Phenotypic plasticity in *Penium margaritaceum*: Experimental manipulation of cell expansion and morphogenesis

Running Title: Phenotypic plasticity and morphogenesis of *Penium*

Josephine G. LoRicco[1], Stuart Malone[1], Abigail Becker, Nichole Xue, Kaylee Bagdan[1], Anika Eastman[1], Gabriel Sgambettera[1], Aaron Winegrad[1], Benjamin Gibeau[1], Lindsay Bauer, Ruby Epstein[1], and David S. Domozych[1]

[1]Department of Biology and the Skidmore Microscopy Imaging Center, Skidmore College, Saratoga Springs, NY, 12866, USA

Email addresses:
Josephine G. LoRicco (jloricco@skidmore.edu), Stuart Malone (smalonea@skidmore.edu), Abigail Becker (danceaby13@gmail.com), Nichole Xue (nichole.y.xue@gmail.com), Kaylee Bagdan (kbagdan1@skidmore.edu), Anika Eastman (anikaeastman@skidmore.edu), Gabriel Sgambettera (sgambettera@skidmore.edu), Aaron Winegrad (awinegra@skidmore), Benjamin Gibeau (bgibeau@skidmore.edu), Lindsay Bauer (lindsaynorabauer@gmail.com), Ruby Epstein (repstein1@skidmore.edu), and David S. Domozych (ddomoz@skidmore.edu)

Date of Submission: 13 March 2024

Number of Figures: 8
Number of Tables: 0
Word Count: 7,721

Number of Supplemental Figures: 7
Number of Supplemental Tables: 0
Number of Supplemental Videos: 7

Highlight: Experimental manipulation of the cell wall in the unicellular zygnematophyte, *Penium margaritaceum*, reveals the importance of subcellular expansion zones and cell wall integrity maintenance.

Abstract:

Phenotypic plasticity in plant cells is often expressed when cell wall integrity monitoring perceives perturbation to the cell wall and initiates multiple subcellular responses. The unicellular zygnematophyte, Penium margaritaceum in a unicellular zygnematophyte (Streptophyta), was employed to elucidate cell expansion and morphogenesis responses when cells were challenged

with the fungal-derived pectinolytic enzyme pectate lyase and microtubule disrupting agent APM. that exhibits distinct phenotypic plasticity when treated with specific stress agents. When the homogalacturonan lattice on the cell wall is removed by treatment with a fungal enzyme, pectate lyase cell size is notable reduced after several cell division cycles. Cell division in maintained but daughter cell products are notably smaller. Microtubule disruption with the agent, APM, results in significant swelling at the central expansion zone that is mostly likely due to an altered cellulosic architecture of the CW that has weakened lessened tensile resistance to turgor. When incubated in a mixture of both pectate lyase and APM, a distinct phenotype consisting of "dumbbell"-shaped cells results. These cells possess an extensive, central chloroplast-free cytoplasmic zone and swollen zone(s) at one or both cell poles result the poles. In these novel phenotypes, a septum containing ß-glycan, arabinogalactan and homogalacturonan epitopes forms grows into the cell center from the side cell wall regions via a furrowing mechanism. The surrounding cytoplasm of this zone contains displaced Golgi bodies, unique stacks of ER, displaced Golgi bodies and an extensive network of vacuoles. These results provide insight into phenotypic plasticity and cell wall integrity monitoring by *Penium* and the subcellular components that are involved in phenotypic plasticity, and their possible roles in the invasion of land leading to the evolution of land plants by zygnematophyte algae.

Keywords: Cell Monitoring, Cell Wall, Endomembrane System, Phenotypic plasticity, Pectate lyase, *Penium*, Tomography, Zygnematophyte

Abbreviations: Amiprophos methyl (APM), Confocal laser scanning microscopy (CLSM), Cell wall (CW), Cell wall integrity (CWI), Damage associated molecular pattern (DAMP), Extracellular matrix (ECM), Extracellular polymeric substances (EPS), Expansion Zone (EZ), Field Emission Scanning Electron Microscopy (FESEM), Fluorescence light microscopy (FLM), Golgi Apparatus (GA), Homogalacturonan (HG), Isthmus microtubule band (IMB), Light microscopy (LM), , Pectate lyase (PL), Preprophase band (PPB, Satellite microtubule bands (SBs), Spurrs low viscosity plastic (SLVP), Trans Golgi network (TGN), Woods Hole Medium supplemented with soil extract (WHS)

Introduction

Expansion, morphogenesis, and division (i.e., cell development) are foundational processes in the life of eukaryotic cells. For plant cells, all of these events must coordinate with the construction, maintenance, and periodic modulations of the cell wall (CW). The cell devotes a significant portion of its genetic machinery and photosynthesis-generated carbon to accomplish this (Carpita and McCann, 2015; Zhang et al., 2021). The CW is also a dynamic component of the extracellular matrix (ECM) as it constantly monitors its structural integrity (CW integrity monitoring or CWI monitoring) and modulates in response to abiotic and biotic stressors (e.g.; Bacete and Hamann, 2020; Codjoe et al., 2021). This may lead to distinct changes in cell morphology and physiology that allow for survival under stress conditions (phenotypic plasticity; Bradshaw, 2006). At the cellular and subcellular levels, plant CW and cell development requires coordinated activities of the endomembrane system and associated membrane trafficking machinery, the cytoskeletal network, the plasma membrane with its diverse assortment of regulatory and signaling components and the CW (Li et al., 2023; Quinn et al., 2023). Multiple internal (e.g. turgor pressure) and external forces (e.g. Calcium, Ca^{2+} gradients) also play key roles in these developmental events (Cosgrove, 2022; Bidhendi and Geitmann, 2019, 2018; Bascom et al., 2017).

The architectural design of the primary CW is central to expansion, morphogenesis, and cell division. The CW consists of a scaffold of load-bearing cellulose microfibrils linked together by a flexible matrix of polysaccharides and proteins (Zhang et al., 2021). The structural integrity of this polymeric composite are products of the biosynthesis and deposition/secretion of new CW polymers and their precise assemblage into a functional unit once deposited in the CW. High turgor pressure that results from the accumulation of osmotically active solutes is the cell is often

the main “deforming” force with which the CW must cope. That is, osmosis draws water into the cell, but the CW limits cell expansion, resulting in a buildup of turgor pressure and its counterforce, the tensile stress within the CW. If turgor pressure exceeds the tensile strength of the CW or regions therein, stretching of the CW occurs leading to expansion and shape changes to both the CW and the cell (i.e., morphogenesis).The biosynthesis and secretion of CW polymers require the activities of the Golgi Apparatus (GA) and inclusive Golgi bodies, vesicle-mediated transport of the Golgi cargo to the cell surface, specific interactions with plasma membrane components and membrane/cargo retrieval via endocytosis (Hoffmann et al., 2021; Zhang et al. 2021). Golgi bodies and the trans Golgi network (TGN) process (i.e., synthesize, sort and package) CW matrix polysaccharides and proteins as well as cellulose synthase or CSC complexes destined for the plasma membrane (Zhang et al., 2016). Vesicles then carry these cargoes to specific points at the cell surface where various proteins contribute to the vesicle fusion process at the plasma membrane and the release of vesicle constituents to the CW (Kim and Brandizzi, 2014, van de Meene et al., 2017; Aniento et al., 2022; Shi et al., 2023; Khoso et al., 2023). The deposition of these new CW polymers and membrane result in CW/cell expansion that occurs throughout the cell (diffuse growth), or at specific areas (polar growth) as well as the formation of the growing cell plate that yields a cross wall during cytokinesis (Cosgrove, 2022; Sinclair et al., 2022; Jawaid et al., 2022).

