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CELL WALL DEGRADATION AND MODIFICATION DURING PROGRAMMED CELL DEATH IN LACE PLANT, *APONOGETON MADAGASCARIENSIS* (APONOGETONACEAE)[1]

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An unusual form of leaf morphogenesis occurs in the aquatic, lace plant, *Aponogeton madagascariensis* (Aponogetonaceae). Early in development, discrete patches of cells undergo programmed cell death (PCD) and form perforations during leaf expansion. In addition to the protoplasts, walls of the dying cells are degraded during PCD. The cuticle of the perforation site is eroded first, followed by dissolution of cell wall matrix components, so that walls appear as loose fibrillar networks as perforations form. Gel diffusion assays of wall-degrading enzyme activity indicated that pectinases are active throughout leaf development, while cellulase activity was restricted to early stages of perforation formation. Alcian blue staining showed that degrading walls remain rich in pectin, and immunolocalization of pectin epitopes indicated that the proportions of esterified and de-esterifed pectins do not change significantly. Walls of perforation border cells are modified by suberin deposition late in development, and reactive oxygen species, thought to have a role in polymerization of phenolic suberin monomers, are present at the same stage. This timing suggests that suberization may limit the spread of PCD and provide an apoplastic barrier against microbial invasion but does not initiate PCD.

Key words: *Aponogeton*; cell wall degradation; cellulose; lace plant; pectin; pectinase; programmed cell death; suberin.

Programmed cell death (PCD) is a normal part of plant development and occurs throughout the life cycle, from death of the embryonic suspensor to senescence of leaves and flower parts. During plant PCD, the breakdown products of the protoplast are thought to be absorbed by surrounding cells, but the cell wall may be partially or completely degraded, or left intact as a corpse-like remnant of the PCD process (Jones, 2001). For instance, during ethylene-induced aerenchyma formation in roots (Campbell and Drew, 1979; Webb and Jackson, 1986; He et al., 1994; Gunawardena et al., 2001) and in certain examples of abscission and dehiscence (Rose et al., 2003), the cell wall is completely disassembled in a coordinated, regulated way. In contrast, only partial degradation of lateral walls occurs during xylem vessel element differentiation (O'Brien, 1970; Fukuda et al., 2000). In other cases, such as pathogen-induced PCD in tobacco leaves or endosperm development in maize, the walls appear to be unmodified and remain as crushed primary walls (Mittler and Lamb, 1995; Mittler et al., 1997; Young et al., 1997).

The development of complex leaf shape through PCD is an unusual but widely cited form of PCD (Greenberg, 1996; Jones and Dangl, 1996; Beers, 1997; Pennell and Lamb, 1997; Dangl et al., 2000; Huelskamp and Schnittger, 2004). Several species of *Monstera* and related genera in the Araceae and a single species of the distantly related Aponogetonaceae, *Aponogeton madagascariensis* (lace plant), are the only vascular plants known to form perforations (openings) by PCD during leaf development (Gunawardena et al., 2004, 2005; Gunawardena and Dengler, 2006). The cell biology of PCD in lace plant and *Monstera* is similar; *Monstera* is distinguished, however, by the simultaneous execution of PCD throughout the perforation site and an apparent absence of cell wall degradation. By contrast, PCD is progressive in lace plant leaves, with PCD being initiated at the center of a perforation site and then propagated toward the periphery until PCD is arrested. In lace plant, initiation sites form in predictable locations and at a predictable developmental stage in young leaves, making it possible to identify which cells are going to die and which of the adjacent cells of the same age will remain alive. An initial study of lace plant indicated that cell wall degradation accompanies changes to the protoplast, such as the cessation of cytoplasmic streaming, cytoplasmic collapse, and DNA fragmentation. Cell wall degradation also provides a zone of weakness that allows mechanical rupture at the perforation site as the leaf expands (Gunawardena et al., 2004); however, the extent of wall degradation during this process is unknown.

During cell wall degradation in other systems, multiple enzymes act in concert to depolymerize and hydrolyze cell wall components (Rose and Bennett, 1999; Carpita and McCann, 2000; Cosgrove, 2000; Rose et al., 2003). The enzyme polygalacturonase hydrolyzes the α-1,4-D-galacturonan backbone of pectic polysaccharides, and this increased activity coincides with pectin depolymerization (Hadfield and Bennett, 1998). During growth, pectins are secreted from the Golgi apparatus in an esterified form and then undergo de-esterification as required at their destination in the wall, catalyzed by the activity of pectin methyl esterase. De-esterification significantly affects the physical properties of pectin, and the creation of free carboxyl groups during de-esterification increases the charge density in the wall, influencing the activities of wall enzymes such as polygalacturonase and pectic lyase (Wakabayashi et al., 2000). Endo-β-

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1,4-glucanase can hydrolyze the 1,4-β-D linkages between unsubstituted linear glucans, and thus cellulose and xyloglucan within plant cell walls are potential substrates (Rose et al., 2003). Expansin enzymes weaken glucan–glucan interactions by disrupting hydrogen bonds at the cellulose–cross-linking glucan interface and appear to act in wall disassembly as well as in cell growth (Cosgrove, 1993, 2000). Similarly, cleavage and re-ligation of xyloglucans through the activity of xyloglucan endotransglycosylase may also function during cell wall degradation (reviewed by Rose et al., 2003). In addition to these and other enzymes, nonenzymatic factors have been identified that may play a role in cell wall degradation. Two groups of reactive oxygen species (ROS), hydroxyl radicals (Fry, 1998; Fry et al., 2001) and hydrogen peroxide (H_2O_2) (Miller, 1986), may disrupt pectin and hemicellulose backbones. ROS degrade cell wall polysaccharides in vitro (Brennan and Frenkel, 1977; Fry, 1998; Fry et al., 2001), and evidence of ROS-mediated breakage of chemical bonds has been observed during fruit ripening (Fry et al., 2001) and during wall expansion in growing leaves (Rodriquez et al., 2002) and roots (Liszkay et al., 2004) of maize.

Developmentally regulated PCD is typically localized in space and restricted in time, and tissues undergoing PCD can be directly adjacent to tissues that do not respond to the cell death signals. This is particularly striking when specific cell walls are degraded, yet neighboring walls retain structural integrity, such as seen for xylem differentiation in vivo (O'Brien, 1970), floral organ abortion (Cheng et al., 1983) and for perforation formation in lace plant (Gunawardena et al., 2004). In the only other developmental study of lace plant, a cell wall modification was described that might play a role in resistance to wall-degrading enzymes. Serguéeff (1907) reported that, before perforations form, a brown pigment is deposited that demarcates the future perforation site. The substance was identified as suberin because the brown regions were the only areas that resisted sulfuric acid digestion. Serguéeff (1907) hypothesized that the suberized zone actually induces perforation formation because the suberin blocks the cells to the interior from receiving adequate nutrients, thus leading to their death. Suberin is a hydrophobic material that impregnates the cell walls of specialized cells (Kolattukudy, 1984; Bernards, 2002) and is thought to prevent the passage of water and other materials through the apoplast. It consists of both a suberin polyphenolic domain and a suberin polyaliphatic domain (Bernards et al., 2004). Specific isoforms of peroxidase have been associated with wound-induced suberization (Bernards et al., 1999; Quiroga et al., 2000; Lucena et al., 2003), and Bernards et al. (2004) have suggested that suberin polyphenolic domain assembly involves cell wall-associated peroxidase enzymes that use hydrogen peroxide (H_2O_2) to oxidize phenolic suberin monomers, resulting in their polymerization.

The primary goal of this study was to characterize cell wall degradation during the leaf perforation process in lace plant. Gel assays of cell wall-degrading enzyme activity and light and electron microscopic observations were made during the complete sequence of perforation formation, including a pre-perforation stage (stage 1), the first cytological evidence of PCD (stage 2), mechanical rupture at the perforation site (stage 3), leaf expansion (stage 4), and maturation (stage 5) (Gunawardena et al., 2004). A second goal was to test Serguéeff's (1907) intriguing hypothesis that deposition of suberin in the cell walls at the periphery of the perforation site might initiate PCD.

