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Rapid changes in cell wall pectic polysaccharides are closely associated with early stages of aerenchyma formation, a spatially localized form of programmed cell death in roots of maize (*Zea mays* L.) promoted by ethylene

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ABSTRACT

Aerenchyma formation in roots of maize (*Zea mays* L.) involves programmed death of cortical cells that is promoted by exogenous ethylene (1 μL L^{-1}) or by endogenous ethylene produced in response to external oxygen shortage (3%, v/v). In this study, evidence that degeneration of the cell wall accompanies apoptotic-like changes previously observed in the cytoplasm and nucleus (Gunawardena *et al*. Planta 212, 205–214, 2001), has been sought by examining de-esterified pectins (revealed by monoclonal antibody JIM 5), and esterified pectins (revealed by monoclonal antibody JIM 7). In controls, de-esterified wall pectins were found at the vertices of triangular junctions between cortical cells (untreated roots). Esterified pectins in control roots were present in the three walls bounding triangular cell-to-cell junctions. After treatment with 3% oxygen or 1 μL L^{-1} ethylene, this pattern was lost but walls surrounding aerenchyma gas spaces became strongly stained. The results showed that cell wall changes commenced within 0·5 d and evidently were initiated by ethylene in parallel with cytoplasmic and nucleoplasmic events associated with classic intracellular processes of programmed cell death.

Key-words: De-esterified pectin; esterified pectin; JIM 5; JIM 7; root growth; waterlogging.

INTRODUCTION

Plant roots growing in waterlogged soils are at risk from anoxia unless they can secure a supply of oxygen to replace that normally provided by well-aerated soil. In species adapted to wet places, oxygen is provided by diffusion through interconnected gas-filled space in porous tissue known as aerenchyma (Jackson & Armstrong 1999). This pathway links the root with the shoot and thus with the oxygen-rich aerial environment (Armstrong 1979). Some species, including maize (*Zea mays* L.), form aerenchyma in response to waterlogging of the soil, an effect mediated by endogenous ethylene. Increased amounts of this gaseous hormone are produced in the roots of waterlogged plants in response to a reduced (but not extinguished) supply of oxygen (Brailsford *et al*. 1993) imposed by the extremely low diffusion coefficient of oxygen in flood water compared with that in gas-filled soil pores (Armstrong 1979). Aerenchyma formation in maize roots begins with the death and lysis of cells of the mid cortex, in a zone behind the root apex. Cell death subsequently spreads radially and longitudinally to form gas spaces separated by radial bridges of living cells (Armstrong & Armstrong 1994) linking the stele and epidermis. We showed recently that the controlled death and absorption of materials of the protoplast occurs in cells less than 1 d old and in a manner resembling the form of programmed cell death in animal cells known as apoptosis (Gunawardena *et al*. 2001). The first changes in intracellular morphology induced by ethylene or 3% oxygen occurred in cell membranes and cytoplasm within 0·5 d, closely followed by chromatin condensation, oligonucleosomal DNA fragmentation and the formation of membrane-enclosed clusters of organelles and other cellular contents. In contrast, the degeneration of cell walls had been thought to occur much later and to be a final clean-up phase that follows cell death. It has been demonstrated that cell walls are digested and leave little trace, except for some residual radial strands (Drew, Jackson & Gifford 1979). This suggests a wholesale breakdown of cell walls that may well be the outcome of activity by a range of cell wall degrading enzymes. These could include expansins, cellulases, xyloglucan endo-transglycosylase (XET) and pectinases (Jackson & Armstrong 1999). In support of this, up-regulation of expansin gene expression has been observed in ethylene-regulated tomato fruit ripening, where wall disassembly occurs (Rose *et al*. 2000). In addition, transcripts of XET increased in maize roots after 12 h of flooding, the