Transport of CW cargo to secretion zones and the regulation of CW deposition also employ the cytoskeletal network (Bashline et al., 2014; Gu and Rasmussen, 2022; Chebli et al., 2021; Zhang and Staiger, 2021). Actin microfilaments and associated proteins (e.g. myosin) serve as tracts for transporting Golgi vesicles to specific sites at the cell surface (Ito and Uemura, 2022). The synthesis of cellulose microfibrils by plasma membrane-based CSC complexes entails rapid and precise spatial positioning of cortical microtubules and microtubule linking proteins (e.g.,

kinesins; Chebli et al., 2021; Nebenführ and Dixit, 2018). Cytoskeletal components also work in concert to form the phragmoplast and to accumulate Golgi vesicles for the construction of the growing cell plate during cytokinesis (Smertenko, 2018; Smertenko et al., 2018).

Previous studies have identified specific zones where division, cell/CW expansion and cell morphogenesis take place in plant cells. These are defined by specialized zones within the cytoplasm, often in growing tips and cortical regions adjacent to the plasma membrane. They typically consist of cytoskeletal components, specific cytoplasmic proteins, plasma membrane protein and lipid constituents, ion gradients (e.g. Ca^{2+}), apoplastic reactive oxygen species (ROS) and endomembrane components (Zhang et al., 2021). Examples of these include the preprophase band or PPB (Dahiya and Bürstenbinder, 2023), the growing tips of pollen tubes (Grebnev et al., 2017), *Physcomitrium* protonemata (Ruan et al., 2023) and root hairs (Mendrinna and Persson, 2015). The activation and control of these expansion and morphogenesis zones have also played a central role in the evolution and manifestation of the great diversity of forms exhibited by plant cells tissues and organs (Buschmann, 2020; Buschmann and Zachgo, 2016).

Land plants evolved 500+ mya from a group of freshwater/subaerial green algae that were ancestors of the modern day Zygnematophyceae (Streptophyta, Virideplantae; de Vries and Archibald, 2018). Extant zygnematophytes exhibit simple phenotypes that include unicells and unbranched/branched filaments. Pioneering studies of their cell division and morphogenesis mechanisms by Jeremy Pickett-Heaps (1975), Ursula Lütz-Meindl (2016) and others described distinct and often complex subcellular zones and associated processes that were responsible for cell developmental events. This research coupled with recent molecular and high-resolution microscopy studies has demonstrated that zygnematophytes can be valuable organisms for understanding fundamentals of plant cell development and evolution (Domozych and Bagdan,

2022; Lütz-Meindl, 2016). Likewise, their simple forms and ease in experimental manipulation allow for detailed investigations of subcellular structures and functions that are often difficult to access in large plants. The unicellular zygnematophyte, *Penium margaritaceum*, provides a highly efficacious model system for understanding cell expansion, cell division, CW development and phenotypic plasticity (Palacio-Lopez et al., 2020; Domozych et al., 2021; Davis et al., 2020; Domozych, 2014) in zygnematophytes. *Penium* exhibits a simple cylindrical shape, a well-defined central expansion zone or EZ (i.e., the isthmus), a well-characterized CW (Domozych et al., 2014), distinct cytoskeletal networks (Ochs et al., 2014) and a recently sequenced genome (Jiao et al., 2020). Of particular interest in *Penium*'s cell development are microtubule bands found in the cell cortex that resemble the preprophase band (PPB) structures of most land plants (Ochs et al. 2014). As important, this alga is easily adaptable for rapid assessment of chemical and abiotic stressors on subcellular dynamics (LoRicco et al., 2023). In this study we employed the pectin-specific fungal enzyme, pectate lyase (PL) and the microtubule-disrupting agent, amiprophos methyl or APM, to investigate CW and cell morphogenetic plasticity in *Penium* in order to identify foundational controls of plant cell development in light of evolution.

Materials & Methods

Cell Culture

Penium margaritaceum was cultured in WHS medium (Woods Hole Medium supplemented with 5% soil extract (Carolina Biological Supply) using previously described methods (Rydahl et al., 2015). Live cells from 7–10 d old cultures were harvested by centrifugation at 700 g for 1 min. The cell pellets were washed three times with fresh WHS and collected by centrifugation. Cells were added to 1 mL cultures of WHS containing an appropriate concentration

of chemical agent/enzyme (Table 1) in a 12-well uncoated tissue culture Petri dish. The wells were gently mixed and the dish sealed with Parafilm. Plates were cultured under 74 µmol photons m^{-2} s^{-1} of cool white fluorescent light with a 16:8 h light–dark cycle for up to 1 week depending on the treatment.

Table 1: Treatment conditions

Treatment	Concentration	Length of Treatment
APM	2µg/mL	1-2 d
Pectate Lyase	3.8 U	4-6 d
APM & Pectate Lyase	See above	4-6 d
Vinblastine	30µM	1-2 d

Amiprophos methyl (APM) and vinblastine were purchased from Sigma Chemical (St. Louis, MO, USA). Pectate Lyase (*Aspergillus sp.*) was purchased from Megazyme in a 50% aqueous glycerol solution. Stock solutions of vinblastine (2mM in DMSO) and APM (1 mg/mL in DMSO) were made shortly before experimental application to cells.

EPS screening

To perform EPS screening 0.5 µm FITC-fluorescent microspheres (Polysciences, USA) were added to 1 mL of WHS in each well of a 12-welled uncoated petri dish (Fisher Scientific). Varying amounts of experimental agents were added to the wells and the plate was gently mixed. 20 µL aliquots containing 500-1,000 washed cells were added to each well. The plate was gently mixed and sealed with Parafilm. Plates were cultured for 24 h under 74 µmol photons m^{-2} s^{-1} of cool white fluorescent light with a 16:8-h light–dark cycle. The wells were then observed with an IX83 inverted light microscope (Olympus, USA) with fluorescence optics and FITC filter. The presence or absence of EPS trails or ensheathments were noted.

Live Cell Labeling

Fluorescent Dyes

Aliquots of treated cells were removed from the 12-well plate and added to a 1.5 mL microcentrifuge tubes. After centrifugation at 4,000 x g for 1 min, the supernatant was discarded from each tube and the cell pellets was resuspended in fresh WHS. The tubes were vigorously shaken (to remove extracellular polymeric substances or EPS) and re-centrifuged. This represented the washing protocol for cells throughout the study. Washing was repeated twice more before labeling. Imaging was attained using wide field fluorescence light microscopy (FLM; Olympus BX-60 FLM) or confocal laser scanning microscopy (CLSM: Olympus Fluoview 1200 CLSM). Specific labeling with each of the fluorescent dyes were as follows:

Calcofluor was used to label β-glucans: The cell pellet was resuspended in 1mL WHS and 1 uL of Calcofluor white (Sigma Chemical) was added to the microcentrifuge tube. Cells were then incubated for 30 min in the dark while gently rotated. Cells were washed 3x with WHS and imaged using the UV filter set.