MATERIALS AND METHODS

Plant material—Sterile cultures of lace plant, *Aponogeton madagascariensis* (Aponogetonaceae, obtained from Dr. Michael E. Kane, University of Florida) were maintained through subculturing in autoclaved Murashige and Skoog (MS) medium (2.15 g MS basal salts, 100 mg myo-inositol, 0.4 mg thiamine-HCl, 30 g sucrose and distilled water to make 1 L). Subdivided corms were placed on solidified MS medium containing 1% agar in autoclaved Magenta GA7 boxes (Bioworld, Dublin, Ohio, USA), and sterile liquid MS (0% agar) was added to fill approximately 75% of the volume (Gunawardena et al., 2006). Cultures were maintained in a Conviron E15 growth chamber (Controlled Environments, Winnipeg, Manitoba, Canada) with 18 h light/6 h dark cycles at 24°C and 290 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from cool white fluorescent lights (Sylvania F72T12 CW/HVO, Enviromate Products, Richmond Hill, Ontario, Canada). Although leaves grown in sterile culture are small, the cell biology of perforation formation does not differ from that in aquarium-grown plants (Gunawardena et al., 2006). Leaves representing developmental stages 1 to 5 were harvested from cultured plants and used for gel diffusion assays or microscopy. All experiments or observations were replicated with leaves from 4 to 16 independent plants. Unless otherwise stated, all chemicals were purchased from Sigma (Sigma, Oakville, Ontario, Canada).

Scanning and transmission electron microscopy—For scanning electron microscopy, tissue samples (5 mm^2) from leaves at stages 1 to 3 were fixed ca. 12 h in 70% FAA (formalin: acetic acid: 70% ethanol; 1:1:18, by vol.). Samples were dehydrated through a graded ethanol series, dried using a Tousimis Autosamdri-814 critical point dryer (Tousimis Research, Rockville, Maryland, USA), mounted on specimen stubs, coated with gold on a Cressington 108 sputter-coater (Cressington Scientific Instruments, Cramberry Township, Pennsylvania, USA), and observed using a Hitachi S-2500 scanning electron microscope (Hitachi, Tokyo, Japan). Images were recorded using a Quartz PCI version 5.1 camera (Quartz Imaging, Vancouver, British Columbia, Canada).

For transmission electron microscopy, samples (2 mm^2) from leaves at stages 1 to 5 were fixed ca. 12 h in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 6.9), washed in the same buffer, then postfixed in 2.5% aqueous osmium tetroxide for 4 h at room temperature. Tissues were dehydrated through a graded ethanol series, infiltrated with ethanol:Spurr resin mixtures (3:1, 1:1, 1:3), embedded in pure Spurr resin, and polymerized at 70°C for 9 h. Sections, 70 nm thick, were cut on a Reichert-Jung ultramicrotome (Reichert, Vienna, Austria), collected onto formvar-coated grids, and stained with 4% uranyl acetate for 7 min and Reynolds lead citrate (Reynolds, 1963) for 4 min. Observations were made using a Phillips 201 transmission electron microscope (Phillips, Eindhoven, Netherlands) and recorded digitally using an AMT CCD camera system (Advanced Microscopy Techniques, Danvers, Massachusetts, USA).

Gel diffusion assays—Tissue samples from leaves at developmental stages 1–5 were ground to powder in liquid nitrogen and incubated for 1 h at 4°C in two volumes of 50 mM sodium acetate and pH 5.0 buffer containing 10 mM EDTA and 3 mM β-mercaptoethanol. The soluble fraction was collected following 10 min centrifugation at 8000 × *g* (modified from Kofalvi et al., 1995). The final gel matrix consisted of 50 mM sodium acetate buffer pH 5.5 with 0.7% phytagel as solidifying agent and with 0.05% cellulose (β-1,4-glucan, Sigma C-5678), 0.2% pectin (polygalacturanic acid methyl ester from citric fruits, Sigma P 9135), or glucuroarabinoxylan (β-1,4-xylan backbone with arabinose and glucose side-chains containing 70–75% xylose, 41% arabinose, 15% glucose; from oat spelts, Sigma X-0627) or xylose (99% xylan from birchwood, Sigma X-0502) as the substrate. Twenty milliliters of the matrix solution was poured into each 15 × 100 mm petri plate, and seven wells were made with a cork borer (#2) after solidification. Samples were pipetted into the wells, and the plates were incubated at 37°C for 12–16 h. Undigested areas were visualized either by staining with 0.25% (w/v) Congo red (for cellulose, glucuroarabinoxylan, and xylose) or 0.5% (w/v) Alcian blue (for pectin). Positive controls were carried out with 0.002 g/mL cellulase, 0.2 g/mL pectinase, or 0.002 g/mL endoxylanase. Negative controls were carried out either with boiled enzyme samples (13 independent experiments) or distilled water (four independent experiments). For pectinase assays, mature green

Arabidopsis leaves were used as an additional negative control (two independent experiments).

Light microscopy and histochemistry—All specimens were observed using a Reichert-Jung Polyvar microscope under brightfield, differential interference contrast (DIC), or epifluorescence (exciter filter BP 330–380, dichroic mirror DS 420, and barrier filter LP 418) optics, as appropriate, and recorded using a Nikon DXM 1200 digital camera (Nikon Canada, Mississauga, Ontario, Canada). For histochemical tests, tissues were usually examined both as fresh, living whole mounts and as cleared leaves. For clearings, leaves were placed in 70% (v/v) ethanol for 6 h, cleared for ca. 16 h in saturated chloral hydrate, and mounted in the same solution. For light microscopy of sectioned leaves, tissue was fixed in 70% FAA (formalin: acetic acid: 70% ethanol; 1:1:18, by vol.), dehydrated in an ethanol series, and embedded in Paraplast (Tyco Healthcare Group, Mansfield, Massachusetts, USA). Sections (7 μm thick) were cut using a Reichert rotary microtome and collected on polylysine-coated slides.

Pectin—Tissues were tested for the presence of pectins (and other acidic polysaccharides including mucilages) by staining with Alcian blue (Ohdaira et al., 2002) or ruthenium red (Ruzin, 1999). For Alcian blue, leaves were stained in a 1% solution in 3% acetic acid (pH 2.5) for 20 min, washed several times with distilled water, and observed for blue staining. For ruthenium red, leaves were stained in a 0.05% solution in distilled water for 10 min, rinsed several times with distilled water, and observed for red staining.

Suberin and cutin—Sudan 7B and fluorol yellow 088 were used to identify the aliphatic components of suberin (Brundrett et al., 1991). For Sudan 7B, a final concentration of 0.1% (w/v) was made by dissolving 0.01 g dye in 50 mL polyethylene glycol (PEG) 400 solution and heating at 90°C for 1 h (Brundrett et al., 1991). An equal volume of 90% (v/v) glycerol solution was added to the PEG staining solution. Whole mounts of leaves were stained for 3 h at room temperature, rinsed several times with distilled water, and observed for a pink-red color. For Paraplast-embedded tissues, paraffin was removed in histoclear, and tissues were hydrated through a graded ethanol series before staining with Sudan 7B.

For fluorol yellow 088, a final concentration of 0.01% (w/v) was made by dissolving 0.01 g of fluorol yellow 088 in 50 mL of PEG 400 solution and heating at 90°C for 1 h (Brundrett et al., 1991). An equal volume of 90% (v/v) glycerol solution was added to the PEG staining solution. Leaves were stained for 1 h at room temperature, rinsed several times with distilled water, and observed with UV light for a bright yellow fluorescence. Unstained cell walls were also examined for autofluorescence as a test for the phenolic constituents of suberin.

Because cell walls impregnated with suberin, cutin, or lignin are resistant to digestion with sulfuric acid (Eastman et al., 1988), mature stage 5 leaves were placed in concentrated sulfuric acid and examined for any digestion with bright field optics.

Lignin—Phloroglucinol staining was used as a test for lignin. For the phloroglucinol stain, 0.1 g phloroglucinol, 16 mL of concentrated HCl, and 84 mL 95% ethanol were mixed, and tissues were incubated in this solution for 60 s on a hot plate (Jensen, 1962). Tissues were examined under brightfield optics for red-stained cell walls.

Condensed tannins—The presence of condensed tannins was tested by staining with dimethoxybenzaldehyde (DMB) (Mace and Howell, 1974). Equal parts of 1% DMB in ethanol and 18% HCl were mixed immediately before use. Tissue samples were stained for 5 min and examined for red coloration of condensed tannins.

Flavonoids—Leaves were stained with 0.1% Naturestoffreagenz A in ethanol solution for 5 min and observed with both UV excitation and bright field optics (Hutzler et al., 1998). Flavonoids give bright yellow fluorescence with UV light.