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effect being antagonized by ethylene biosynthesis inhibitors (Saab & Sachs 1996). Thus, modification of xyloglucan (a hemicellulose) may occur, although direct measurements of XET enzyme activity have yet to be undertaken (Jackson & Armstrong 1999). Increased carboxymethylcellulase activity in response to ethylene was observed in dicot abscission zones (Horton & Osborne 1967), in which cells separate without collapsing, in swelling dicot stems during flooding (Kawase 1979) and in aerenchyma formation in maize roots (Grineva & Bragina 1993; He, Drew & Morgan 1994, He *et al*. 1996a, He, Morgan & Drew 1996b; Bragina & Grineva 1996). He *et al*. (1994) observed this activity after 12 h of treatment with ethylene or low oxygen. However, microscopy studies by Campbell & Drew (1983) and ourselves (Gunawardena *et al*. 2001) suggested that visible cell wall degeneration was evident rather late in the development of aerenchyma. However, microscopy can only reveal late changes in cell wall structure when degradation has proceeded sufficiently for an effect to become visible. Thus, it remains likely that changes in wall composition during the formation of aerenchyma occur much earlier, possibly in tandem with the rapidly triggered apoptotic events in cytoplasm and nucleus already reported by us (Gunawardena *et al*. 2001).

Thus, in the present study, we sought evidence for very early changes in the cell wall, that precede events previously revealed by the electron microscopy of Campbell & Drew (1983) and ourselves (Gunawardena *et al*. 2001). Since changes in wall pectins had not previously been examined during aerenchyma formation we decided to examine this class of wall polysaccharide. Pectins are known to help maintain the structural integrity of cell walls. They can do so even in conditions where much of the cellulose has been removed (Shedletzky *et al*. 1990, Shedletzky *et al*. 1992) and have been implicated in many wall functions, including regulation of cell wall ionic status, cell wall porosity, cell-to-cell adhesion and cell expansion (Willats *et al*. 1999). There are three major classes of pectic polysaccharides: polygalacturonic acid (PGA) and rhamnogalacturonans I and II (RG I and RG II; Levy & Staehlin 1992). Although monocots contain less pectins than dicots (Masuda 1990), all three classes are represented in monocots such as maize. The degree of esterification can vary. PGAs comprise long chains of 1,4 α-D-galacturosyl residues each of which can be esterified at the C-6 position. It is deposited into the wall fully esterified and may subsequently become de-esterified by pectin methyl esterases (PME; Kauss & Hassid 1967; Jarvis 1984; Zhang & Staehlin 1992). Accordingly, two monoclonal antibodies, JIM 5 and JIM 7 (Knox *et al*. 1990) were selected to study changes in the state of de-esterified and esterified pectins, respectively, during the development of aerenchyma in hydroponically grown roots treated with ethylene or 3% (v/v) oxygen. The daily increments in extension were marked onto the roots in a manner that allowed segments of root axis of similar age to be compared.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. Ltd, Poole, UK and were of the highest available grade. Maize caryopses (*Zea mays* L. cv LG-20–80; Force Limagrain Ltd, Market Rasen, UK) were washed with running tap water for 2–3 h, and soaked overnight in aerated distilled water in a growth cabinet at 18 °C. Imbibed caryopses were sown in dampened vermiculite medium for germination at 18 °C for 3 d. Seedlings with uniform root length (about 20 mm) were selected and marked 10 mm from the root tip with a spot application of charcoal slurry using a fine brush. Then, 7–10 seedlings were transferred to a stainless steel mesh over 500 mL of 500 μM $Ca(NO_3)_2$ solution bubbled with either a gas stream of 3% oxygen in nitrogen gas (BOC Ltd, London, UK), 1 μL L^{-1} ethylene in 18–21% oxygen (BOC Ltd), or with air (21% oxygen) at a rate of 150 mL min^{-1}.

Seedlings were grown in a growth cabinet for 6 d at 18 °C (16 h photoperiod). The experiment was repeated at least three times with three replicates per treatment. The oxygen concentration in the gas flow and in solution was measured using a membrane inlet quadrupole mass spectrometer (VG Micromass, Chester, UK). Roots were marked 10 mm from the root tip every 24 h to establish the age of tissue along the root axis and measurements taken to estimate the rate of elongation. Also, every 24 h, two roots were selected at random from each treatment, and transverse sections made by hand at various distances from the root tip and observed by light microscopy to determine where aerenchyma was forming. Three days after the treatments, root sections at 0·5 d (induction of aerenchyma), 1·5 d (developing aerenchyma) and 2·5 d (developed aerenchyma) were fixed for light microscopy.