Syto9 was used to label nucleic acids: Washed cell pellets were resuspended in 1 mL of deionized water or dH_2O, and incubated for 30 min in the dark with 2.5 μM Syto9 (Invitrogen). Cells were then washed 3x with dH_2O and imaged using FITC filter set.

MDY-64 was used to label Golgi bodies (Domozych et al., 2021): Washed cell pellets were resuspended in 1mL of dH_2O, and incubated for 30 min in the dark with 1 μM Yeast Vacuolar Membrane Marker MDY-64 (Invitrogen) and 1uL Pluronic acid (Sigma). Cells were then washed 3x with $dH_2O$and imaged using FITC filter set.

2,7 CFDA was used to detect ROS (Kim and Xue, 2020): Washed cell pellets were resuspended in 1mL of dH_2O, and incubated for 30 min in the dark with 2.5 μM 2,7 CFDA (Sigma Chemical). Cells were then washed 3x with dH_2O and imaged using FITC filter set.

Fluorescein diacetate (FDA; Santa Cruz Biotechnology) was used to stain starch (Ichikawa et al., 2024): Washed cell pellets were resuspended in 1mL of dH_2O, and incubated for 30 min in the dark with 1 μM FDA (Sigma Chemical). Cells were then washed 3x with dH_2O and imaged using FITC filter set.

Mitotracker® Orange CM-H2TMRos or CMT MRos were used to stain mitochondria: Washed cell pellets were resuspended in 1mL of dH_2O, and incubated for 30 min in the dark with 1 μM Mitotracker® (Invitrogen) and 1uL Pluronic acid (Sigma). Cells were then washed 3x with dH_2O and imaged using TRITC filter set.

Antibody labeling of CW

Washed cells were resuspended in WHS containing a 10:1 dilution primary antibody (JIM5, JIM7) in WHS. Cells were incubated for 90 min in the primary antibody and vortexed every 30 minutes. JIM5 incubation was performed in the dark on a rotating platform. JIM7 incubation was performed under 74 μmol photons m^{-2} s^{-1} of cool white fluorescent light on a rotating platform. Cells were then washed 3x with WHS and resuspended in a 1:50 dilution of secondary antibody (Anti-Rat TRITC or Anti-Rat FITC; Sigma Chemical). Cells were incubated with the secondary antibody for 90 min, in the dark on a rotating platform. Cells were vortexed every 30 min. Cells were then washed 3x with WHS and imaged using either the TRITC or FITC filter set.

To monitor cell wall expansion JIM5 labeled cells could be placed back into WHS (with or without chemical/enzymatic treatment) and placed back under the lights for 24-48h. New growth was detected by the presence of unlabeled regions of CW.

Fixed Cell Labeling

Control and treated cells were washed 3x with WHS, and treated as follows:

Rhodamine Phalloidin

The cell pellets were resuspended in WHS + 0.1 mM MBS (conc. and full name: Sigma Chemical) and incubated for 30 minutes in the dark. Cells were then washed 3X with a Phalloidin Wash solution containing 50 mM PIPES, 5 mM $MgCl_2$, 25mM KCl, 5 mM $CaCl_2$ and 5 mM EGTA (pH 6.9) and subsequently fixed in the fixative solution containing Wash + 1.9% formaldehyde (Sigma Chemical) solutions. The cells were washed 3X in the Phalloidin Wash and then incubated in a solution of 1:500 – 1:250 dilution of rhodamine-phalloidin (Abcam) in Phalloidin Wash for 90 minutes in the dark. The cells were washed 3X with Phalloidin Wash and imaged on an Olympus Fluoview 1000 confocal laser scanning microscope (CLSM).

Anti-tubulin

Washed cells were incubated for 30 min in the dark in a solution of a Microtubule Wash consisting of 20 mM PIPES, 3.5 mM $MgCl_2$ and 2mM EGTA (pH 6.9) containing fixative 4% formaldehyde and 2.7% glutaraldehyde (Electron Microscopy Sciences, EMS; Fort Washington, PA, USA). The cells were then washed 3X with the Microtubule Wash solution. Cells were then freeze shattered using the protocol of Wasteneys et al. (1997). The shattered cells were then incubated for 30 minutes in phosphate buffered saline or PBS containing 1% Triton-X. The cells were washed 3X with PBS and then incubated for 30 minutes in PBS containing 13.2 mM $NaBH_4$ (Sigma). The cells were washed 3X with PBS and incubated for 10 minutes in PBS containing 10mg/mL Driselase (Sigma). The cells were washed 3X and incubated for 10 minutes in PBS containing 35uM Trypsin (Sigma). The cells were washed 3X with PBS and then incubated for 10 min in PBS

containing 50 mM Glycine (Sigma). The cells were washed 3X with PBS and incubated overnight in a solution of 1 part Anti-Tryosine Tubulin (Sigma T-9028)/800 parts PBS (i.e., 1:800 dilution). The cells were washed 3X with PBS and then incubated for 90 minutes in a solution of 1 part Anti Mouse-TRITC (Sigma)/200 parts PBS (i.e., 1:200 dilution). The cells were washed 3X with PBS. Imaged using CLSM with a TRITC or Rhodamine filter set filter set.

Carbohydrate-binding module (CBM)-GFP

Washed cells were fixed in a solution of 0.1 % Tween-20 (Sigma) + 0.6% paraformaldehyde in WHS for 20 min. The cells were washed 3X with WHS and resuspended in a solution containing 3.8 U pectate lyase (PL; Megazyme) per mL of WHS for 1h. The cells were washed 3X in a solution of 0.1% Tween-20 in WHS. The cells were then incubated in a solution of 1 part CBM-GFP (CBM64-GFP or CBM3a-GFP; NZYtech)/100 parts WHS containing 0.1% Tween 20 for 90 minutes. The cells were washed 3X with WHS imaged using CLSM with a FITC filter set. For dual labeling with JIM5 and CBM-GFP, cells were first labeled with JIM5 (see above) and then CBM-GFP.

Cryofixation

Washed cells were spray frozen using a commercial artist's airbrush into 10 mL of liquid propane cooled to −185° C in liquid nitrogen. The frozen cells were poured into precooled (−80° C) glass scintillation vials containing 0.5 % glutaraldehyde/0.2 % tannic acid in acetone. The vials were placed in a −80° C freezer for 24 h. After 24h, 0.1 g of osmium tetroxide was added to the scintillation vial and the vial was placed back in the −80° C freezer for another 24 h. After this time, the vial was slowly warmed to room temperature over 16 h. The cells were then collected into a pellet by centrifugation at 700 x g for 1 min. The supernatant was discarded and the pellet

was washed with acetone and recentrifuged. This was repeated twice more. The cells/pellet was then infiltrated for 3 h each in cocktails of 25 % Spurrs Low Viscosity Plastic (SLVP; EMS, USA)/75 % acetone, 50 % SLVP/50 % acetone and 75 % SLVP/25 % acetone at room temperature (RT). The cells were then placed in 100 % SLVP for 2 h at RT. The cells were then pelleted into Beem capsules (EMS, USA) and polymerized at 55° C for 8 h.

