁻¹ EDTA, pH 8.0, 0.5 % Triton X-100), and incubated for 10 min. The mixture was centrifuged at 16000 rpm for 20 min at 4 °C. RNase A (24 μL of 20 mg L⁻¹) was added to the supernatant and the mixture was incubated for 60 min at 37 °C. Proteinase K (24 μL of 10 mg L⁻¹) was added to the mixture and incubated for 60 min at 37 °C. NaCl solution and isopropanol were added so that the final NaCl concentration was 0.4 mol L⁻¹ and isopropanol was at 50 %, and this mixture was held for 15 min at 37 °C. The precipitate was resuspended in 40 μL of Tris/EDTA buffer. Ten microlitres of each sample was loaded onto a buffered (Tris/borate/EDTA, pH 8.3, 2 %) agarose gel with 7 mmol L⁻¹ ethidium bromide incorporated as marker. Electrophoresis was conducted at 50 V for about 30 min. Results were photographed under UV excitation using Fuji Neopan SS 100 ASA film.

**Transmission electron microscopy (TEM)**

Two-millimetre-long root tips and 2-mm-long elongation zone segments (n = 10–15 roots per treatment) were excised and fixed overnight in 2.4 % glutaraldehyde and 0.3 % paraformaldehyde in 0.03 mol L⁻¹ phosphate buffer (pH 7.2). After fixation, the segments were rinsed in four changes of 0.2 mol L⁻¹ phosphate buffer adjusted to pH 7.2 (rinse buffer) for 30 min each, then post-fixed with 0.025 % tannic acid in rinse buffer for 3 h with agitation. The segments were then rinsed three times with DW for 10 min each and post-fixed with 0.25 % osmium tetroxide in rinse buffer for 1 h at 4 °C. Following fixation, the segments were rinsed once in rinse buffer for 10 min followed by three 10-min DW rinses. The segments were stained overnight with 3 % uranyl acetate in DW. After rinsing twice in DW for 20 min, the segments were dehydrated through a graded ethanol series as above. The segments were then embedded in Spurr resin, sectioned at 0–0.9 μm on a Sorvall MT-1 ultramicrotome (Research and Manufacturing Co., Tucson, AZ, USA) and mounted on copper grids. Three or four embedded tip segments and 3–4 elongation zone segments from each treatment were selected at random for sectioning. The segments were observed with a Hitachi H-300 transmission electron microscope (Hitachi Ltd., Tokyo, Japan) at 75 kV.

**RESULTS**

**Growth responses**

Primary roots of plants grown for 4 d after imbibition (4 d old) and then flooded continued to elongate. After 24 h under flooded conditions 4-d-old primary roots grew at 42 % the rate of unflooded control plants of the same age and continued to grow after 48 h of flooding (data not shown). Primary roots of 5-d-old plants stopped growing in as little as 6 h after the time of flooding or slowed dramatically and ultimately ceased growing within 24 h. Many of the root tips curled before ceasing growth. The tips of 5-d-old flooded roots often narrowed and sometimes yellowed (data not shown).

**Histological and cell-level responses to sudden flooding**

With the exception of cells in mitosis, the nuclei of ground meristem cells in the control roots and in roots of plants that were 4 d old then flooded were usually round or oval in shape and had a uniformly granular appearance (Fig. 1A, B). In primary roots that were 5 d old then flooded for 6, 12 or 24 h, DAPI staining revealed that nuclear condensation had occurred and was primarily limited to groups of cells in the ground meristem. The affected cells were usually clustered together (Fig. 1C). At high magnification it could be seen that these nuclei were often flattened (usually transversely), lobed, invaginated or otherwise misshapen. Many appeared to contain large aggregates of brightly stained chromatin (Fig. 1D, E). In 5-d-old roots flooded for 12 or 24 h some nuclei had disintegrated, and chromatin had dispersed across the cells. In many cases the cells had also shrunken away from their walls and fragmented into irregularly shaped bodies (Fig. 1F). This was probably not an artefact of osmotic-induced plasmolysis as the present methods included osmotically balanced fixatives and buffers. At least one, but not more than two, roots in each treatment group of the 5-d-old flooded roots contained no affected nuclei, even after 24 h of flooding.