Reactive oxygen species (ROS)—3–3′-Diaminobenzidine (DAB) and 5-(and-6)-carboxy-2′, 7′-dichlorodihydro-fluorescein diacetate (CH_2DCFDA, Molecular Probes, C-400, Eugene, Oregon, USA) stains were used to test for ROS (Cathcart et al., 1983; Yoda et al., 2004). Fresh leaves were placed in 1 mg DAB/mL H_2O at pH 3.2 and incubated under vacuum for 3 h. DAB deposits were revealed after washing leaves in boiled 100% (v/v) ethanol for 15 min to decolorize the leaves except for the deep brown polymerization product from the reaction of DAB with H_2O_2 (Yoda et al., 2004). For the fluorescent probe, fresh leaves were placed in 10 μM CH_2DCFDA in H_2O and incubated in a vacuum oven for 2 h. CH_2DCFDA easily diffuses across the cell membrane and is then converted to the deacetylated form CH_2DCF, which has very low membrane permeability. CH_2DCF is oxidized by various ROS to form carboxy-dichloro-fluorescein, which fluoresces when examined with a 450–495 excitation filter and a 520 nm emission filter (Cathcart et al., 1983).

Immunostaining for esterified and de-esterified pectins—Tissue samples (5 mm^2) from leaves at developmental stages 1 to 5 were fixed in 70% FAA for 2 h, washed in 70% ethanol, and then either processed for Paraplast embedding or used as whole tissue pieces. Whole tissue pieces were blocked using 0.1% Tween 20 in 1% bovine serum albumin (BSA), and 0.002% sodium azide in phosphate buffered saline (PBS) or 15 min (Gunawardena et al., 2001). Samples were incubated with primary antibodies JIM 5 (John Innes monoclonal antibody 5, preferentially binding pectins lacking methyl esters; Knox et al., 1990; Willats et al., 2000; Clausen et al., 2003) and JIM 7 (preferentially binding pectins that are highly methyl esterified) at 4°C for ca. 16 h without dilution, washed 3 × 10 min with phosphate-buffered saline (PBS), and incubated with goat anti-rat FITC (fluorescein isothiocyanate)-conjugated secondary antibody (1:40) for 2 h at room temperature. Specimens were then rinsed in PBS and mounted in Antifade mounting medium. Paraffin-embedded tissues were prepared as described for Sudan 7B staining, before immunolocalization experiments were conducted. Controls lacking primary antibody were performed for both types of tissue preparation.

RESULTS

Electron microscopy of wall degradation during perforation—Changes in cell wall structure during perforation formation were observed using scanning and transmission electron microscopy (Fig. 1). In stage 2 leaves, epidermal cells adjacent to the perforation site were turgid, having primary walls about 0.25 μm thick (Fig. 1A, B) and a continuous, homogenous cuticle. Light micrographs of sectioned tissue revealed a conspicuous Sudan 7-positive layer on the surface of these epidermal cells (Fig. 1I). Within the perforation site, epidermal cells were flattened and had thinner cell walls (Fig. 1C, D), and the surface cuticle was degraded and fibrillar (Fig. 1A, C). Sudan staining of epidermal cells within the perforation revealed a thin, disrupted layer (Fig. 1J). During stage 3, epidermal cells outside the perforation site appeared rounded with distinct wall sculpturing, and walls were slightly thicker than at stage 2 (Fig. 1E, F). By contrast, within the perforation site epidermal cell walls appeared highly degraded with only a fibrillar structure remaining, suggesting that cell wall matrix components had been removed (Fig. 1G). In sectioned tissue, these walls were highly disrupted with thicker, electron-dense regions interspersed between thin fibrillar regions (Fig. 1H). Based on the ultrastructural appearance of these walls, we predicted that cutin-degrading enzymes would act early during perforation formation, followed by increased activity of pectin- and then cellulose-degrading enzymes.

Gel diffusion assays for pectinase and cellulase enzyme activity—Gel diffusion assays were performed using extracts from leaf tissues at stages 1 to 5 leaves to evaluate changes in pectinase, cellulase, and hemicellulase enzyme activities during wall disassembly. Pectinase activity was present in extracts from all five stages of leaf development, as revealed by cleared zones in the Alcian blue-stained pectin substrate (Fig. 2A), but not in negative controls including boiled lace plant enzyme extracts or extracts from green, nonsenescent *Arabidopsis*

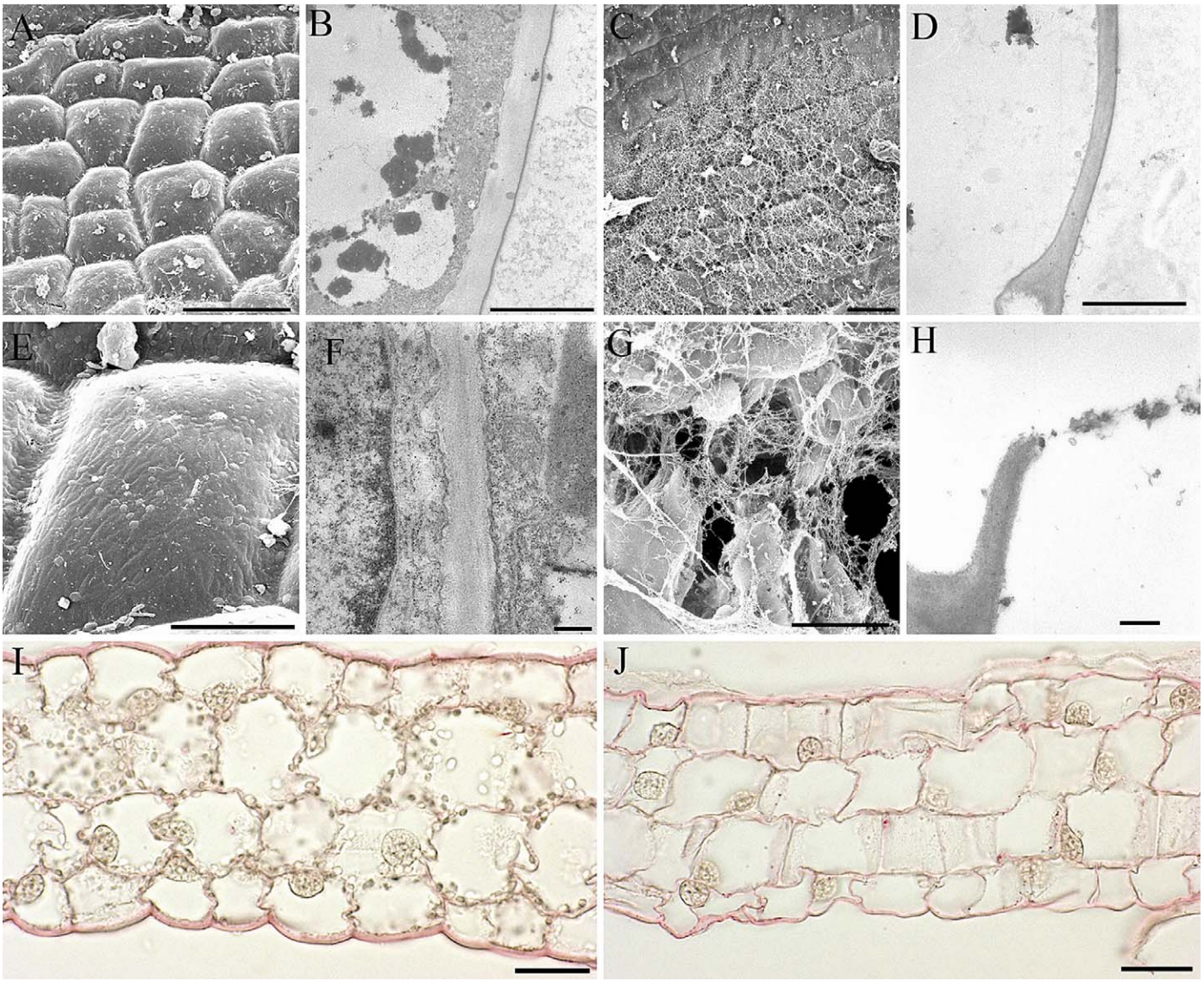

Fig. 1. Scanning and transmission electron microscopy of epidermal cell walls during perforation formation in developing leaves of lace plant. (A–D) Stage 2 leaves comparing tissue (A, B) outside the perforation site with (C, D) tissue within. Note (C) degraded appearance of cuticle and (D) thinner walls of cells within the perforation site compared with those outside. (E–H) Stage 3 leaves comparing tissue (E, F) outside the perforation site with (G, H) tissue within. Note that (G) remnants of cells within the perforation site appear fibrillar in SEM views and (H) highly disrupted in TEM. (I, J) Light microscope images of late stage 2 tissue stained with Sudan 7B. Tissue (I) outside the perforation site has a conspicuous Sudan 7B-positive surface layer, while tissue (J) within the perforation site lacks this layer. Bars = 15 μm in A, C, G; 0.5 μm in B, D, F, H; 5 μm in E; 20 μm in I, J.

leaves (data not shown). By contrast, only extracts from stage 2 and 3 leaves possessed cellulase activity (Fig. 2B). Glucuroarabinoxylanase and xylase activity were not measurable in any of the leaf extracts (data not shown). These results suggest that pectins degrade or turnover throughout all five stages, with cellulose degrading primarily during stages 2 and 3. This last agrees with the timing of cell wall degradation observed in the ultrastructural studies.