TEM and immunogold labeling

For ultrastructural analysis, 70–100-nm sections were cut using a Diatome diamond knife with a LeicaUltracut microtome, collected on Formvar-coated copper grids (EMS, USA) and stained with Uranyless (EMS)/0/1% lead citrate and imaged on an Hitatchi 7800 TEM at 120 kV. For immunogold labeling, 70 nm thick sections were collected on Formvar-coated nickel grids. The protocol of Domozych et al., (2014) was followed using JIM5 (Kerafast, USA), CCRCM-80 (Complex Carbohydrate Research Center or CCRC, Georgia, USA) or 400-2 (BioSupplies, Australia). Controls included omission of the primary antibodies.

Scanning Electron Microscopy of Thin Sections

Thin sections (70nm thickness) were cut using a Diatome diamond knife with a Leica Ultracut microtome, and collected on 1 cm x 1 cm squares of silicon wafer (EMS). Sections were stained with uranyless/lead citrate (EMS), and imaged with a QUATTRO S Field Emission Scanning Electron Microscope (FESEM) using backscattered electrons and the inner ABS detector. The electron beam voltage was set to 3.5 kV and the spot size set to 2.5 (11 pA). Individual images were collected with a dwell time of 30µs, at a magnification of 8,000-12,000x depending on the sample. A grid of images was taken and assembled into an ultrastructural collage using MAPS 3.0 software (Thermofisher) such that the entire cell was captured at a resolution of ~6-8 nm/pixel.

For 3D reconstructions, grids of images were collected for cells over 50-100 serial sections using the Array tomography plug-in of the MAPS 3.0 software (Thermofisher). Reconstruction of 3D models was performed using the IMOD software (https://bio3d.colorado.edu/imod/) developed by the University of Colorado Boulder (Kremer et al., 1996). First, the images collected from each section were aligned using the "Align Serial Sections" option of the etomo graphical user interface. The images were then segmented manually using 3dmod.

Cryo-FESEM

Penium cells were spotted onto a piece of NitroBind nitrocellulose transfer membrane (Micron Separations Inc.). The nitrocellulose sheet was then plunged into liquid nitrogen to freeze the sample, and mounted onto the cryostage which had been pre-cooled in liquid nitrogen. Cells were imaged with a QUATTRO S Field Emission Scanning Electron Microscope (FESEM) in low vacuum mode. The chamber pressure was set to 50 Pa. The electron beam voltage was set to 5 kV and the spot size set to 3. Individual images were collected with a dwell time of 10 µs.

Results

Mapping the Expansion/Morphogenesis centers in Penium

Penium margaritaceum is a unicellular zygnematophyte whose phenotype is a long, narrow cylinder with rounded edges (Fig. 1A). The nucleus resides in the cell center or isthmus and is surrounded on both sides by 1-2 chloroplasts that fill most of the cell volume. The expansion zone (EZ) is found at the isthmus. *Penium* is distinguished by a unique cell wall (CW) whose outer surface is covered with projections that extend outward (Fig. 1B). This lattice-like covering

consists of Ca^{2+}-complexed homogalacturonan (HG; Domozych et al., 2014; 2021) that is readily labeled in live cells with the monoclonal antibody (mAb), JIM5 (specificity: relatively low esterified HG; Fig. 1C). The load-bearing component of the CW is cellulose, which can be visualized with carbohydrate binding module, CBM64-GFP (specificity; crystalline cellulose) (Fig. 1D) or CBM3a-GFP. A longitudinal cross section of a cell co-labeled with both CBM64-GFP and JIM5, shows that cellulose can be observed throughout the CW including in the expansion zone (EZ) (dashed line) while JIM5 is excluded from this zone (Fig. 1E). This can be clearly illustrated by plotting the intensity of CBM64-GFP (magenta) and JIM5 (green) labeling at the isthmus (dashed lines). When intensity elsewhere in the CW is compared (solid lines) the peak intensity of JIM5 (green) is found on the outer wall, whereas the CBM-GFP intensity (magenta) is reaches its maxima toward the interior of the wall.

TEM imaging shows that the cellulosic inner layer occupies the center of the EZ of the isthmus (Fig. 1F). Outside this zone, electron dense fibrils form patches on the new CW surface followed by the emergence of the HG lattice. This suggests that cellulose is an initial CW polymer deposited at the EZ and serves as a platform for the deposition and organization of the other CW layers, including the HG lattice. Likewise, this cellulosic layer, prior to the formation of the HG lattice, maintains the cylindrical shape of the EZ. During cell expansion, highly esterified HG, labeled with the mAb, JIM7 (specificity: high esterified HG), is secreted in a narrow band at the isthmus (Fig. 1G). Previous studies (Domozych et al., 2014; 2021) have shown that after secretion to the EZ, the HG is de-esterified, complexes with Ca^{2+} and forms the lattice that subsequently displaces the previously formed HG lattice outward toward the two poles. After the cell reaches a certain length, the cell divides and yields two daughter cells, followed by expansion of each daughter cell at the isthmus zone (Movie S1; see also LoRicco et al. 2023).

Within the EZ, a band of microtubules, the isthmus microtubule band (IMB), is aligned perpendicular to the longitudinal axis of the cell (Fig. 1H, I; see also Ochs et al., 2014). During pre-division phases of the cell cycle, cells also contain two satellite bands (SBs) of microtubules that are found on either side of the IMB (Fig. 1H). 10-15 microtubules are typically found in the IMB when labeled with an anti-tubulin antibody (Fig. 1I). The location of the IMB also corresponds with the center of the EZ as labeled by JIM7 (Fig. 1G) and the unlabeled-JIM5 zone (Fig. 1C). TEM imaging of cryofixed cells reveals the parallel band of microtubules of the IMB located just inside the plasma membrane of the EZ (Fig. 1J). The orientation of the IMB microtubules is the same as the alignment of the cellulose microfibrils of the innermost layer of the CW at the EZ (Fig. 1K; both are perpendicular to the plane of expansion).

When cells are labeled with the actin-binding label, Rhodamine-phalloidin, parallel bands of actin cables/bundles line the outer periphery of the cell and are positioned parallel to the long axis of the cell (Fig. 1L). These cables/bundles are located in, and most likely are a main component that is involved in cytoplasmic streaming found at the cell periphery. During cell expansion, a band of actin cables/bundles lines the center at the EZ (Fig. 1M) and is located at the same location as the IMB and secreted HG as labeled by JIM7. These observations highlight the presence of a narrow CW-cell EZ at the isthmus. Fig. 1N is an ultrastructural collage of a longitudinal view of the cell. The two semi-cells are filled each with a chloroplast and the secretory machinery for the production of new CW components. The EZ occupies a small zone in the cell center and is almost always associated with the nucleus.

Cellular responses to remove of the CW's HG lattice

The HG lattice is the most distinct component of the *Penium* CW. In order to resolve its role in maintaining CW/cell architecture, the HG was compromised (e.g. removed or significantly altered) by culturing cells in WHS containing 2.8 U of pectate lyase (PL). PL catalyzes the midchain cleavage of the α-1,4-glycosidic bonds of the pectin back bone via a β-elimination reaction to produce pectin oligosaccharides (Zheng et al., 2021). After 1-2 d of incubation, removal of some of the CW HG is evident. Cryo-FESEM images show three bands where the HG lattice is missing, including a large band at the isthmus and two smaller bands at peripheral regions (Fig. 2A). Labeling with the JIM5 antibody, confirms that the low-esterified HG epitope is missing at these three regions (Fig. 2B). TEM imaging of cells after 2 days of PL treatment shows the HG lattice "peeling" away from the inner CW layers (Fig. 2C, D).