The percentage aerenchyma area (i.e. area of the cortex occupied by collapsed cell space) was measured on fixed, paraffin wax-embedded specimens. Segments of root tissue (5 mm) were fixed in 5% formalin, 5% glacial acetic acid, 45% aqueous ethanol for 3 h and dehydrated in a graded ethanol series before embedding in paraffin wax (Raymond A Lamb, London, UK). Sections (5 μm) were cut using a Spencer 820 microtome (Leica, Milton Keynes, UK) and either stained with haemotoxylin and eosin (H and E) or dried onto poly-lysine-coated slides for immunocytochemistry. Percentage area of aerenchyma was estimated by capturing images of haematoxylin-and eosin-stained sections with an Olympus BX 40 microscope [Olympus Optical Co. (UK) Ltd, London, UK] fitted with a JVC 3-CCD colour video camera [JVC (UK) Ltd, London, UK] and analysing them using an Optimas 6 image analysis package (Optimas Corporation, Washington DC, USA). To correct for possible alteration of section volume during processing, aerenchyma area is expressed as a percentage of total cortex.

Data were tested for normality using the Shapiro–Wilk test using SAS software (SAS Institute Inc., Cary, NC, USA). Arcsin data transformation was required for aerenchyma area analysis (but was not required for other data).

Repeated measures two-way ANOVA followed by mean comparisons using SAS established the significance of the data.

Indirect immunofluorescence microscopy was carried out using sections mounted on poly-L-lysine-coated slides. Antibodies JIM 5 and JIM 7 (obtained from the Monoclonal Antibody Unit at the John Innes Centre for Plant Science Research, Norwich, UK) were used without dilution. After the section had been rehydrated, it was blocked using 0·1% Tween 20 in 1% BSA, 0·002% sodium azide in TBS for 15 min, and incubated with primary antibody at 4 °C overnight. It was then washed 3 × 10 min with phosphate-buffered saline (PBS) and incubated with goat antirat secondary antibody for 2 h at room temperature. Finally, specimens were rinsed 3 × 10 min in PBS and stained 2 min with 3 μg ml^{-1} propidium iodide before washing and mounting in Citifluor glycerol-based antifade (Agar Scientific, Stansted, UK). Specimens were observed using a Zeiss (Welwyn Garden City, UK) Axioplan fluorescence microscope fitted with an FITC filter set and images recorded using Ilford HP5 monochromatic film (Ilford, Cheshire, UK) rated at 400 ASA. Controls without primary antibody were carried out for all experiments and showed no staining.

RESULTS

Roots grown in 3% oxygen or 1 μL L^{-1} ethylene had slower root extension than those grown in 21% oxygen over 4 d (Fig. 1). Repeated measures two-way ANOVA indicated that the effects of treatments, time and interactions were statistically significant (all $P < 0{\cdot}001$). Mean comparisons showed that root lengths in 3% oxygen and 1 μL L^{-1} ethylene were significantly less (all $P < 0{\cdot}05$) than in the control (21% oxygen) by day 1 with ethylene being more inhibitory than 3% oxygen by day 4 ($P = 0{\cdot}04$).

When roots were marked every 24 h 10 mm from the tip, measurements revealed that all elongation took place in the apical 10 mm for all treatments (data not shown). Aerenchyma was first detected in 0·5-d-old tissue in roots given either 3% oxygen or 1 μL L^{-1} ethylene for 3 d (Fig. 2). More fully developed aerenchyma was observed in 1·5-d-old tissue whereas 2·5-d-old tissue showed well-developed aerenchyma in both treatments. Tissue age (0·5, 1·5, 2·5 d) is given as the true age (i.e. time from formation in the meristem) of that section of tissue, as derived from charcoal marks placed on the root surface at 24 h intervals.