Histochemistry of cell wall pectins—Because the gel diffusion assay results indicated that pectinases are active throughout perforation development, we attempted to monitor changes in pectin levels using Alcian blue staining. No detectable staining was observed in stage 1 leaves, presumably because the dye is excluded by the intact cuticle (Fig. 3A–C); all cell walls stained, however, in sections of Paraplast-embedded tissue (data not shown). Walls of dying cells become Alcian blue positive in leaves at stage 2 (Fig. 3D–F), stage 3 (Fig. 3G–I), and stage 4 (Fig. 3J–L), coincident with the loss of the cuticle. The density of Alcian blue staining did not diminish in cells within the perforation zone with developmental stage, although it was clearly evident that many cells were completely lost as perforation progresses; thus there is an overall loss of pectin. However, pectins were evident in the remnants of the dead cells at the periphery of mature perforations (stage 5; Fig. 3M–O).

Immunostaining with JIM antibodies for pectin—Although pectins were present throughout the development of

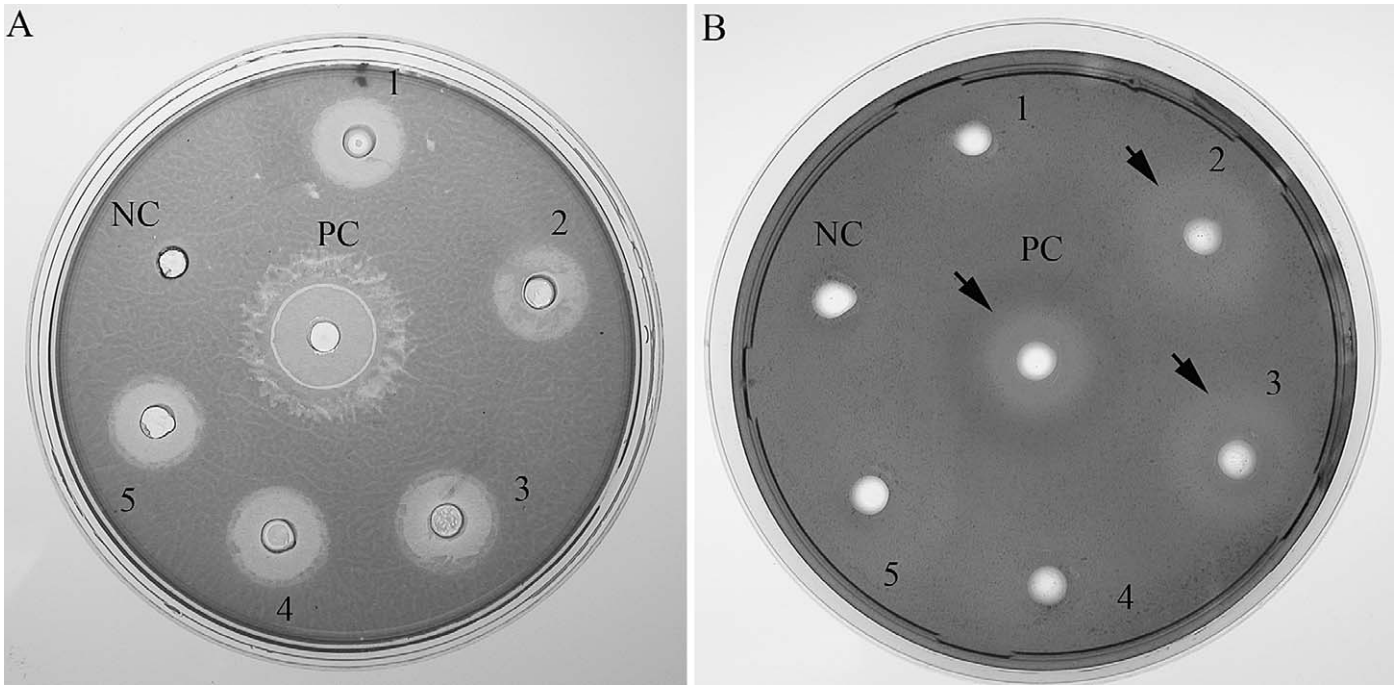

Fig. 2. Gel diffusion assays for pectinase and cellulase enzyme activity during perforation development in lace plant leaves. Tissue extracts from stages 1–5 were loaded into wells of plates containing either (A) 0.2% pectin or (B) 0.05% cellulose and stained with Alcian blue for pectins or Congo red for cellulase after incubation for 16 h at 37°C. NC, negative controls (boiled enzyme extracts); PC, positive controls (commercially available pectinase or cellulose). Arrows mark cleared areas indicating enzyme activity in B.

perforations, they could be modified through de-esterification as part of the wall degradation process. Leaves from stages 1 to 5 were immunostained with JIM 5 (specific for de-esterified pectin) and JIM 7 (specific for esterified pectin) antibodies to reveal any changes in esterification. At stage 1, no detectable antibody staining was observed with JIM 5 (Fig. 4A–D). At stages 2 (Fig. 4E–H) and 3 (data not shown), leaves had antibody staining only in the dying cells of the perforation site. At stages 4 (data not shown) and 5 (Fig. 4I–L), JIM 5 antibody staining was present only along the border of mature perforations. The staining pattern observed with JIM 7 antibody (data not shown) was identical, indicating that walls of cells undergoing PCD maintain similar proportions of esterified and de-esterified pectins throughout development. Sections through the border of perforation sites at early (Fig. 4M, N) or late (not shown) stage 2 showed no differences between cells undergoing PCD and those outside the perforation site.

Modification of cell walls at the perforation border—In cleared, unstained stages 2 and 3 leaves, the vein-associated laticifers had a brown pigmentation, but cell walls at the border of the perforation site were clear (Fig. 5A, B). Deposition of the brown pigmentation in cell walls along the perforation border first appeared at stage 4 (Fig. 5C) and became dense and continuous in both cleared (Fig. 5D) and living leaves (Fig. 5E, F) by stage 5.

Histochemistry of cleared and fresh leaf tissue was used to identify the brown pigmentation within cell walls at the perforation border in stage 5 leaves. Bright yellowish-green fluorescence with fluorol yellow 088 (Fig. 6A–D) and a pinkish-red color with Sudan 7B (Fig. 6E, F) suggested that the substance causing the brown pigmentation was at least partially composed of aliphatic compounds. Transmission electron micrographs only showed diffuse electron-dense areas in the cell walls at the perforation border (Fig. 6H), in contrast to the more uniform, electron-transparent walls of neighboring epidermal cells (Fig. 6G). Phenolic wall components were autofluorescent under UV excitation, and cells at the perforation border emit a weak autofluorescence (Fig. 6I–L). DMB for tannin (Fig. 6M–N) and phloroglucinol staining for lignin (Fig. 6O–P) were negative. Staining with Naturstoffreagenz A for flavonoids did not cause any fluorescence at the perforation border (Fig. 6Q–T). Treatment with concentrated sulfuric acid digested all cell walls except for the brown walls at the perforation border (data not shown). Thus, epifluorescence microscopy and histochemical tests indicated that both aliphatic and phenolic constituents were present and coincident with the brown pigmentation, and, along with acid digestion, strongly suggested that the substance is suberin. These constituents could be detected only at stages 4 and 5, however, well after the cytoplasmic events of PCD are complete.

