Hypoxia induced non-apoptotic cellular changes during aerenchyma formation in rice (*Oryza sativa* L.) roots

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ABSTRACT

The stress of low oxygen concentrations in a waterlogged environment is minimized in some plants that produce aerenchyma, a tissue characterized by prominent intercellular spaces. It is produced by the predictable collapse of root cortex cells, indicating a programmed cell death (PCD) and facilitates gas diffusion between root and the aerial environment. The objective of this study was to characterize the cellular changes take place during aerenchyma formation in root of rice that accompany PCD. Scanning electron microscopy and transmission electron microscopy were used for cellular analysis of roots. Aerenchyma development was observed in both aerobic and flooded conditions. Structural changes in membranes and organelles were examined during development of root cortex cells to compare with previous examples of PCD. There was an initial collapse which started at a specific position in the mid cortex, indicating loss of turgor, and the cytoplasm became more electron dense. These cells were distinct in shape from those located towards the periphery. Mitochondria and endoplasmic reticulum appeared normal at this early stage though the tonoplast lost its integrity. Subsequently it underwent further degeneration while the plasmalemma retracted from the cell wall followed by death of neighboring cells followed a radial path. However, pycnosis of the nucleus, blebbing of plasma membrane and production of apoptotic bodies were not found which in turn indicated nonapoptotic PCD during aerenchyma formation in rice. [*Physiol. Mol. Biol. Plants* 2010; 16(1) : 99-106] E-mail : pramodk63@yahoo.com

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INTRODUCTION

The stress of low oxygen concentrations in a waterlogged environment is minimized in wetland plants that produce aerenchyma, a tissue characterized by continuous gas spaces (Colmer, 2003; Drew *et al.*, 2000; Malik *et al.*, 2003; Gunawardena *et al.*, 2001). Ample evidence has been presented to show that aerenchyma provides a diffusion path of low resistance for the transport of oxygen from aerial plant parts to roots or rhizomes (Laan *et al.*, 1989). Development of gas spaces in many wetland species is constitutive because this process occurs whether or not plants are growing in aerated or waterlogged soils (Schussler and Longstreth, 1996). Restricted water supply as well as waterlogging may limit yields of upland rice in many areas and in

deep-water and non-irrigated paddy systems. Rice roots are more sensitive to anoxia than nontolerant plants (Vartapetian *et al.*, 1970). Gas-filled spaces in rice arise from the separation of cell walls from adjacent cells so that the radial walls from the collapsing cells aggregate together, forming “forks”, leaving a large gas-filled space or lacuna between them (Clark and Harris, 1981). In anaerobic conditions, mitochondrial destruction was observed in rice roots which were found to be absent under aerobic conditions (Vartapetian and Andreeva, 1986). Aerobic cultivars maintain many semi aquatic adaptations i.e., development of aerenchyma in roots and high levels of non-stomatal water loss from leaves (Lafitte and Bennett, 2002).

In 20th century many scholars depicted mainly two patterns of aerenchymatous origin: either lysigenous which develops as a consequence of senescence of specific cells followed by their autolysis and disintegration or, schizogenous which develops by cell

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separation and cell division (Kawai *et al.*, 1998; Evans, 2003). Present study has been focused on the lysigenous gas-spaces produced in the primary roots of rice. During the normal course of development, many cells in the cortex collapse to form aerenchyma. In monocots, lysigenous aerenchyma develops in the cortical region where cell expansion growth has been completed, behind the root tip (Ranathunge *et al.*, 2003; Jung *et al.*, 2008).

The predictable lysis of root cortex cells suggests aerenchyma production in roots depends on a genetically controlled program of cell death (Jones and Dangl, 1996; Drew, 1997; Kawai *et al.*, 1998). Programmed cell death (PCD) is recognized as an important developmental mechanism in a wide range of plant tissues (Greenberg, 1996; Jones and Dangl, 1996). PCD in animal cells has been characterized in various forms including apoptosis and cytoplasmic cell death, distinguishable by the order of events in cell degradation i.e., chromatin clumping, disruption of nuclear membrane, pyknosis of the nucleus and subsequent engulfment by the vacuole, crenulation and blebbing of plasma membrane, production of apoptotic bodies, loss of cytoplasmic contents, and collapse of cell. Any deviation, in aforementioned events is considered non-apoptotic degradation. PCD has also been observed in higher plants during various developmental processes resembling apoptosis. Other characteristics such as retention of intact organelles, membrane invagination and the presence of membrane-bound bodies have not been reported in plants at cellular level (Gunawardena *et al.*, 2001). By comparison, necrotic cell death is characterized by initial changes in mitochondria and cell swelling prior to death (Kerr *et al.*, 1972). The presence and absence of these later changes in cell structure depend on the type of cell death examined (Greenberg, 1996).