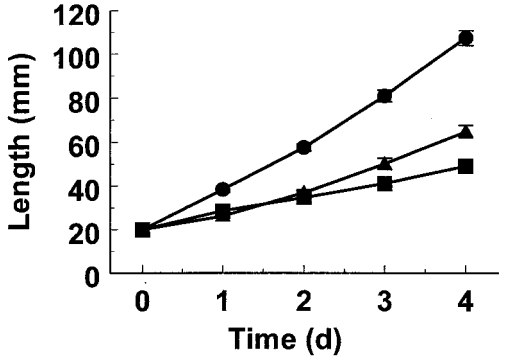

Figure 1. Time course of root length increase in 21% oxygen, 3% oxygen, 1 μL L^{-1} ethylene at 18 °C. Mean % ± SE of five experiments, two replicates per treatment for 21% oxygen and 3% oxygen and three experiments, two replicates for ethylene treatment). ●, 21% oxygen; ▲, 3% oxygen, ■, 1 μL L^{-1} ethylene.

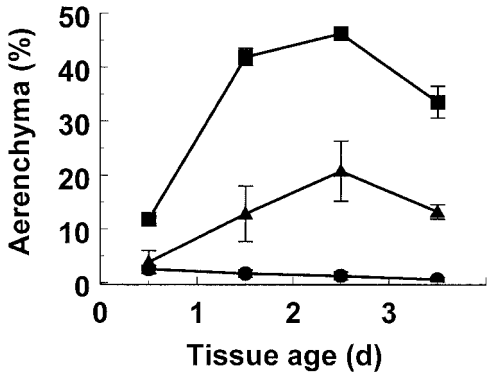

Figure 2. Development of aerenchyma over a 3·5 d period in 21% oxygen (●) 3% oxygen (▲), and 1 μL L^{-1}ethylene (■). The area of aerenchyma is given as a percentage of the area of the cortex. Mean ±1 SE of four replicates (four roots – three sections from each root) of each treatment.

Percentage aerenchyma area increased over time along the root and then decreased in older tissue close to the caryopses (Fig. 2). Repeated measures two-way ANOVA indicated that the effects of treatments, time and interactions were statistically significant (all $P < 0{\cdot}001$). Mean comparisons revealed that the percentage of aerenchyma was significantly higher ($P < 0{\cdot}001$) in 3% oxygen- and 1 μL L^{-1} ethylene-treated roots than in controls for all ages of tissue. Ethylene was significantly more effective than 3% oxygen as from 1·5-d-old tissue. Small intercellular spaces, but not developing aerenchyma, were observed in control roots after 3 d but this represented the interstitial space between intact cells and was not created by cell lysis.

Figure 3 shows the distribution of de-esterified pectin detected by indirect immunofluorescence using the monoclonal antibody JIM 5. In control roots treated with 21% oxygen, punctate labelling was restricted to points of contact between adjacent cell walls within three-way junctions of cortical cells (arrows). Labelling was stronger in 1·5-d-old tissue (Fig. 3b,c) than 0·5-d-old tissue (Fig. 3a). Walls of cortical cells taken from 0·5-d-old tissue in roots treated with either 3% oxygen or 1 μL L^{-1} ethylene in air in which aerenchyma was developing (Fig. 2) showed enhanced labelling compared with 21% oxygen controls (Fig. 3a,d,g). Not only were the three-way cell junctions labelled, but also much of the rest of the walls of some cortical cells. Cell walls in older (> 1·0 d) tissue in which aerenchyma was more fully developed showed loss of labelling of three-way cell junctions, but stronger and more extensive labelling of