ROS accumulation during wall degradation and modification—H_2O_2 was detected in lace plant leaves using DAB as a substrate. DAB staining occurs throughout the dying cells of

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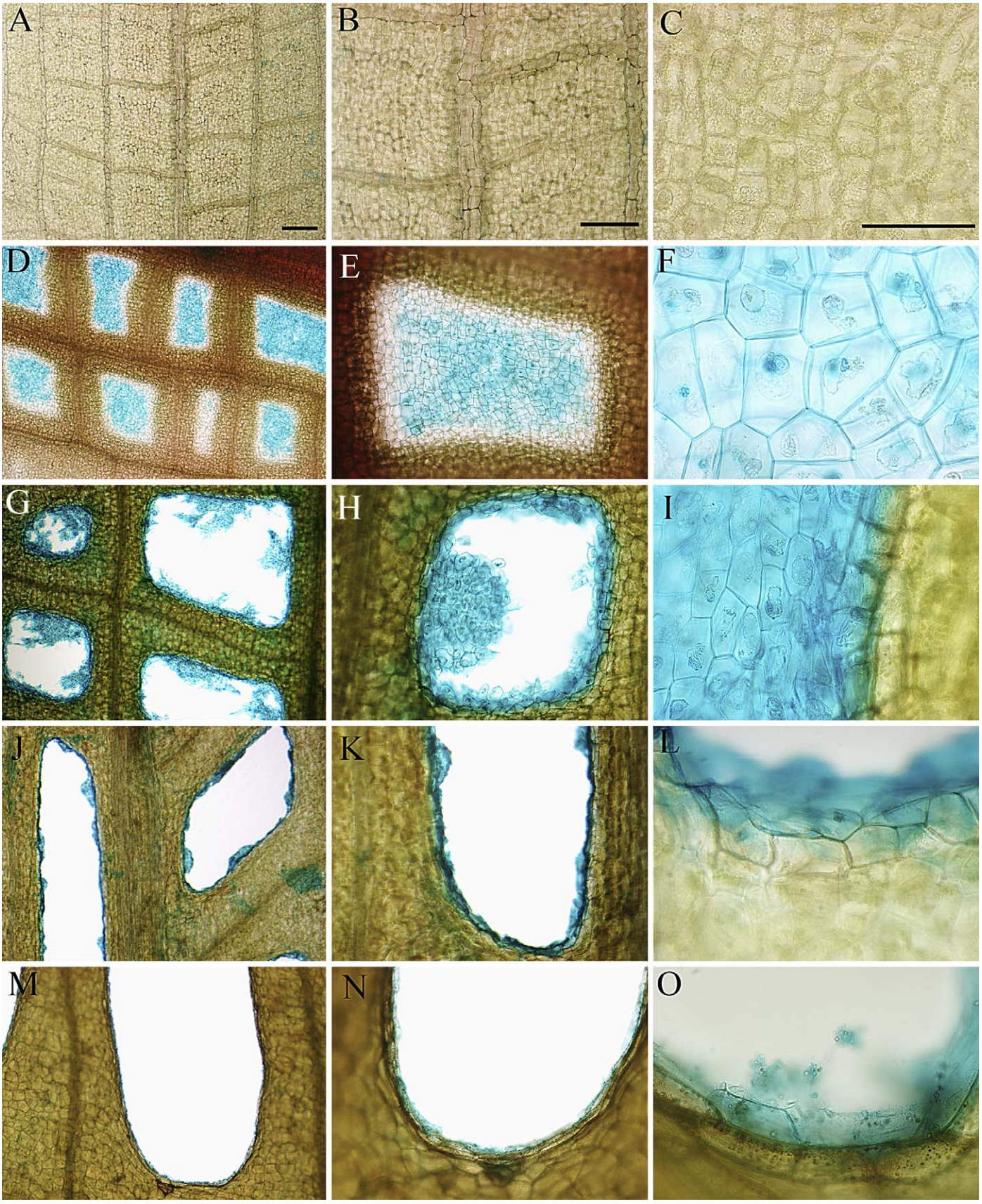
Fig. 3. Alcian blue staining for pectin during perforation formation in developing lace plant leaves. (A–C) Stage 1 leaf showing absence of positive staining. Positive Alcian blue staining is seen within the perforation site in (D–F) stage 2 and (G–I) stage 3 leaves but not in tissue outside. (J–L) Stage 4 and (M–O) stage 5 show Alcian blue staining of cells at the perforation border. Bars = 100 μm in A, B, D, E, G, H, J, K, M, N; 50 μm in C, F, I, L, O.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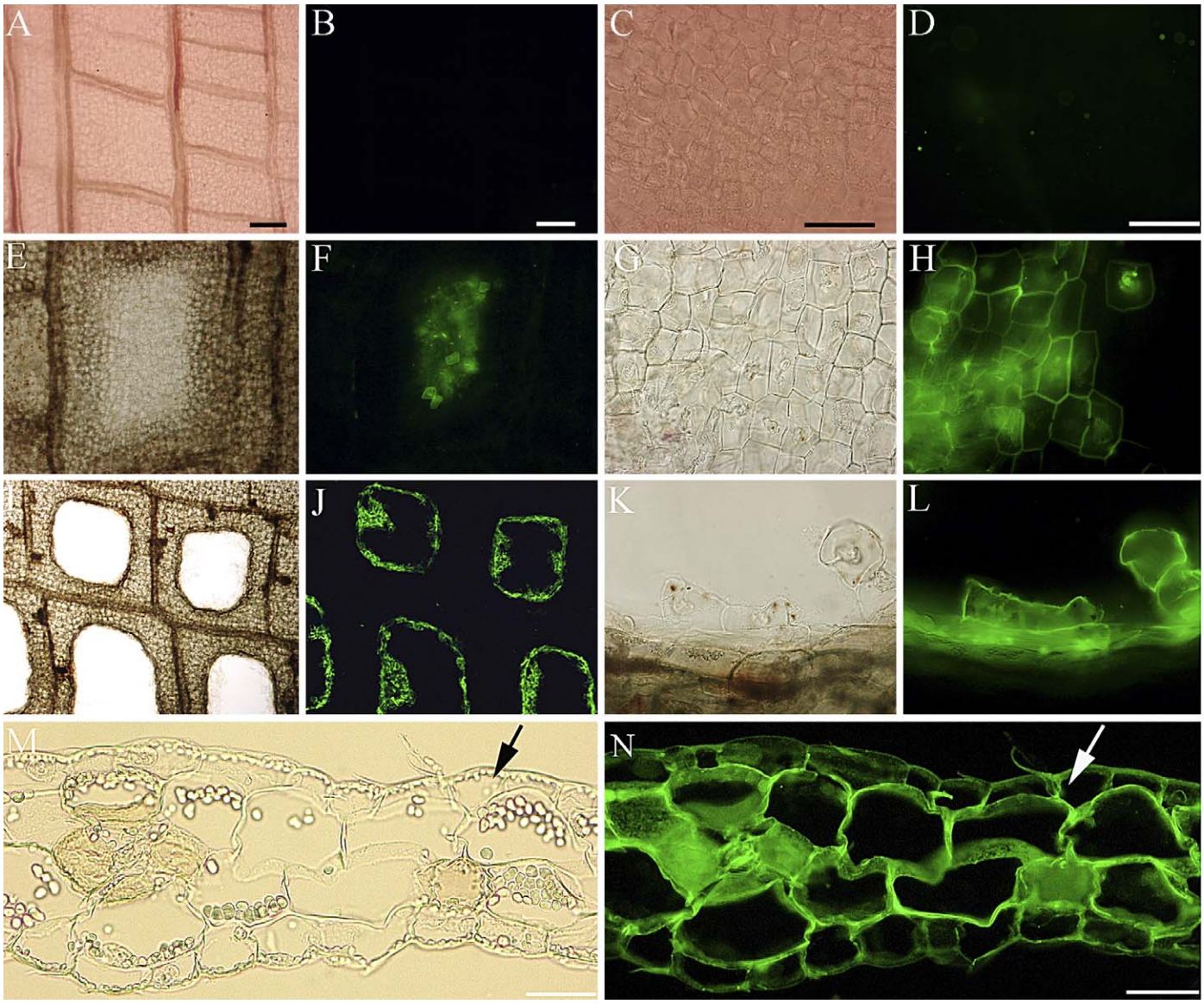