Webb and Jackson (1986) proposed that the processes of aerenchyma formation differ in rice and maize with regard to the order of events. In maize roots, cellular collapse was followed by the loss of tonoplast integrity. However, in rice, middle lamella degeneration preceded by cell wall disintegration and loss of tonoplast integrity. The detailed mechanisms of aerenchyma formation are not well understood yet. In this report, hypothesize that aerenchyma development is nonapoptotic based on earlier studies of gas space formation in roots of *Zea mays* and *S. lancifolia*. Further, the present research was to determine the sequence of changes in membrane and organelle structure that occur during cell lysis. Aerobic and flooded root aerenchyma development was examined by scanning electron

microscopy (SEM) and transmission electron microscopy (TEM) to meet these objectives.

MATERIALS AND METHODS

Plant material and growth condition

For the present study, a field experiment was conducted in split plot design (S.P.D) using three replications at Crop Research Center, G.B. Pant University of Agriculture and Technology, Pantnagar, India. Main plot comprised flooded and aerobic and sub plot consisted of four genotypes namely DRRH-2, PA6444, KRH2 and JAYA. Seedlings were raised in nursery by dry bed method and were irrigated on alternate days. Transplanting was done after 20-25 days in plots of 2×3 m size in total size of 544 m² in *Kharif* season 2006. During transplanting plant-to-plant distance was 10 cm and row-to-row distance was 20 cm. 2m gap was maintained between flooded and aerobic plots to avoid water flow through seepage. Bunds were remained opened in aerobic plots. Apical root segments, 2 to 3 mm long, were cut about 2-5 cm behind the tip sampled from different stages of rice plants.

Scanning electron microscopy

For observations through scanning electron microscope (SEM), fresh tissues (1cm) were fixed for 4 h at 4 °C, using a modified Karnovsky's fixative (2 % paraformaldehyde and 2 % glutaraldehyde in 0.05 M sodium cacodylate buffer). These primary tissues were then post-fixed for 2 h at 4 °C using 1 % osmium tetroxide in 0.05 M sodium cacodylate buffer before being washed with distilled water. Afterward, they were dehydrated in a graded ethanol series, then placed in a critical point dryer (Polaron Jumbo Critical Point Dryer) and liquid carbon dioxide. The dried tissues were mounted on stubs and sputtercoated with gold under reduced pressure in an inert argon gas atmosphere by Agar Sputter Coater P 7340. After sputter coating the tissues were examined under Variable Pressure Scanning Electron Microscope (Leo 435VP) operated at 15-25 KV. Grids were made of copper and coated with 3 % formavar (poly vinyl formaldehyde) in ethylene dichloride for lifting the sections.

Transmission electron microscopy

Roots of rice collected from aerobic and flooded plots were cut properly (2 mm), washed and then fixed in

2 % para-formaldehyde, 1 % glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 6.9), for 2 h at room temperature (Gunawardena *et al.*, 2001). The segments were washed in the buffer, and post-fixed in 1 % aqueous osmium tetroxide for 1h, before being stained in 0.5 % uranyl acetate at 4 °C overnight. Tissue sections were dehydrated in a graded ethanol series (30 min each) and then embedded through ethanol: spur resin mixtures (3:1, 1:1, 1:3) for 1 h each. They were finally embedded in 100 % Spurr resin and polymerized at 60 °C for 9 h in flat silicon rubber moulds. Ultra thin sections (60–80 nm) were cut on a Reichert Jung Ultracut E microtome (Leica, Milton Keynes, UK), collected on Formavar-coated grids (200-mesh copper hexagonal mesh: Agar scientific, Stansted, UK), stained in lead citrate and examined with a transmission electron microscope (Phillips CM-10) operated at 60–80 kV.

RESULTS

Aerenchyma formation was examined in roots of rice seedlings which were aerobically and flooded treated. Aerenchyma formation was started very rapidly and hypoxia conditions stimulated the rate of cavity formation in roots. The area of porosity increased toward the basal portion of each root. At a distance of around 20 mm from the root tip, cortical cells started to collapse and aerenchyma gradually developed along roots (Fig. 1A&B). Fully developed aerenchyma was observed at a distance of about 100 mm (Fig. 1C). Aerenchyma was located in the central cortex separating the inner part of the root from the outer part of roots (OPR). Well-defined OPR contained only four cell layers. Rhizodermis was the outermost layer, which surrounded an exodermis (hypodermis with Casparian bands). A single layer of dead sclerenchyma fibre tissue was laid down below

Fig. 1. Scanning electron microscopy of roots of rice (*Oryza sativa*) plants. **A–C.** Development of aerenchyma at 20 (A), 50 (B) and 100 mm (C) from the root tip. **D.** The outer part of roots (OPR) contained four cell layers (rhizodermis, exodermis, sclerenchyma and one cortical cell layer). Ae Aerenchyma, co cortical cells, ex exodermis, rh rhizodermis, scl sclerenchyma fibre tissue, rl radial lysigeny. **E.** Cross section of roots that emerged under aerobic conditions (15–20 cm from the root base) showing less aerenchyma. **F.** Cross section of roots that emerged under flooded conditions for two weeks (0.5 cm from the root-shoot junction) showing fully developed aerenchyma.