After flooding, condensed nuclei and abnormal cytoplasms could even be observed in flooded 5-d-old root tips with conventional toluidine blue ‘O’-stained sections. In abnormal cells, nuclei and cytoplasms stained
more darkly with toluidine blue ‘O’. Serial cross-sections of roots in the earlier stages of response to flooding (6 h post-flooding) showed that nuclear condensation and distortion and other cell abnormalities were usually limited to cells of the pericycle, endodermis and inner cortex at first (Figs 2 and 3). These cells were usually found in groups asymmetrically distributed across roots (Figs 2A and 3). In roots that did not curl, affected cells were more or less symmetrically distributed around the procambial tissue (data not shown). It appeared that the first cells to be altered by flooding were in the pericycle and endodermis adjacent to or in protoxylem poles that were just beginning to differentiate. Even at an early stage of the response to flooding, cytoplasmic shrinkage was observed (Fig. 2B). The asymmetrical distribution pattern of affected cells was always found in curled root tips, which were sometimes so strongly affected that lysigenous lesions appeared in the inner cortex (Fig. 3).

**DNA fragmentation**

Except for a few nuclei of peripheral rootcap cells, control roots (Fig. 4A) and roots from 4-d-old plants flooded for 24 h (data not shown) had only background levels of reaction to the TUNEL procedure. Elongation zones in 5-d-old primary roots flooded for 6, 12 and 24 h usually contained groups of cells in the ground meristem whose nuclei reacted to the TUNEL antibodies, producing bright green to white nuclei in patterns consistent with those seen in the DAPI-stained roots (Fig. 4B, C). Nuclei in cells of the vascular cylinder, except those of the pericycle cells, were never seen to react positively to the TUNEL antibodies. A few roots of 5-d-old flooded seedlings did not show a positive reaction in any tissue except for a few peripheral rootcap cells (data not shown).

Nuclear DNA extracted from tips of roots of control plants and 4-d-old plants flooded for 24 or 48 h did not migrate significantly through 2 % agarose gels in response to electrophoresis (Fig. 5A). In 5-d-old plants gel electrophoresis showed that nuclear DNA from root tips had been fragmented in response to flooding for 6, 12 and 24 h, which caused the appearance of a DNA ladder in the gel. The smallest DNA fragment was less than 80 bp in length. The second smallest fragment was about 180 bp. Flooding for 3 h was not sufficient to cause DNA fragmentation in the root tips of 5-d-old plants. The amount of fragmentation usually increased from 6 to 24 h so that laddering became less distinct and fragment size became smaller over time (Fig. 5B).

**Morphological changes in nuclei and cell ultrastructure**

In the ground meristem of primary roots of control plants, nuclei were usually spherical in shape and contained many small bodies of condensed chromatin uniformly distributed across them (Fig. 6A). At comparable locations in root tips of 5-d-old flooded plants, nuclei were flattened, lobed, invaginated or otherwise irregular in shape. Nuclei of some of the ground meristem cells contained large aggregates of condensed chromatin. Sometimes the condensed chromatin was associated with the nuclear envelopes of these cells, and often it was asymmetrically distributed within the nucleus (Fig. 6B). Some of the nuclei were noticeably smaller than others in the same root and smaller than nuclei of comparable cells in control plants. Other

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**Fig. 1.** DAPI-stained longitudinal sections through the elongation zones of pea root tips. (A, B) Five-day-old control primary root. Scale bar = 25 μm. (A) Inner cortical cells; a mitotic figure can be seen (arrowhead). (B) Outer cortical cells. Nuclei are spherical; chromatin is dispersed. (C–F) Root suddenly flooded for 24 h after growing for 5 d at 25 °C. (C) Tangential section. Condensed nuclei appear in a band of cells (between arrows) in the inner cortex. Procambium is to the right of the band. Cells to the immediate right of the lower left-hand arrow and above the upper left-hand arrow have undergone cytoplasmic shrinking and fragmentation. Scale bar = 50 μm. (D) Detail of inner cortical cells with very condensed and distorted nuclei (arrows). Scale bar = 25 μm. (E) Outer cortical cells. Shown are normal nucleus (N), nucleus with condensing chromatin (small arrowhead), and very condensed and lobed nucleus (arrow). Scale bar = 15 μm. (F) Cortical cells in late stage of apoptotic collapse. Nuclei have fragmented, and chromatin has dispersed throughout the cells. In some cases cells have shrivelled and fragmented within their walls. Scale bar = 25 μm.
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organelles and cytoplasm appeared normal in cells with distorted nuclei (Fig. 6B), except in cells at advanced stages of deterioration (Fig. 6C). Endoplasmic reticulum (ER) often presented as concentric cisternae, tonoplasts began failing and some cell walls thinned in the later stages of the response to flooding (Fig. 6C).