Fig. 4. Immunostaining with JIM 5 antibodies for de-esterified pectin during perforation development in lace plant leaves. (A–D) Stage 1 leaf. (E–H) Stage 2 leaf. (I–L) Stage 5 leaf. Same tissue region shown with (A, C, E, G, I, K) differential interference contrast (DIC) or with (B, D, F, H, J, L) epifluorescence microscopy. Antibody staining is not detected at (B, D) stage 1 but is present throughout the perforation site (F, H) at stage 2 . Antibody staining is present in dead cells at the perforation border (J, L) at stage 5. (M, N) JIM 5 antibody staining of sectioned early stage 2 tissue (M) under DIC or (N) fluorescence. Arrows mark boundary between cells within perforation site (right) and outside perforation site (left). Bars = 100 μm in A, B, E, F, I, J, M, N; 50 μm in C, D, G, H, K, L.

the perforation site at stages 2 and 3 (Fig. 7A, B). At stages 4 and 5, DAB staining was present at the periphery of expanding or mature perforations and appears to be confined to dying (Fig. 7C) or dead (Fig. 7D) cells at the perforation margin. Because the restriction of DAB staining to the perforation site at stage 2 might simply reflect the impermeability of the leaf cuticle to some stains and reagents (e.g., Alcian blue, JIM antibodies) but not to others (e.g, propidium iodide, 4′,6-diamidino-2-phenylindole [DAPI], Gunawardena et al., 2004), we also tested for the presence of ROS using CH_2DCFDA. In stage 2 leaves the fluorescent carboxy-dichloro-fluorescein was restricted to the perforation site (Fig. 7E, F), while in parallel experiments, fluorescein diacetate itself readily penetrated the entire leaf tissue (Fig. 7G, H). Thus, histochemical staining provided evidence that ROS accumulates in cells undergoing PCD during stages 2 and 3 and in the remnants of dying cells during stages 4 and 5.

DISCUSSION

In lace plant, one of the programmed cell death processes has a highly unusual developmental function. It results in the formation of tiny openings in young, expanding leaves that later enlarge 10–20-fold to form conspicuous holes or perforations in mature leaves. These perforations are formed

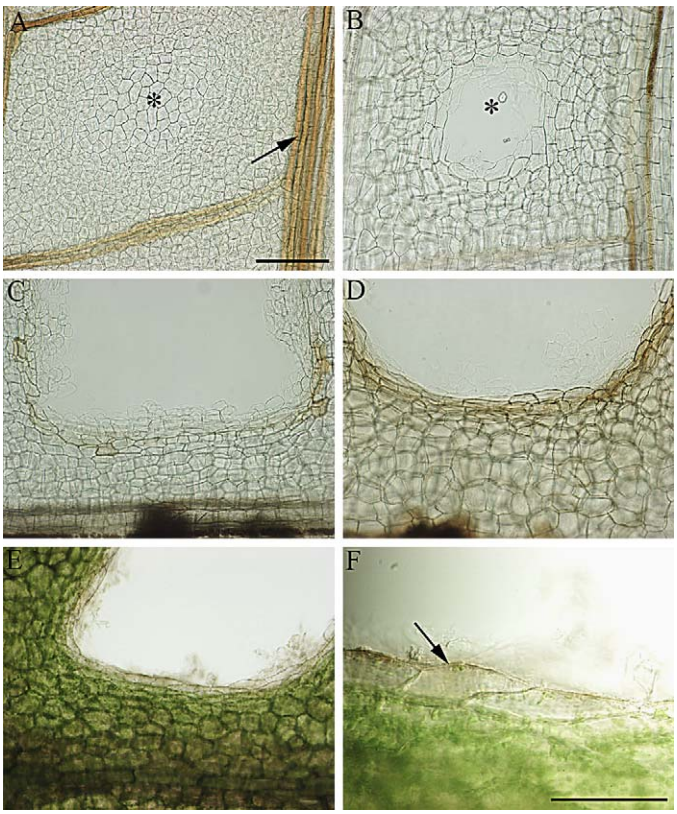

Fig. 5. Development of brown pigmentation at perforation border in lace plant leaves. (A–D) Cleared leaves from stages 2–5. (E, F) Stage 5 fresh leaves. (A) Stage 2 showing clear area of perforation site (asterisk). (B) Stage 3 showing newly opened perforation (asterisk). (C) Stage 4 showing brown pigmentation in walls of cells at periphery of perforation. (D–F) Stage 5 leaves showing brown pigmentation in walls of cells at periphery of perforation. Bars = 100 μm in A–E; 50 μm in F.

in highly predictable positions with respect to the lattice-like vein pattern and at a predictable stage of leaf expansion, making them suitable material for studying developmentally regulated PCD in plants (Gunawardena et al., 2004, 2006). PCD is initiated in a localized population of about 100 epidermal and mesophyll cells, propagates to neighboring cells at the periphery of the perforation site, and is finally arrested about five cells from the vascular tissue. The developmental environment for perforation formation by PCD in lace plant is striking because PCD is precisely localized and occurs adjacent to and concomitant with cells that are proliferating and growing (Gunawardena et al., 2004). Thus, cells outside the perforation site are undergoing wall expansion and remodeling, presumably through the activity of wall-loosening enzymes (Cosgrove, 2000), but they appear not to be affected by the processes that result in the complete wall degradation accompanying PCD within the perforation zone. These events raise two important developmental questions: (1) how are cell walls degraded within the perforation site, and (2) how is PCD, including wall degradation, prevented in the surrounding cells?

Timing of wall degradation during PCD—During lace plant perforation formation, protoplast alteration consistent with the initiation of PCD and features consistent with the initiation of wall degradation occur coincidentally. Perforation zones are first evident microscopically as the loss of vacuolar anthocyanin coloration and an erratic pattern of cytoplasmic streaming, both thought to reflect changes in tonoplast permeability and a resultant lowering of cytoplasmic pH (Gunawardena et al., 2004). At the same time, nuclei in cells in the perforation zone become positive for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), an indicator of DNA fragmentation, a characteristic feature of PCD (Gunawardena et al., 2004). At this stage of development (early stage 2), cell walls in the perforation zone have signs of surface erosion and loss of Sudan 7B staining, indicating that cuticular material has degraded. Ultrastructurally, walls of cells within the perforation zone have thinned. During late stage 2, cytoplasmic streaming ceases in cells at the center of the perforation zone, and the protoplast collapses, although the nucleus and organelles initially remain intact (Gunawardena et al., 2004). Cell walls show striking signs of degradation at the ultrastructural level and presumably represent zones of mechanical weakness, because the perforation sites rupture during stage 3, forming regular breaks in the previously continuous leaf blade. Wall remnants of late-dying cells at the periphery of the perforation may be retained in mature leaves, but cell walls of the initial population of cells undergoing PCD in each perforation zone ultimately are entirely degraded. Thus, complete wall degradation is initiated at a comparatively early stage and is an integral part of lace plant PCD, as shown for the formation of cortical aerenchyma (Campbell and Drew, 1979; He et al., 1994; Gunawardena et al., 2001), abscission (Osborne and Sargent, 1976), and differentiation of xylem vessel elements (O'Brien, 1970; Fukuda, 2000; Ohdaira et al., 2002). This is not surprising because the process of wall degradation depends entirely on the production and secretion of wall-mobilizing enzymes by the protoplast. This synthesis and delivery must occur before the death of the cell.

In lace plant, thinning of the cell wall is one of the first indicators of wall alteration during early stage 2 of PCD and might simply reflect an absence of the normal processes of wall loosening and deposition of wall components in living adjacent cells. By late in stage 2, however, scanning electron micrographs of perforation zones indicate that matrix wall components have been degraded, exposing a fibrillar material, and transmission electron micrographs give the appearance of "beads on a string" that correspond to the dimensions of the larger fibrillar material seen in the SEM. These images are similar to those for differentiating tracheary elements, where the early stages of perforation formation (degradation of the end wall of a single cell in this case) give first a pitted and then a fibrillar appearance before disappearing completely (Nakashima et al., 2000; Ohdaira et al., 2002). Electron micrographs of differentiating vessel elements indicate that the remaining primary cell wall of a differentiating tracheary element is also highly modified, becoming more fibrillar and less electron-dense between the secondary wall thickenings (O'Brien, 1970). Similarly, during the formation of cortical aerenchyma in maize roots, degrading walls become thinner, less electron dense, and more fibrillar (Campbell and Drew, 1979; Webb and Jackson, 1986), as do cell walls in the abscission layer of *Phaseolus* (Sexton and Hall, 1974). This suggests that cell wall matrix components such as pectins and hemicelluloses are degraded early, initially exposing the fibrillar cellulose component.