During incubation with PL, cells continue to grow and divide. Prior to division, a slight swelling is followed by formation of an expanded cytoplasmic zone near the isthmus. Cells eventually narrow and divide leaving two slightly misshapen daughter cells (Movie S2). The average cell length after 4 days of treatment was ~20% less than control cells. The average cell length, after 8-10 d treatment with PL, cells were ~34% shorter than control cells (Fig. 2E,I). Labeling of cells with the anti-HG mAb, JIM5, after 5 d of PL treatment shows that HG is greatly reduced on the CW surface (Fig. 2F). TEM imaging also reveals the notable changes to cell shape in PL-treated cells. In zones where the HG lattice was removed, the semi-cell exhibits some swelling (Fig. 2G). Closer examination of the swollen areas shows that the HG lattice is missing, leaving just the inner CW layers and the outer adhesive fibrils (Fig. 3H).

It is also important to note that EPS production (Suppl. Fig. 1B), the positioning of the Golgi bodies in the cell (Suppl. Fig. 2D) and Golgi architecture (Suppl. Fig. 2E) were not affected by PL incubation. However, the peripheral cytoplasm of treated cells was packed with secretory

vesicles (Fig. 2G) and in some cells, stacked layers of ER tubules/sheets are observed in the cytoplasm (Suppl. Fig.3A). The mitochondria after 3d (Suppl. Fig. 6B) are notably shorter and rounder. Under the stress of PL treatment, starch is seen to accumulate within the choloroplast (Suppl. Fig. 7B). When PL is removed through extensive washing and placement of cells in fresh growth medium, the regular cell shape and size returns after several cell divisions (Suppl. Fig. 4A-D).

APM Treatment Causes Significant Swelling at the Expansion Zone

When cells are incubated in 1 µg/mL of APM (amiprophos-methyl), a phosphoric amide herbicide that disrupts microtubule dynamics in plants (Murthy et al., 1994) swelling occurs at the EZ within 24-48 h (Fig. 3A, Movie S3). When JIM5-labeled cells were treated with APM for 48h, a reduction in typical cell expansion is noted at the swollen isthmus region (Fig. 3B) and no cell division is observed. JIM5 labeling and cryo-FESEM imaging of the swollen isthmus reveals significant disruption to the HG lattice (Fig. 3C, D). JIM7 labeling of APM-treated cells shows that highly esterified HG is secreted not in a narrow band as seen in control cells but in irregular fashion upon the swollen isthmus (Fig. 3E).

TEM imaging was used to investigate ultrastructural changes at the swollen zone of the APM-treated cells. These regions had CW zones with a highly disorganized HG lattice. The swollen zones have large and branched vacuoles dispersed between the lobes of the chloroplast (Fig. 3F). High-resolution imaging of the CW formed during APM treatment imaging (Fig. 3G) reveals only remnants of the HG lattice attached to the medial and inner CW layers. An ultrastructural collage of an APM-treated cell demonstrates the changes in the EZ (compare with Fig. 1N).

411 2,7-CFDA labeling of APM-treated cells reveals the presence of ROS at the swollen zone
412 (Fig. 4C). During APM incubation, while CW expansion was curtailed, EPS secretion was not
413 inhibited (Suppl. Fig. 1D), the architecture of Golgi bodies remain intact (Suppl. Fig. 2G) but
414 Golgi bodies become scattered in the swollen zones (Suppl. Fig. 2F). Mitochondria structure did
415 not appear to be strongly impacted towards in the poles of the cell (Suppl. Fig. 6D) but exhibited
416 alterations in mitochondria structure was observed in the swollen zone with some mitochondria
417 exhibiting branching (Suppl. Fig. 6E). Treatment with APM also led to an accumulation of starch
418 within the chloroplast (Suppl. Fig. 7C). Recovery experiments show that cell division rapidly
419 occurred after removal of APM, the cylindrical shape was re-established and the HG lattice
420 reformed (Suppl. Fig. 4E-I , Movie S4).
421
422 *The Nucleus is not the Main Control or Marker for the site of Cell Expansion/Morphogenesis*
423 In this and previous studies, we have observed the nucleus in the EZ (Fig. 5A). In APM-
424 treated cells, the nucleus was also observed in the EZ which corresponds with the swollen zone
425 (Fig. 5B). To examine whether the EZ was "fixed" around the nucleus, cells were centrifuged at
426 12,000 x g for 20 minutes to displace the nucleus to one side of the cell. Cells with displaced nuclei
427 were grown for 2d in WHS and the location of the EZ assessed by either the growth of an unlabeled
428 zone in JIM5 labeled cells, or the location of newly secreted HG assessed by JIM7 labeling. The
429 location of the nucleus was not correlated with the location of CW expansion (Fig. 5C, D). We
430 also examined whether the APM-induced EZ was "fixed" around the nucleus, by centrifuging cells
431 to displace the nucleus, followed by a 2d treatment with APM. Expansion/swelling of cells
432 typically occurs at the cell center and is independent of the position of the nucleus (Fig. 5E).

We confirmed that the location of the nucleus did not "fix" the location of the EZ by treating cells with another microtubule disrupting agent, vinblastine. Treating cells with 30 μM vinblastine does not greatly disrupt cell expansion and division although slight alterations to the pectin lattice and slight swelling at the EZ is detected (Fig. 5H). Treatment of cells with Vinblastine for two days, resulted in cells with polarly localized nuclei. In cells treated with both vinblastine and cytochalasin E which has been shown previously to inhibit cytokinesis in *Penium* (Ochs et al., 2014), the nucleus is replicated but the two nuclei are not partitioned into the two pseudo-cells (Fig. 5I). The nuclei of vinblastine treated cells became displaced following cell division, however CW expansion still occurred at the at the isthmus zone of JIM5 labeled cells, irrespective of nuclear placement (Fig. 5D). Highly esterified HG is secreted at the isthmus, irrespective of the location of the nucleus (Fig. 5E).

Pectate Lyase & APM: the Cocktail of Dumbbells

The most distinct changes to cell phenotype occurred in cells incubated in both 3.8 U PL and 2 μg/mL of APM. After 4-6 days of treatment, cell swelling occurred at one or both polar zones, the latter producing a dumbbell shape (Fig. 6A). Little or no symmetry was noted in the swellings as some swellings formed at the polar ends while others occurred just interior to the poles. The HG lattice of these cells either peels away from the inner cell wall or is significantly altered (Fig. 6B-D). A distinct feature that was noted in the majority treated cells was the formation of a CW septum or apposition at the cell center which was stained with calcofluor white which recognizes β-glucans (Fig. 6E). Rhodamine phalloidin labeling highlights the actin network of treated cells except the actin-free zone observed in the isthmus surrounding the septum (Fig. 6F).