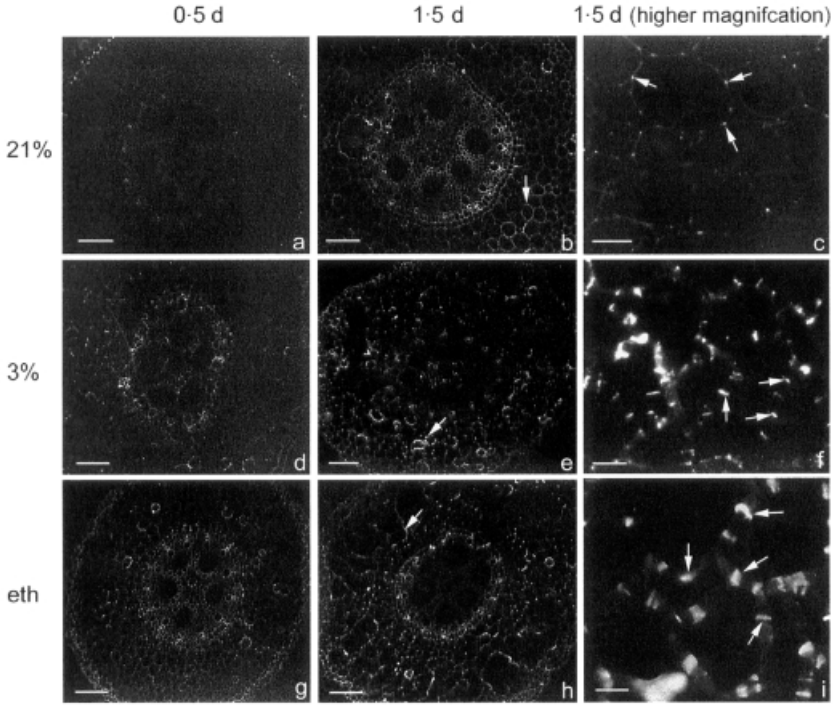

Figure 3. Distribution of de-esterified pectin revealed by immunofluorescence microscopy using JIM 5. Root material treated with 21% oxygen (a, b, c), 3% oxygen (d, e, f) or 1 μL L^{-1} ethylene in air (g, h, i) was embedded in wax and sections of 0·5-d-old (a, d, g) and 1·5-d-old (b, c, e, f, h, i) tissue prepared and stained with antibody. Note staining of vertices of triangular junctions in controls (b, c, arrows) and characteristic banding pattern in cell walls surrounding gas spaces in sections from tissue treated with both 3% oxygen and ethylene (e, f, h, i, arrows). Scale bars – (low magnification) a, b, d, e, g, h = 100 μm; (high magnification) c, f, i = 20 μm.

cell walls of the inner and mid cortex, where aerenchyma was forming, whereas other regions retained the characteristic pattern of the three-way cell junctions (Fig. 3e,h). At higher magnification, this more extensive labelling appeared as bands across the wall (Fig. 3f,i, arrows). By this stage, the walls appeared either swollen or had tipped over during sectioning to reveal part of the face of the wall as well as the cut surface (Fig. 3f,i). The bands were most clearly evident in 2·5-d-old tissue treated with ethylene (Fig. 4).

Indirect immunofluorescence detection of esterified pectin with JIM 7 in controls (21% oxygen) in 0·5 d and 1·5 d tissue was restricted to triangular cell junctions in the cortex and uniform wall labelling in the stele (Fig. 5a,b). Unlike JIM 5 (which, in control roots labelled only wall contact points at the vertices of triangular junctions), JIM 7 strongly labelled the walls at all three sides of the triangular space (Fig. 5c). Cortical cells taken from 0·5 d tissue in roots treated with either 3% oxygen or 1 μL L^{-1} ethylene in air showed altered labelling compared to controls (Fig. 5d–g). Cell walls in older (> 1·0 d) tissue showed loss of labelling of three-way cell junctions, but more intense labelling of cells of predominantly the inner and mid cortex, where aerenchyma was forming. Other, adjacent, regions retained the characteristic pattern of the three-way cell junctions (Fig. 5e,f,h,i). Labelling persisted throughout the formation of aerenchyma and increased to include all the cell wall material surrounding the gas space (Fig. 5e,h). Higher magnifications revealed wall zones of more intense labelling (perhaps representing areas of wall previously within triangular junctions) in the cortex of treated roots. These zones were interspersed with less well-labelled areas (Fig. 5f,i).