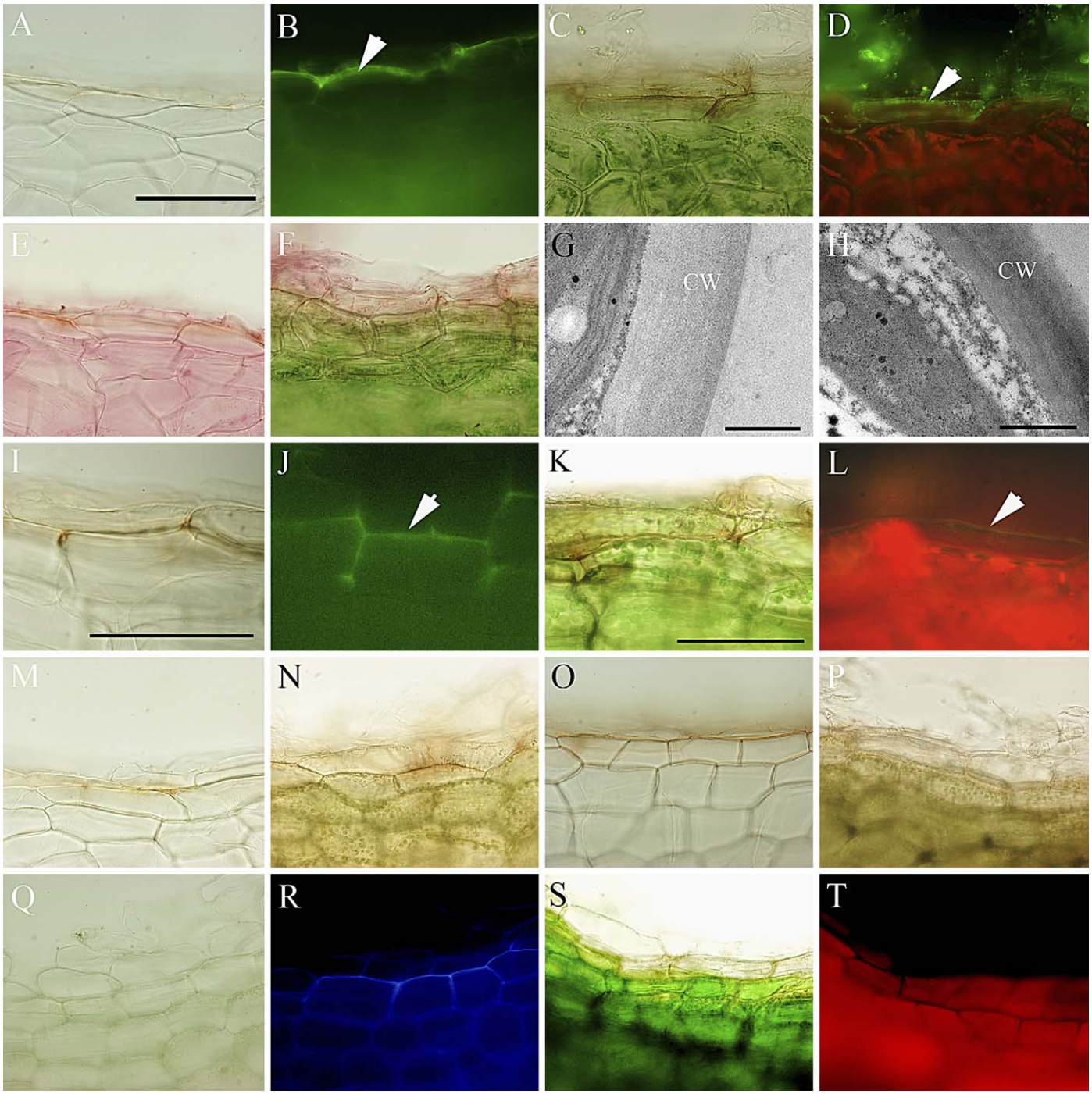

Fig. 6. Histochemistry of cell walls at perforation border in mature (stage 5) lace plant leaves. (A–D) Fluorol yellow 088 staining for aliphatic substances. (A, B) Cleared leaves. (C, D) Fresh leaves. (A, C) Differential interference contrast (DIC) images showing brown pigmentation and (B, D) epifluorescence images showing positive staining (arrows). (E, F) Sudan 7B staining for aliphatic substances in (E) cleared and (F) fresh leaves under bright field optics. Note positive staining in all cell walls (obscured by chloroplasts in fresh leaves). (G, H) Transmission electron micrographs of epidermal cell wall (CW) (G) outside the perforation area and (H) at the border of the perforation. Note greater electron density of border cell. (I–L) Autofluorescence of unstained leaf tissue, either (I, J) cleared or (K, L) fresh and viewed with (I, K) DIC or (J, L) epifluorescence microscopy. Note weak autofluorescence in walls of border cells (arrows). (M, N) DMB staining for tannins in (M) cleared and (N) fresh leaves. No positive staining was observed. (O, P) Phloroglucinol staining for lignins in (O) cleared and (P) fresh leaves. No positive staining was observed. (Q–T) Staining with Naturstoffreagenz for flavonoids in (Q, R) cleared or (S, T) fresh leaves with (Q, S) DIC or (R, T) epifluorescence microscopy. No positive staining was observed. Bars = 50 μm in A–F, I–T; 500 nm in G, H.

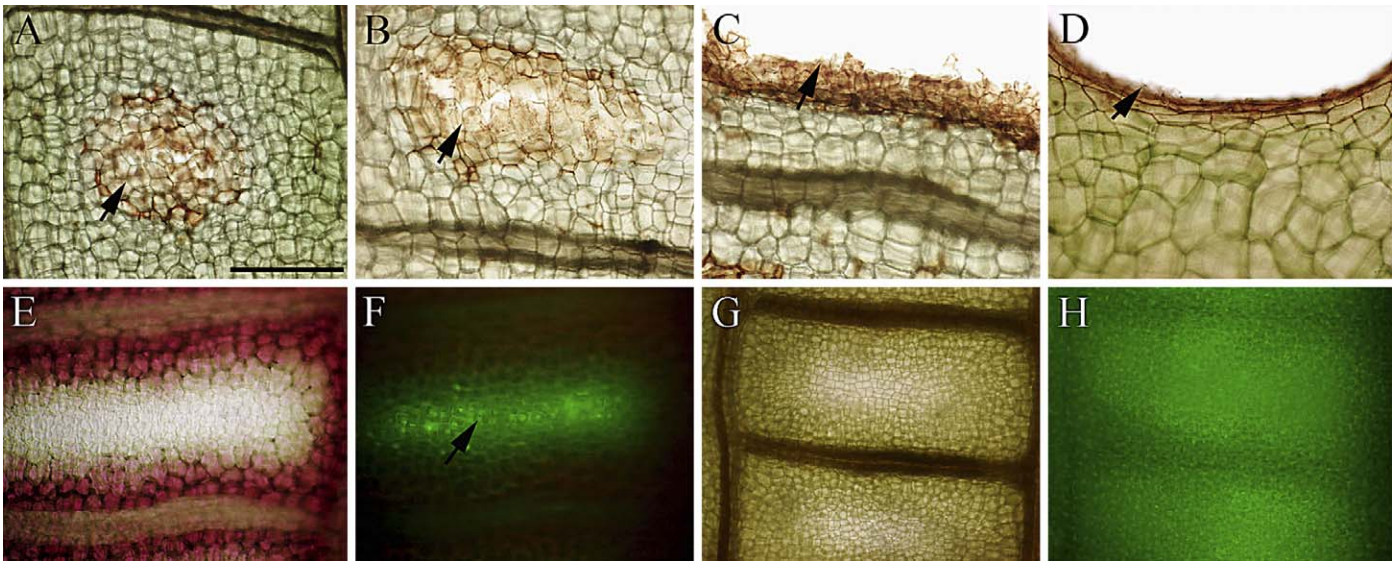

Fig. 7. Detection of reactive oxygen species in developing lace plant leaves. (A–D) Staining using the 3–3′-diaminobenzidine method indicates the presence of H_2O_2 throughout the perforation site of (A) stage 2 and (B) stage 3 leaves, as well as at the border of the (C) expanding and (D) mature perforation. (E, F) Staining with CH_2DCFDA with (E) differential interference contrast (DIC) and (F) epifluorescence microscopy indicates the presence of reactive oxygen species in stage 2 leaves (arrow in F). (G, H) Control observations of leaves under (G) DIC and (H) epifluorescence microscopy using FDA only indicate that the dye can penetrate all leaf tissue. Bars = 100 μm.