**DISCUSSION**

A previous study (Niki and Gladish, 2001) showed that pea seedling root systems have different strategies to accommodate different flooded conditions. Seeds can germinate and produce healthy primary roots at 25°C under constant flooded conditions, but they grow plagiotropically and contain vascular aerenchyma. Seeds of *P. sativum* germinated at 25°C under relatively dry conditions produce primary roots that grow orthotropically and do not usually produce vascular cavities. Niki and Gladish (2001) showed that if suddenly flooded 4 d after imbibition, primary roots grown at 25°C rapidly produce vascular cavities and continue to grow orthotropically, albeit more slowly. If the plants are 5 d old, however, their roots apparently are unable to produce vascular cavities that are effective at providing a path for oxygen and the primary root rapidly stops growing, often after curling. This raised the question of whether this response is necrosis or PCD. The results of the current study suggest that the root tip arrest reported by Niki and Gladish (2001) is not necrosis, and they show that the process has much in common with apoptosis, a specific type of PCD.

Potten and Wilson (2004) described necrotic cell death as involving groups of cells rather than individual cells.
necrosis, cells swell in size. Membranes fail. Mitochondria swell; in general, organelles lose integrity. Chromatin aggregations develop irregular edges, and DNA is not fragmented or is randomly fragmented. Potten and Wilson (2004) describe apoptosis as a process affecting individual cells. Membranes remain intact; organelle integrity generally is maintained. Cytoplasm condense and shrink; cell-to-cell contact is lost. Chromatin condenses into masses with sharp margins, and DNA is hydrolysed into a series of more or less regular fragments.

By contrast, some have argued that, although there are similarities, PCD in plants is not apoptosis (Hengartner and Bryant, 2000), and some prefer to refer to the process in plants as autophagy (Gray, 2004). Others think that the argument is largely semantics, and that the distinction derives from differences in the length of time over which cell death occurs in animals versus plants. For example, following an interrupted PCD and a period of dormancy, endosperm cells of seeds, or rather their cytoplasmic fragments, are ultimately resorbed by the embryo as a consequence of hydrolytic activity of the aleurone tissue, which breaks down endosperm cell walls during seed germination (Gallie 2004; D. Gallie, pers. comm.).

Misshapen nuclei that contain condensed chromatin with sharply defined margins associated with the nuclear envelope, such as seen in the present study, are characteristic of PCD in plants (Katsuhara and Kawasaki, 1996; Kossak et al., 1997), and fragmented nuclei are characteristic of apoptosis, as are shrunken and fragmented cytoplasms (Cohen, 1993; White, 1996; Cook, 2001; Potten and

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**Fig. 4.** Longitudinal sections through the elongation zone of pea root tips stained using the TUNEL procedure to indicate locations of nuclei with fragmented chromatin. (A) Cortices of 5-d-old control roots. (B) A root flooded for 12 h after growing for 5 d. Positively stained nuclei (arrows) appear in the outer cortex. Non-reacting nuclei (arrowheads) can be seen in the inner cortex. (C) Positively stained nuclei (arrows) can be seen in the mid and outer cortex of this curled root tip flooded for 24 h after growing for 5 d. Non-reacting nuclei (arrowheads) can be seen in the inner cortex and vascular cylinder. Scale bar = 50 μm.

**Fig. 5.** Electrophoresis gels of DNA extracted from pea primary root tips. (A) DNA from 5-d-old control roots (C) or roots flooded for 1 or 2 d after 4 d of growth (4+1 d and 4+2 d, respectively), migrated little in the gel. Roots flooded for 1 d after 5 d of growth (5+1 d) had fragmented DNA in their tips that ladder. (B) Time-course experiments showed that DNA fragmentation occurred within 6 h after sudden flooding in tips of roots grown for 5 d. Fragmented DNA that generates a ‘ladder’ pattern in a gel, as seen here, is typical of apoptosis. M, marker DNA; C, unflooded 6-d-old control root tips.
Concentric circles of membranes in cells of Zea mays and Oryza sativa undergoing PCD have been reported (Schussler and Longstreth, 2000; cf. figs 16 and 18). In a previous report on the responses of pea root tips to flooding, concentrically distributed ER membranes could be seen in cells containing distorted and condensed nuclei in tips of flooded roots (Niki and Gladish, 2001, cf. fig. 10B), as were also seen in the present study. We found all of these characteristics in the cortical cells of elongation zones (ground meristems) of 5-d-old suddenly flooded root tips.