Fig. 2. Ultrastructure of root cross sections of *O. sativa*, showing cells of the cortex region. **A–B.** Cortical cells with intact structure and large cytoplasmic area. **C.** Nuclei were found to be absent from the lysed cortical cells. Empty membrane-bound structures or granular material instead of recognizable organs in the cells. **D.** Nucleus breaking apart and mixed with the vacuole. Bars = 1 µm

the exodermis (Fig. 1D). An innermost unmodified cortical cell layer was adjacent to large air lacunae in the root. These were separated from each other by radial, monolayered walls, which appeared as spokes in cross-sections (Fig. 1C). The number of cortical cells in rice roots was less than that offered some advantages of distinctive cell positions in relation to cell death. However, not all of the cortical cells collapsed; the outermost and innermost cells remained intact. The remains of the collapsed cortical cells formed “forks” or “spokes of a wheel” (Fig. 1C).

Scanning electron microscopy showed that the presence of unusually large cells in the cortical region and aerenchyma development was analyzed in both aerobic and flooded conditions. But aerenchyma was less developed in aerobic conditions in comparison to flooded conditions (Fig. 1E&F). Transmission electron microscopy showed that few of these cells contained protoplasm, indicating that they had no metabolic function (Fig. 2A). Nuclei were found to be absent from the lysed cortical cells. There were empty, membrane-bound structures or granular material instead of recognizable organs in the cells (Fig. 2C&D). Particulate matter occasionally appeared between the plasma membrane and cell wall. In some cells, the tonoplast of the cell had disintegrated and altered the appearance of the cytoplasm.

Fig. 3. Abnormal nuclear structures in cross sections of cortex cells in *O. sativa*. **A.** Nucleus with disintegrated nuclear membrane (arrows). **B&C.** Changes in the cytoplasm and membranes of cross sections of cortex cells. Condensation of the cytoplasm against the edges of the cell (arrows). **D.** Numerous electron-lucent regions (arrows) within the cytoplasm. **E.** Crenulation of the plasma membrane (arrows) in a cell with a disintegrated tonoplast. Bars = 1 µm

Structural features of aerenchyma formation in roots were the same whether plants were grown in aerobic or flooded conditions. Just behind the root tip, cortex cells possessed normal nuclei with intact membranes, smooth surfaces, and dispersed chromatin. The earliest divergence between normal and lysing cells was the appearance of unusual-looking nuclei. Deterioration of nuclear membranes, general nuclear fragmentation (Fig. 3A), and the apparent mixing of nuclei and vacuoles were also observed as lysis progressed (Fig. 2D). These changes in nuclear structure were observed in cortex cells found just behind the root tip to as far as 2 cm behind the root tip. Nuclei were noticed only in few lysed cortex cells (Fig. 4B), presumably because nuclear degradation had occurred prior to full development of the aerenchyma. Often at this late developmental stage, tonoplast membranes appeared to have disintegrated and organelles were swollen and distorted. The cytoplasmic contents in lysing cells often appeared abnormal at 2 cm and farther behind the root tip. These abnormalities included condensation of the cytoplasm, electron-lucent

(clear) regions in the cytoplasm (Fig. 3B&C) and crenulation of the plasma membrane (Fig. 3D). Unusual concentric structures of membranes were also observed (Fig. 4A). Many of the cortex cells had collapsed, creating the gas spaces characteristic of aerenchyma tissue (Fig. 4C&D). Cell walls were intact in these cells, but were detached from neighboring radial files of cells in the cortex (Fig. 4E). Cells with collapsed walls remained attached to one another within the same radial file of cells (Fig. 4F).

DISCUSSION

Present investigation contributes to understand the cellular events leading to cortical cell death, which eventually results in the lysigenous formation of aerenchyma in primary roots of rice. The most important finding is that cell death began at a specific cortical region. Cell collapse never initiated at peripheral cortical cells, instead the first cells to collapse were located at

Fig. 4. Cross sections of cortex cells during late stages of cell lysis in *O. sativa*. **A.** Cell of *O. sativa* with concentric structure of membranes (arrows). **B.** Nucleus was observed after degradation of all organelles. **C.** Membrane degradation before the collapse of the cells. **D.** Completely lysed cells (arrows) next to the degraded, but not collapsed, neighbouring cell. **E.** Two adjacent files of cells with collapsed cell detached from the neighbouring cells. **F.** Degradation of cells in continuation with the radial file neighbour. Bars = 1µm.