Pectinase and cellulase activity—The electron micrographs illustrating a progression of cell wall breakdown suggest that enzymatic degradation of pectin would be an early event during perforation formation in lace plant leaves. Gel diffusion assays showed that leaves at all stages of development had pectinase activity comparable to that of the exogenous pectinase positive control, in contrast to the distilled water, boiled enzyme extracts, and nonsenescent *Arabidopsis* leaf negative controls. Therefore, some pectinase activity is present in the leaf tissues of lace plant even at stage 1, before other physical evidence of PCD was detected. Pectinase activity during stages 2 and 3 corresponds well with the hypothesized removal of wall matrix components during these stages, and continued endogenous pectinase activity during stages 4 and 5 may reflect the degradation of cell walls of late-dying cells at the periphery of the perforation and continued activity, perhaps turnover, even in leaves judged to have completed expansion. Alcian blue staining of whole mounts of living leaves also indicates the presence of pectins throughout the perforation formation process, as is seen for differentiating tracheary elements (Ohdaira et al., 2002). Similarly, immunostaining indicates the presence of both esterified and de-esterified (Knox et al., 1990; Willats et al., 2000; Clausen et al., 2003) pectins within the perforation zone (stages 2 and 3) and at the periphery of the zone (stages 4 and 5). No shift in the relative proportions of esterified and de-esterified pectins or differences between dying and healthy adjacent tissues was detected, indicating that de-esterification may not be a predominant pathway for pectin removal. Rather, other enzymes with pectinase activity, such as polygalacturonase and pectic lyase, may be operating, as often observed for the removal of pectins during fruit ripening (Stolle-Smits et al., 1999; Karakurt and Huber, 2002; Rose et al., 2003). In combination with the electron micrographs, these results indicate that pectinase activity could contribute to the disassembly of lace plant cell walls during stage 2 of perforation formation and could underlie the mechanical weakening of the cell walls that allows rupture during stage 3. In addition, pectinase activity could contribute to cell wall removal during the late stages of perforation expansion. But the lack of specificity of pectinase activity to stages 2 and 3, when wall degradation is most conspicuous, indicates that it is not the primary pathway for wall removal. Under natural growth conditions, numerous microorganisms are associated with lace plant leaves undergoing PCD during perforation formation, and these may contribute to wall removal (Gunawardena et al., 2004). In the present study, however, plants were grown in sterile culture, and cell wall degradation reflected endogenous cellular processes.

Gel diffusion assays also indicated that cellulase activity is present in stages 2 and 3 lace plant leaves, at a level similar to the cellulase positive control, but is absent earlier (stage 1) and later (stages 4 and 5). While it is perhaps surprising that detectable cellulase activity does not follow the same time course as pectinase activity, cellulose must be disassembled for mechanical disruption of the perforation site at stage 3. Once the cell walls in the perforation site are weak enough for rupture, cellulase activity may no longer be required, and the cell walls may disassemble more gradually over later stages through the activity of an unidentified pectinase or other enzymes. If cellulose microfibrils are short and/or have weakened hemicellulose cross-linking, they might be released into the aquatic medium in nature or into the culture medium under experimental conditions. If so, analysis of the carbohydrate content of the liquid medium might be useful for determing the products of cell wall breakdown, as was done for differentiating tracheary elements in the *Zinnia* mesophyll system (Nakashima et al., 2000). Additionally, cellulase activity might be detected with the viscometric method used to detect cellulase activity during formation of aerenchyma in maize root (He et al., 1996) and the abscission layer in bean

(del Campillo et al., 1990). Cellulase activitity could also be detected by immunolocalization, which has been used to detect cellulase activity during abscission layer formation in *Phaseolus vulgaris* (Gonzalez-Carranza et al., 1998).

Deposition of suberin in cell walls at the perforation border—Perhaps the most intriguing feature of developmentally regulated PCD is that specific cells and tissues seem to be fated to die, while adjacent cells do not perceive or respond to the cell death signals. This is most dramatic in instances where, prior to PCD, the tissue appears homogeneous, as in gynoecium primordia in male flowers of maize (Cheng et al., 1983; Calderon-Urrea and Dellaporta, 1999) or in the unfurling leaves of lace plant (Gunawardena et al., 2004). In both cases, a sharp boundary between living cells and those slated to die is revealed only as PCD progresses. An early observer of lace plant development, Serguéeff (1907) noticed that perforation zones are ringed with cells with brown-appearing cell walls. Because these brown cell walls resisted sulfuric acid treatment, Serguéeff hypothesized that the walls were modified by the deposition of suberin and that isolation of the perforation zone cells from surrounding living cells by the suberin barrier might cause the cell death within the perforation zone. To investigate this hypothesis, we used both acid digestion and a histochemical approach to test for suberin and other modifications of primary cell walls. Like Serguéeff (1907), we found that the walls of cells forming the margin of the perforation were brown, unlike the colorless walls of living cells to the exterior. The same walls were resistant to acid digestion and positive for Sudan 7B and fluorol yellow 088, and they had a weak autofluorescence, indicating that both the aliphatic and phenolic components of suberin, respectively, were present. Staining for flavonoids with Naturstoffreagenz A, for lignin with phloroglucinol, and for tannins with DMB were all negative, indicating that the primary wall modification is the deposition of suberin. In addition, suberized walls appeared more electron dense than nonsuberized walls in adjacent tissue, but there was no indication of a suberin lamella as in suberized endodermal, bundle sheath, or cork tissues (Kolattukudy, 1984; Eastman et al., 1992; Schreiber et al., 2005). Although we found additional evidence for the presence of suberin in the walls of cells at the perforation periphery, the timing of suberin deposition does not support the hypothesis that suberization plays a causal role in the death of cells within the perforation zone (Serguéeff, 1907). Thus, it is more likely that suberin deposition plays other roles, such as forming an apoplastic barrier against the loss of organic solutes from the leaf apoplast or an antimicrobial barrier against microorganisms attracted to the nutrients that escape into the surrounding medium from cells undergoing PCD (Bernards, 2002; Razem and Bernards, 2003). In addition, wall suberization may protect against endogenous cell wall disassembly enzymes, such as pectinase, that appear to act throughout perforation formation and leaf growth.

ROS are closely associated with PCD and may both provide an inductive signal and be one of the first manifestations of PCD (Orozco-Cardenas and Ryan, 1999; Houot et al., 2001; Dat et al., 2003; Gechev and Hille, 2005). In lace plant, histochemical evidence suggests that ROS are present within the perforation site at early stage 2 and throughout the perforation zone by late stage 2. Staining is especially strong in cells at the periphery of the perforation during stage 4, the stage at which suberin deposition appears to be initiated, and is still present in stage 5 leaves, indicating that H_2O_2 may be involved in the oxidation of phenolic suberin monomers, resulting in their polymerization (Razem and Bernards, 2003; Bernards et al, 2004). Thus, ROS could have multiple roles, including PCD initiation, wall degradation, and wall suberization in perforating lace plant leaves.

Conclusions—Although perforation formation during leaf expansion in lace plant represents an unusual form of developmentally regulated PCD, the predictability of time and place of PCD and the accessibility of tissue undergoing this process makes lace plant highly suitable material for observing and experimentally manipulating PCD and its developmental regulation (Gunawardena et al., 2006). In this study we show that cell wall degradation accompanies the events of protoplast degradation, including alteration of cytoplasmic streaming and tonoplast permeability, degradation of nuclear DNA, and collapse of the protoplast (Gunawardena et al., 2004). Cell wall degradation involves erosion of the cuticle, removal of matrix polysaccharides, and some degradation of cellulose. Thus the walls are weakened sufficiently for the mechanical rupture of the perforation sites and eventual degradation of the entire cell wall. Deposition of suberin in cell walls at the border of newly formed perforations presumably provides an apoplastic barrier against nutrient loss or microbial invasion, but the timing of suberization precludes a causal role in PCD, refuting the hypothesis of 100 years ago (Serguéeff, 1907).

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