LM and TEM analyses of treated cells revealed that the chloroplasts were no longer present in the cell center and were displaced toward the polar swellings (Fig. 6E-J, K; Suppl. Fig. 5A; Movie S5). This created a clear zone whose cytoplasm contained dispersed needle-like components (Fig. 6G). This zone labeled with 2,7-CFDA as did the swelling at the polar zones indicating the presence of ROS (Fig. 4D). When treated cells were labeled with MDY-64, a live cell stain used for labeling acidic organelles including Golgi bodies in *Penium* (Domozych et al., 2021), Golgi bodies (i.e., fluorescent needle-like components) were observed in the clear zone as well as in their typical location, i.e., the valleys of cytoplasm formed by the chloroplast lobes in this cytoplasm (Fig. 6H, Suppl. Fig. 2K). The fluorescent components of the clear zone equated to the needle-like entities observed with DIC imaging (Fig. 6I). In this location the Golgi bodies were scattered and not in the typical linear orientation. The nucleus or nuclei of daughter cells remained in the cell center (Fig. 6J,K). It should also be noted that mitochondrial structure appeared to be noticeably altered with rounded and branched structures (Suppl. Fig. 6F). Occasionally clusters of mitochondria can be seen in the cytoplasmic zone (Suppl. Fig. 6G).

Treatment with PL-APM led to an accumulation of starch throughout the chloroplast (Suppl. Fig. 7G,H). Additionally, an increase in the number and decrease in the size of pyrenoids found within the chloroplast was noted. Typically, one to as many as three pyrenoids are seen in the central mass of the chloroplast, but following PL-APM treatment pyrenoid structures could also be found within the lobes of the chloroplast (Suppl. Fig. 7G-I).

Another feature of PL-APM-treated (and PL-treated cells-see above) cells is the presence of stack sheets/tubules of ER which were also found sporadically in cells treated with PL or APM alone (Suppl. Fig. 3). These stacks range in size from 0.5 to 1.5 µm and consist of up to 6-10 parallel-aligned rough ER sheets/tubules. The individual ER components are separated by 30-35

nm of cytoplasm that is free of other organelles. Only one or two of these stacks are found in swollen isthmus zone of APM-or PL-treated cells (Suppl. Fig. 3A) while as many as 10+ are found in the chloroplast-free cytoplasmic zone of the isthmus of PL-APM-treated cells. Interestingly, some of the stacks are pressed up against the septum or side CWs of PL-APM-treated cells (Suppl. Fig. 3C, D).

An ultrastructural collage of a PL-APM treated cell (Fig. 6K) highlights the unique phenotype especially in comparison to control cells, or cells treated with PL or APM alone (Figs. 1N, 2G, 3H). Serial array tomography was used to reconstruct ~1/3 the volume of the PL-APM treated cell found in figure 5K, and was able to provide additional structural information (Movie S7). Notable features compared to the control cell include: (1) the loss of the outer pectin layer (purple), (2) the formation of an ingrowth/septum at the center of the cell (blue), (3) irregularly positioned chloroplasts within the swollen regions (green) which pull away from the central cytoplasmic zone, (4) two daughter nuclei (cyan), and (5) altered mitochondria (magenta). Within the central cytoplasmic zone we also see (6) stacks of ER, and (7) a complex network of vacuoles (red).

We next focused on the formation of the septum or apposition formed during treatment. This septum sometimes forms during the later stages of mitosis (e.g. telophase, Fig. 7A). The septum initiates at the cross-wall region of the isthmus and grows inward. Septum growth may be complete or incomplete whereby cytoplasm fills the open zones where the septum growth is incomplete. During septum formation clusters of Golgi bodies and large numbers of vesicles are found in the surrounding cytoplasm (Fig. 7B). This central cytoplasmic zone (i.e., clear zone) can be large (30-40 μm in length) as it extends outward toward the poles. Vesicles fill the central part of this cytoplasm and irregular clusters of Golgi bodies are found around its edges (Fig. 7C). In

some cells, this altered cytoplasm is layered with mitochondria, Golgi body- and vacuole-rich zones surrounding the nucleus (Suppl. Fig. 5B). In the Golgi zone, sometimes small groups of Golgi bodies associated with ER appear (Suppl. Fig. 5C). Close examination of the zone found at the tip of the growing CW septum reveals branched, membrane-bound, compartments that have a fibrillar lumen (Fig. 7D;). In cells treated for longer than 6 days, large numbers of elongate vacuoles intersperse with vesicles in the cytoplasm of the cell center (Fig. 7E).

The CW septum possesses a mottled appearance (Fig. 8A). A distinct feature of the septum is a network of electron dense fibrils found in the septum interior (Fig. 8B). This is similar to the medial layer of the CW (Domozych et al., 2021). CCRC-M80 labels the interior region of the septum (Fig. 8C) that corresponds with the fibrillar network observed in Fig. 8B. This mAb also labels the interface of the septum CW and plasma membrane and the inner CW-plasma membrane interface in typical CWs (Fig. 8E). Additionally, CCRC-M80 labels the lumen of autophagous vacuoles on treated cells (Suppl. Fig. 5D). JIM5 also labeled the septum (Fig. 8D) as well as the HG lattice (Fig. 8F). No labeling was noted with the 400-2 antibody (specificity: (1→3)-β-glucan).

Discussion

This study demonstrates that *Penium* exhibits notable phenotypic plasticity when cells are challenged with two agents, PL and APM. Under normal or control conditions, *Penium*'s elongate cylindrical phenotype is maintained by a "bipolar" CW expansion mechanism that centers around a narrow 1-2 µm zone (i.e., EZ) at the cell center or isthmus. New CW polymers are deposited at this zone and form new CW that in turn, displaces pre-existing CW toward the two poles. During expansion, the cellulosic inner layer is formed first in the EZ that then is followed by the secretion/formation of the distinct HG. When a cell reaches a certain size, it divides and yields two

daughter cells (see also Domozych et al., 2014; 2009). The cortical or peripheral cytoplasm of the EZ contains both a narrow band of microtubules, the IMB, and actin cables during cell expansion. Two satellite bands (SBs) of microtubules form on either side of the IMB before cell division and translocate to the future isthmus zones of daughter cells (see also Ochs et al., 2014). The nucleus resides in the center of the isthmus and post-mitotic daughter nuclei move to the centers of the daughter cells marked by the SBs. Centripetal furrowing and a cell plate contribute to the cytokinetic separation of daughter cells (Davis et al., 2020).

The IMB: the marker of expansion/morphogenesis

The EZ/morphogenetic center of *Penium* is marked both by the IMB and a corresponding perpendicular band of actin cables. We posit that both cytoskeletal components participate in CW polymer secretion and deposition during expansion. Ultrastructural images show the same alignment of the innermost cellulose microfibrils with the cortical IMB microtubules at the EZ. These microtubules most likely serve as tracks for and/or regulate the synthesis of cellulose microfibrils at the innermost layer of the newly forming CW. This corresponds with previous studies that show a similar microfibril-microtubule orientation and describe the functional roles of these cytoskeletal components in secretion and regulation of the cellulose synthesis machinery (e.g. Cellulose synthase A or CesA; Liu et al., 2023; Chebli et al., 2021; Gu and Rasmussen, 2021; Cosgrove, 2022; Zhang et al., 2023; Zhang et al., 2016). Previous studies have also demonstrated the actin cytoskeleton and microtubules playing central roles in the delivery of matrix CW components to EZs of plant cells (Khoso et al., 2023; Hoffmann et al, 2021; Zhang et al., 2023**;** Nebenführ and Dixit, 2018; Kim and Brandizzi, 2014; van de Meene et al., 2017; Bashline et al., 2014). The IMB and associated actin cables of *Penium* most likely have a similar role in the

delivery of CW matrix components to the EZ of the isthmus. *Penium* possesses a secretory membrane trafficking network that is notably different than those described in many land plants. This alga does not contain mobile Golgi bodies that travel on the cytoskeleton, then dock onto ER and synthesize/package matrix components destined for EZs. Rather, linear arrays of stationary Golgi bodies line the cytoplasmic valleys of the cell and produce secretory vesicles containing CW cargo that enter cytoplasmic streaming channels situated in the cortical cytoplasm (Domozych et al., 2020). These vesicles are transported around the cell periphery. The IMB and associated band of actin cables mark the narrow band of cortical cytoplasm at the EZ where the CW vesicles are removed from the cytoplasmic streaming network, fuse with the plasma membrane and release their cargo to the expanding CW. The structural and functional features of this zone require future studies especially those that will focus on cytoskeletal dynamics (e.g. assembly-disassembly mechanisms of microtubule and actin cables) and vesicle movement and fusion with the plasma membrane at this secretory targeting zone.