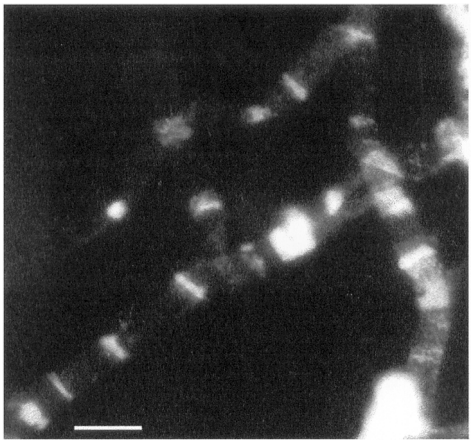

Figure 4. Root material (2·5-d-old) treated with 1 μL L^{-1} ethylene in air and stained with JIM 5 (as Fig. 3). Note distinct banding patterns evident across cell walls. Scale bar = 12 μm.

DISCUSSION

Our previous work showed that the development of aerenchyma occurred by a process that, in part, resembled apoptosis in mammalian cells (Gunawardena *et al*. 2001). The first observable changes were in cytoplasmic membranes, followed by chromatin condensation, oligonucleosomal DNA fragmentation and the formation of membrane bodies enclosing organelles. These events were triggered both by reduced oxygen supply and by exogenous ethylene, strongly suggesting them to be intrinsic to aerenchyma formation. From microscopic examinations cell wall degrada-

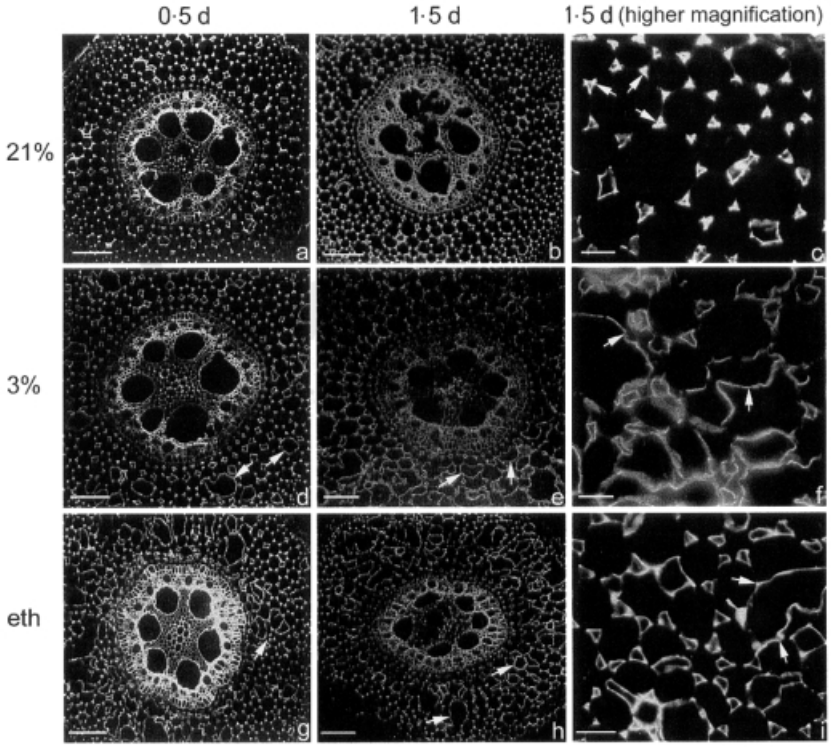

Figure 5. Distribution of esterified pectin revealed by immunofluorescence microscopy using JIM 7. Root material treated with 21% oxygen (a, b, c), 3% oxygen (d, e, f) or 1 μL L^{-1} ethylene in air (g, h, i) was embedded in wax and sections of 0·5 d (a, c, d, g) and 1·5 d (b, e, f, h, i) tissue prepared and stained with antibody. Note staining of walls within triangular junctions in controls (c, arrows) and of cell walls surrounding aerenchyma in sections from tissue treated with both 3% oxygen and ethylene (d, e, f, g, h, i, arrows). Scale bars a, b, d, e, g, h = 100 μm, c, f, i = 25 μm.