The TUNEL fluorescent complex binds to the 3' end of DNA molecules. Few DNA 3' ends are present in normal healthy nuclei (as in Fig. 4A), but cells undergoing apoptosis produce endonucleases that hydrolyse DNA between nucleosomes (Potten and Wilson, 2004). This results in an abundance of 3' ends, and therefore the TUNEL procedure is a good indicator of hydrolysed DNA in situ (as in Fig. 4B, C). DNA fragmented in this manner is characteristic of PCD in plants (Mittler and Lam, 1995; Kossak et al., 1997; Gunawardena et al., 2001) and apoptosis (Cohen, 1993; White, 1996; Potten and Wilson, 2004). Although a nucleus in a necrotic cell might also be positive for TUNEL due to random fragmentation of DNA, in the present study DNA fragmentation was not random (below). That is why it is important not to rely on TUNEL alone.

Fragmented DNA is also revealed by gel electrophoresis. Necrotic tissue that contains randomly fragmented DNA smears rather than forms a ladder pattern during electrophoresis (Cohen, 1993; Katohara and Kawasaki, 1996; Potten and Wilson, 2004). DNA fragmented during apoptosis forms a ladder pattern in the gel (as in Fig. 5; Gunawardena et al., 2001, cf. fig. 5) because endonuclease activity results in fragmentation of DNA into oligonucleosomes of periodic lengths. Although this is not absolutely required for PCD (Walker and Sikorska, 1997), DNA laddering is good evidence for apoptosis (Cohen, 1993; Walker and Sikorska, 1997; Walker et al., 1999; Gunawardena et al., 2001; Potten and Wilson, 2004). The results presented here indicate that the cell death that occurred in the ground meristems in suddenly flooded 5-d-old pea primary roots was probably PCD and, like the maize aerenchyma response (Gunawardena et al., 2001), had many of the characteristics of apoptosis.

Presumably, the above-described response of pea root tips to flooding is a result of hypoxia. Hypoxia is implicated in the formation of vascular cavities (Gladish and Niki, 2000), which is an important part of the overall response to flooding in pea primary roots. Subbaiah and Sachs (2003) suggested that programmed root tip death in maize provided an adaptive advantage by reducing the local oxygen demand by the root, thus preserving the bulk of the root axis for regeneration in a post-anoxic period. The root tip death response is even more rapid in pea than in maize, further reinforcing the idea that it is a programmed response. Because pea primary roots can grow at rather low ambient oxygen levels at low temperatures (Gladish and Niki, 2000), perhaps the difference in respiratory demand between 10 and 25°C is sufficient in primary roots to require an aerenchymatous tissue. And if such tissue cannot be adequately produced in a particular root, it is possible that a rapid, apoptosis-like ‘autodecapitation’ response to sudden flooding quickly reduces hypoxic stress by reducing oxygen demand. Alternatively, autodecapitation may reduce sink strength in the primary root tip to allow rapid diversion of resources to lateral roots growing at shallower levels in the soil where oxygen will probably be more abundant. This might provide a slight advantage in competitive situations if such a shift allows for more efficient overall mineral absorption by roots during flooding.

The fact that the first cells to begin PCD in response to sudden flooding in these root tips are cells in or adjacent to immature protoxylem may be significant, given that differentiation of xylem tracheary elements has itself been shown to be a PCD process (Mittler and Lam, 1995; McCann et al., 2000). Subbaiah and Sachs (2003) reported the propagation of a diffusible ‘death signal’ into the growth media during their root tip experiments. The hypersensitive response to pathogens involves a
propagating ‘death signal’ (Goodman and Novacky, 1994). It is easy then to imagine a ‘death signal’ propagating from protoxylem cells already predisposed to PCD to adjacent cells of the pericycle and inner cortex if the cells become subjected to hypoxia. Perhaps the frequent asymmetrical pattern of responding cells results from the three protoxylem regions not being synchronized in their maturation within the procambium (Rost et al., 1988).

The current study showed that some flooded pea primary root tips contain cells that apparently cannot benefit from effective aeration via vascular aerenchyma. These cells undergo several degradative changes in their ultrastructures and DNA that are consistent with the process of apoptosis. Cells undergoing apoptosis in animals also show several specific physiological and molecular characteristics (Stennicke, 2000; Potten and Wilson, 2004). It will be interesting to see if there are similarities in plants with respect to these as well.

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LITERATURE CITED