The subcellular zones that direct CW/cell expansion, morphogenesis and division in plants are structurally diverse (Chebli et al., 2021; Cosgrove, 2022). These zones consist of complex networks of cytoskeletal components and associated regulatory proteins (e.g. microtubule-and actin-associated proteins), Golgi- and trans Golgi network (TGN)-derived vesicles and proteins specifically positioned in the plasma membrane that control vesicle fusion (Gu and Rasmussen, 2022). One of the most well-studied of these zones is the PPB. The PPB is a transient structure that marks the future plane of cytokinesis prior to mitosis and consists of a dense cortical ring of microtubules, actin filaments and associated proteins (Dahiya and Bürstenbinder, 2023). The PPB disappears before mitosis and imprints a narrow zone that marks the site of the future phragmoplast and cell plate. Similar to the PPB in plants, the IMB of *Penium* predicts the site of cytokinesis

570 (Ochs et al., 2014; Buschmann and Zachgo, 2016) in addition to its role in CW/cell expansion and
571 morphogenesis. These multi-functional roles of the IMB of *Penium* paint a picture of a simple but
572 efficacious means of streamlining the development of an organism with a simple phenotype and a
573 need for rapid growth and division in habitats where there is limited optimal time for its lifecycle
574 (i.e., ephemeral wetlands). This simplified system may reflect one example of evolutionary
575 reduction believed to have occurred in zygnematophyte evolution. Elucidation of the
576 multifunctional IMB and its integration in whole cell development and physiology will greatly
577 benefit from use of stable transformed cell lines expressing fluorescent proteins bound to specific
578 proteins of the cytoskeleton, endomembrane system and plasma membrane. These do not yet exist
579 in zygnematophytes but will be critical to deciphering the ways in which the IMB directs cell
580 development, responds to stress, contributes to phenotypic plasticity and processes large and
581 complex secretory products (e.g. CW, extracellular polymeric matrix (Domozych and LoRicco,
582 2023).

583 In this study we demonstrate the value of employing the microtubule-depolymerizing
584 agent, APM (Wymer et al., 1996) in identifying sites of cell expansion and morphogenesis. That
585 is, APM-induced swelling marks the specific cellular zones where expansion occurs. In untreated
586 cells, expansion occurs at the isthmus in order to create an expanding cylinder. When treated with
587 APM for 1-2 days, cells also expand at the isthmus but in a swelling. The disruption of
588 microtubules of the IMB at the isthmus by APM most likely alters cellulose microfibril
589 synthesis/deposition (Wymer et al., 1996). The cellulosic inner layer is the first CW component
590 formed at the isthmus EZ (Domozych et al., 2014) and is the load bearing component of the CW.
591 When compromised by APM treatment, the tensile strength of the forming CW at the EZ is
592 compromised and this weakened CW zone then yields to internal turgor pressure resulting in the

swelling of the cell at the isthmus. This APM-induced swelling may also be used to identify other EZs when cells are treated with other agents, e.g. PL and APM together (see later).

During APM treatment, HG is still secreted but no longer forms the regular lattice of the outer CW at the swollen EZ. APM-based alteration to the IMB that leads to disruption of the architecture of the inner cellulosic layer and cell swelling may not provide the proper structural platform for the secreted HG to organize into the branched lattice. For example, as the composition of the CW changes and the surface area of the CW greatly expands at the swelling, the rate of HG deposition into this CW zone may not reach the quantitative threshold that allows the typical HG lattice to form (i.e., insufficient amounts of HG to form a lattice). Our data show that the HG deposited at the swelling must still de-esterify and complex Ca^{2+} but cannot form the regular lattice architecture. During recovery, the HG lattice reforms as cell shape returns to its cylindrical form in newly expanding areas of the cell. Interpolymeric interactions of the plant CW such as pectin-cellulose linkages are central to expansion dynamics but current limitations of imaging technology do not allow for detailed examination of these interactions in live cells (Baez and Bacete, 2023). *Penium*'s unique HG lattice and its connection to the inner cellulosic layer of the CW as well as its rapid response to stress agents make this alga a potentially valuable model organism for elucidating the chemical and physical interactions between CW polymers.

The positioning of the nucleus and IMB.

In untreated cells, the location of the IMB corresponds with the placement of the nucleus. During cell division, the daughter nuclei are positioned at the SBs which ultimately become the IMBs of the newly generated daughter cells. Displacement of the nucleus in *Penium* can be achieved via centrifugation. Following this nuclear displacement, cell expansion still occurs at the

isthmus, irrespective of the nuclear location. The nucleus could also be displaced by treatment with the microtubule disrupting agent, vinblastine. Vinblastine-treated cells continued to expand and divide, unlike cells treated with APM. However, the daughter nuclei remained at the polar zones of the daughter cells rather than being relocated to the isthmus during cell division. CW expansion continued to occur at the central isthmus zone, irrespective of nuclear positioning in vinblastine-treated cells. Interestingly, the location of the nucleus did not recover within 1-2 d recovery period following displacement. In *Penium* some level of connection exists between the nucleus and the IMB/expansion zone. Aligning the nucleus with the dynamic IMB is undoubtedly necessary for competent cell division. However, the relative independence of the IMB from the nucleus suggests that this microtubular band and associated actin band are firmly fixed in the EZ, perhaps to a specific zone of the plasma membrane/CW of the isthmus. CW/cell expansion continue at the EZ even after centrifugation and not at a nuclear location.

Cell expansion after removal of the pectin in the CW

Cell development was also analyzed when CW architecture was compromised in cells incubated in PL derived from the fungus, *Aspergillus sp.*. This enzyme removes the HG lattice from the CW starting first at the isthmus and lateral growth zones and then whole cell surface. Little if any of the HG lattice remains upon the inner cellulosic layer. After several days incubation in medium with PL, cell size is notably reduced by nearly a third. During this time, the long cylindrical cell shape transforms into a small cylinder with a slightly swollen center. These smaller cells are products of several cell division cycles (i.e., cell size decreases as more cell divisions occur). Cell length is an important determinant for initiating cell division in *Penium*. Cells with a compromised HG composition likely have a smaller size threshold that signals initiation of

division. When the HG lattice reforms in the new CW during recovery periods, the normal cell size and shape return (i.e., several division cycles).