tion appeared to occur at a late stage of the process, long after the intracellular events listed above. However, we thought it likely that wall degradation is initiated at a much earlier stage, and before structural changes become clearly visible under the microscope. The present study set out to establish evidence of early wall changes and to establish whether these took place in parallel with the cytoplasmic and nuclear apoptotic events. We used JIM 5 and JIM 7 antibody staining, together with root marking and tissue ageing that permitted us to establish the timing of wall changes in relation to intracellular events reported previously (Gunawardena *et al*. 2001). JIM 5 binds to de-esterified pectins and JIM 7 to esterified pectins and were chosen on the basis of earlier developmental studies. The JIM 5 antibody is known uniformly to stain cell walls of non-dividing cells in the root tip of *Arabidopsis* but not those of dividing cells (Dolan, Linstead & Roberts 1997) whereas JIM 7 uniformly stains most cells. In carrot root apices, de-esterified pectin was found at the inner surface of the primary cell wall, adjacent to the plasma membrane, in the middle lamella and at intercellular spaces, whereas esterified pectin was located evenly throughout the wall (Knox *et al*. 1990). In oat and other gramineae, JIM 5 and JIM 7 staining was limited to specific cells and not spread throughout the root. JIM 5 staining occurred in the cell walls of intercellular spaces, whereas JIM 7 was most abundant in the cells of the cortex and stele (Knox *et al*. 1990). Neither stained the epidermis or root cap.

In our present studies, changes in both JIM 5 and JIM 7 staining were evident in cortical cells exposed to only 0·5 d of low oxygen or ethylene. No staining changes were noted in 0·5 d tissue grown in 21% oxygen. It seems likely therefore that changes in the walls of those cells that are to die to form aerenchyma occur at a very early stage. Comparison with the data of our previous study (Gunawardena *et al*. 2001) shows this to be at a stage when abundant vesicles are visible at an invaginating plasma membrane and when the first stage of oligonucleosomal cleavage of DNA is detectable by TdT mediated dutp nick-end labelling (TUNEL) reagents. It precedes chromatin condensation and the formation of membrane-bounded bodies containing organelles, first visible at 1–2 d and the first wall changes detectable by electron microscopy at >2 d. JIM 5 and JIM 7 are therefore effective early stage markers for cell wall changes in aerenchyma formation and reveal that cell wall degradation begins alongside cytoplasmic and nuclear disassembly.

The most striking change in pectin staining was the presence of a banding pattern of de-esterified pectin in cell walls forming aerenchyma, revealed by JIM 5. This was in contrast to 21% oxygen controls, where de-esterified pectin was detected only at the vertices of three-way cell junctions, as previously observed in *Arabidopsis thaliana* roots (Dolan *et al*. 1997). Banding occurred in 3% oxygen or 1 μL L^{-1} ethylene-treated tissue, commencing at 0·5 d and being fully developed by 1·5 d. The bands appeared to be where points of contact existed within three-way junctions prior to wall degradation. Murdoch *et al*. (1999), in a study of cell wall degradation by *Erwinia,* noted JIM 5 labelling at three-way junctions between cells which remained after loss of labelling of surrounding wall material. Cell separation at the middle lamella of three-way junctions results in the formation of intercellular spaces in non-aerenchymatous tissue (Jeffree, Dale & Fry 1986). Jauneau, Quentin & Driouich (1997) also observed JIM 5 staining at cell junc-

tions and walls surrounding air spaces in cortical cells of flax.