the center of the cortical tissues (Fig. 1, A). The location of cells undergoing lysis was observed to be precise, indicating the existence of a targeting mechanism for initiating the first cell death. Cells in this position were characterized by being shorter but enlarge radially in diameter than other cortical cells. The development of aerenchyma was observed to be associated with the enlargement of cortical cells. The cells undergoing lysis were noted larger in diameter than other cortical cells and similar observations were also reported earlier in *Sagittaria lancifolia* roots (Schussler and Longstreth, 2000). Kawase (1979) also reported that cortical cells of sunflower stem treated with cellulase enlarged radially, and disintegrate leading to an intercellular space. Root porosity was greater in rice plants grown with continuous flooding, and rate of aerenchyma development varies among genotypes as also reported earlier (Kawai *et al.*, 1998).

In our study, central cortex cells were degraded earlier, indicating that vacuoles may mature more rapidly in central cortex than in other regions. The specific step involved in the first cell death in aerenchyma formation of rice root cannot be identified on the present evidence, but the program leading to tonoplast disruption and first cell death might be initiated in response to developmental signals. The signal which provokes vacuole disruption is still unknown. In relation to the mechanism of PCD, it is necessary to speculate that the sequential spread of cell death may be due to H_2O_2 produced as an oxidative burst, since a high dose of H_2O_2 induces cell death in higher plants (Tenhaken *et al.*, 1995). Further the interaction of these signals in conjunction with ethylene has been shown to stimulate aerenchyma formation (Pierik *et al.*, 2004; Voesenek *et al.*, 2004; Millenaar *et al.*, 2005; Colmer *et al.*, 2006). van der Weele *et al.* (1996) observed that earlier stage of cell death began with the loss of vacuolar solutes and collapse of cells in maize. This hypothesized that dying cells release a message initiating the process of cell death in neighboring cells through a cytoplasmic syncytium termed the symplast, which facilitates transport through apoplastic barriers as in the root cortex. Symplastic transport is investigated to be mediated by specialized trans-cell wall structures called plasmodesmata. It was observed that the fates of both meristematic cells and those undergoing successive cell deaths in plant roots are under strict spatial-related regulation (Berg *et al.*, 1995).

Collapsing cells lost contact with neighboring (tangential) cells, and sequentially destroyed in a radial

direction in cortical parenchyma tissues. The highly selective death in the central cortical cells shows similarity with programmed cell death or apoptosis in animal cells, which offers an active control mechanism that is important in developmental and pathological process (Kawai *et al.*, 1998). In the early phase of aerenchyma formation in rice roots, nuclei remain intact. Concentric structures of membranes were observed in lysing cells of *Zea mays* and *O. sativa*, but not in *Sagittaria lancifolia* (Schussler and Longstreth, 2000). *Pisum sativum* roots growing at low O_2 concentrations also showed similar membrane structures. These membranes are hypothesized to be endoplasmic reticulum that has become reorganized at energy charge values below that found in cells with normal-appearing endoplasmic reticulum (Davies *et al.*, 1992).

Several features of root aerenchyma formation reported in *O. sativa* are consistent with PCD, i.e., targeting of certain cortex cells for death (Schussler and Longstreth, 1996), obligate production of aerenchyma under a variety of environmental conditions (Schussler and Longstreth, 2000; Ranathunge *et al.*, 2003; Vartapetian *et al.*, 2003; Jung *et al.*, 2008), and early changes in nuclear structure as found in our study. These changes in nuclear structure included clumping of the chromatin, fragmentation, disruption of the nuclear membrane, and apparent engulfment by the vacuole followed by crenulation of plasma membrane, formation of electron-lucent regions in the cytoplasm, tonoplast disintegration, organellar swelling and disruption, loss of cytoplasmic contents, and collapse of cell as also reported earlier (Borrás *et al.*, 2006). These observations are not consistent with cell necrosis where initial changes are commonly destruction of mitochondria followed by swelling of the cell; instead the changes during cell death are structural features of apoptosis (Kerr *et al.*, 1972). However during the present investigation, other characteristics of apoptosis i.e., pycnosis of the nucleus, plasma membrane blebbing, and subsequent production of apoptotic bodies were not observed in lysing cells. Thus PCD during aerenchyma formation in rice (*O. sativa*) roots indicated a nonapoptotic degradation. To date, there is no classification system available for nonapoptotic PCD in plants as in animals (Clarke, 1990). While aerenchyma formation is apparently an example of PCD, there are distinct differences among plant species in the mechanisms that produce gas spaces (Borrás *et al.*, 2006; Lee *et al.*, 2007). Therefore in future, studies on aerenchyma development in more wetland plant species

are needed to understand the range of mechanisms that produce this important adaptation to flooded soils.

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