The distribution of esterified pectin revealed by JIM 7 labelling also altered during aerenchyma formation. In controls (21% oxygen) JIM 7 binding was restricted to triangular cell junctions in the cortex where it strongly labelled walls on all three sides of the triangle. This labelling not only persisted during formation of aerenchyma in hypoxic or ethylene-treated roots but also increased to include cell wall material surrounding the newly formed aerenchyma gas space. Where structure was badly disrupted by cell death, strands of JIM 7-binding wall material were present within the gas space. Persistent triangles of labelled cell junction regions were also evident where cortical cells remained intact. Triangular cell junctions are important in root development. They are frequently distorted by cortical cell growth and intercellular spaces form when the cell walls separate within the junction. Such spaces are surrounded by cell walls characterized by high levels of JIM 5 labelling (Dolan *et al*. 1997). It is believed that in these circumstances, pectins in the middle lamella prevent separation of the adjacent walls beyond the vertices of the junction. The high levels of labelling observed during aerenchyma development may result from either increased pectin deposition in the area or increased exposure of pectins to antibody binding. As pectins are abundant in the middle lamella, loss of one sister cell wall might result in exposure of the pectin in that region to antibodies. Jauneau *et al*. (1997) observed immunogold labelling with JIM 5 in the middle lamella of cortical cells of flax hypocotyls. Strongest labelling occurred along the inner edge of the wall surrounding normal (non-aerenchymatous) intercellular spaces. A similar pattern was observed at cell junctions in *Arabidopsis* (Dolan *et al*. 1997). This would explain enhanced labelling of small intercellular spaces caused by cell separation or (as in our studies) large spaces caused by lysigeny as a result of a loss of one of the two cell walls. It would also suggest that wall digestion takes place from the inner face until the middle lamella is reached (suggesting the process is initiated and controlled by the cell undergoing programmed cell death) and ceases there, possibly because it is resistant to degradation.

It has been shown previously that aerenchyma formation induced by low oxygen or ethylene is initiated in the inner cortex and spreads from these cell layers. However, there is no published evidence that there are intrinsic differences between the cell walls of those cells which go on to form aerenchyma and those which do not. When cell wall degradation occurs in both fruit ripening and abscission, marked changes in the solubility of pectic polysaccharides occur at an early stage (Rose *et al*. 1998). Thus, it is to be expected that those walls which are to be digested during aerenchyma formation would also show altered pectin distribution, prior to the loss of the cell contents, in preparation for digestion by cellulases and hemicellulases. Evidence of actual loss of total wall pectin was not obtained in the study. The techniques currently available would neither detect small percentage losses nor pick-up subtle changes that would alter exposure of pectins to antibody binding. However, the sensitive labelling of both de-esterified (JIM 5) and esterified (JIM 7) pectin was found to be much altered by treatments promoting aerenchyma formation. This indicates a marked qualitative change in wall pectins of cortical cells. The degree of esterification of the network of pectic polysaccharides within the cell wall may well affect its structural strength and resistance to degradation by pectin methylesterases as pectic polysaccharides influence matrix properties such as porosity, calcium binding and adhesion (Willats *et al*. 2000).

JIM 5 and JIM 7 staining, together with root marking and tissue ageing, enabled us to establish the timing of wall changes in relation to steps in intracellular apoptosis. Changes in both JIM 5 and JIM 7 staining were evident in cortical cells after 0·5 d of treatment with low oxygen or ethylene. No staining was noted in 0·5 d tissue grown in 21% oxygen. It seems likely therefore that changes in the walls of those cells that are to die to form aerenchyma occur at a very early stage. Comparison with the data of our previous study (Gunawardena *et al*. 2001) shows this to be at a stage when abundant vesicles are visible at an invaginating plasma membrane and the first stages of oligonucleosomal cleavage of DNA is detectable by TUNEL reagents. It precedes chromatin condensation and the formation of membrane-bounded bodies containing organelles, first visible at 1–2 d and the first wall changes detectable by electron microscopy at >2 d. JIM 5 and JIM 7 are therefore effective early stage markers for cell wall changes in aerenchyma formation and reveal that cell wall degradation begins alongside cytoplasmic and nuclear events.

The results of this study indicate that cell wall changes involving both esterified and de-esterified pectins are induced by hypoxia or ethylene in the roots of maize within 12 h. They appear to be the fastest wall changes observed to date in hormone-regulated aerenchyma formation. Taken together with our previous data (Gunawardena *et al*. 2001) it seems likely that a dying cell synthesizes and/or releases degrading enzymes into the wall at an early stage, as it begins the changes that will lead to nuclear and cytoplasmic degradation. Wall degradation is therefore an inherent part of the initial stages of aerenchyma development and involves controlled expression and release of wall-degrading enzymes as an integral part of programmed cell death.

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