Removal of *Penium*'s HG lattice demonstrates two important points about its CW. First, the lattice is not primarily responsible for its species-specific cell shape but does have some role in maintaining shape integrity. Second, the phenotypic plasticity that results from the loss of the HG from the CW may be a product of *Penium* monitoring and maintaining CWI. Plant cells constantly monitor the integrity of their CWs and alterations to them lead to responses mediated by a CWI maintenance mechanism (Baez et al., 2022; Rui and Dinneny, 2020). Receptor-like kinases and ion channels are typically involved in sensing CWI impairment and activating responses that include hormone biosynthesis and signaling, ROS, Ca^{2+} transport and transcriptional regulation (Pontiggia et al., 2020). At present, very little is known about CWI sensing in streptophyte algae especially in response to abiotic and biotic stressors. The loss of a major CW component (HG lattice) in *Penium* may signal a cascade of events that lead to a reduction of cell size. While HG may not be the main load bearing component of the CW, it may be important in maintaining a certain cell size threshold. The loss of HG may compromise this threshold and limit cells to a reduced size. PL is a fungal enzyme that removes the HG. This change in CW composition/integrity by a biotic stressor may signal the cell to divide faster in order to survive fungal attack, i.e. a type of a damage-associated molecular pattern or DAMP immunity. DAMP has been widely observed in many plants (Pontiggia et al., 2020). Abiotic or biotic-based attack on a plant CW often releases elicitors that are perceived by plasma membrane immune receptors. These then trigger signaling cascades that activate multiple defense responses. Oligogalacturonides resulting from breakdown of pectin due to pathogen attack (e.g. or with fungal PL) or physical wounding may act as DAMPs in plant cells (Ferrari et al., 2013; Bethke at al.,

2016, Wan et al., 2021). Are oligogalacturonides released from *Penium*'s CW upon fungal PL treatment the signals for a DAMP-based response? We are only in an infancy stage in elucidating interactions of streptophyte algae with surrounding microorganisms. However, recent analyses of the genomes of streptophyte algae have identified key genes that are parts of plant-microbiome interactions including receptor-like kinases (RLKs) and nucleotide-binding domain leucine-rich repeat (NLR) proteins (Fürst-Jansen et al., 2020; Han, 2019; Jiao et al., 2020). *Penium*'s response to both PL and PL/APM treatments may serve as an impetus for future investigations of streptophyte algae, their potential DAMP immunity and its potential role in colonization of land and the evolution of land plants.

Incubation in dual agents result in a distinct phenotype

When *Penium* is treated with both PL and APM, a distinct phenotype results. Cell swelling occurs at one or both poles and the cell center is filled with cytoplasm that contains a mix of various organelles. ROS, a marker of cellular stress, is detected using 2,7 CFDA in both the swollen zones at the poles in the central cytoplasmic zone. Cell division is inhibited in these treated cells and expansion at the isthmus is much reduced. Furthermore, a CW-derived septum forms quickly (e.g. 2 d) at the center of the PL/APM-treated cell that partially or completely separates the two halves of the cell. A timelapse that highlights this event can be seen at Movie S5. The presence of swelling in polar-adjacent regions by APM suggests that the EZs are repositioned under PL/APM-induced stress most likely at the location of the SBs. When the agents are removed in recovery experiments, cell expansion and division return to the cell's central zone and after several rounds, normal cell shape returns in daughter cells.

During treatments with PL/APM, distinct stacks of ER appear in the extensive cytoplasmic zoner of the isthmus. ER is a biosynthetic center of the plant cell and modulates its structure and function during the life cycle of the cell and in response to stress (Brandizzi, 2021; Srivastava et al., 2018; Howell, 2013). Stacked sheets or tubules of ER have been described in many eukaryotes especially those involved in active protein processing/secretion (Terasaki et al., 2013). Constructed like a "parking garage", this ER architecture is thought to allow the cell to effectively pack more ER membrane into more confined spaces and consequently enhance the ER's biosynthetic machinery (e.g. more membrane-bound polysomes for protein synthesis). The structural architecture and movement of the ER network has also been shown to be regulated by the actin and microtubule cytoskeleton, specifically through actin motor proteins and membrane-cytoskeleton adaptors (Zang et al., 2021). In *Penium*, ER typically forms elongate tubules or sheets that line the valleys of cytoplasm and underlie the large network of Golgi bodies. The stacked sheets observed in cells treated with PL/APM may also be a stress-induced response to the loss of the HG in the CW whereby increased amounts of ER are needed to produce functioning CW components via the ER-Golgi-vesicle trafficking network. Likewise, it may also be that the disruption of the microtubule network by APM results in local disruption of ER architecture leading to the formation of multiple ER stacks. It should also be noted that ER stacks were also found in PL- and APM-treated cells but were fewer in number. Further work will be needed to elucidate these unusual ER stacks and the functional roles that they serve in stressed cells.

Tomographic analysis of phenotypic plasticity

The major changes to the cell can be illustrated via array tomography (Figure 6). The asymmetrically swollen or "dumbbell" shaped cell has most if not all of its HG lattice stripped

away from the CW. The central cytoplasmic zone contains tubules of ER, irregular clusters of Golgi bodies and large numbers of vesicles and branched vacuoles. Two daughter nuclei are often present but are not properly segregated to the proto-daughter cells, similar to the effect elicited by vinblastine treatment. While APM likely inhibits several sub-types of microtubules, vinblastine appears to specifically affect the population of microtubules associated with separation and partitioning of the daughter nuclei. (e.g. phragmoplasts).

The septum

The septum is an ingrowth or apposition of the CW that grows inward into the cell. Our ultrastructural imaging of PL/APM treated cells shows that it grows via vesicle fusion at the growing tip of the septum, in a manner similar to furrowing that occurs during cytokinesis. Furrowing is a common cytokinetic mechanism of many eukaryotes but the subcellular dynamics of this process in zygnematophytes remain to be resolved. In other green algae like *Chlamydomonas*, furrowing may require actin but not type-II myosin (e.g. animal cells; Pollard, 2020) and is also associated with microtubules (Onishi et al., 2020). During cell division in *Penium*, furrowing along with cell plate formation (Davis et al., 2020) contribute to the rapid separation of cytoplasmic masses, i.e., daughter cells. In PL/APM-treated cells, cell plate-like components are not found in this septum zone. Rather the furrowing-like mechanism is activated and appears to serve as a rapid default mechanism for separating two "cytoplasmic masses" at the isthmus.

Our cytochemical labeling showed that the septum contains ß-glucan (Calcofluor), arabinogalactan (CCRC M80), HG but not callose. These results indicate that the septum, while structurally different, is composed of similar constituents to those found in the CW. The formation

of these CW-derived ingrowths may be another manifestation of CWI monitoring and a DAMP-based stress response, in this case, one that responds to and enhances defense against biotic stress (i.e., exposure to fungal-derived pectate lyase).

The formation of a septum in *Penium* could be a rapid means of reinforcing the CW at the EZ under stress conditions that include diverse assaults on the CW. This would be similar to the local depositions of new CW materials (e.g. papillae or appositions) observed in plants that have been wounded or attacked by biotropic fungi (Wan et al., 2021; Chowdhury et al., 2014). These ingrowths act as physical barriers that effectively halt or impair the penetration of the fungus and/or serve as chemical barriers that accommodate a variety of defense chemicals. It is intriguing to speculate that stress which includes the digestion of *Penium*'s HG lattice by an exogenous pectinolytic enzyme (PL), and the alteration of the cellulose infrastructure (APM), may elicit the hyper-response of septum formation. As the cell senses the significant damage to its CW its CWI response mechanism activates its CW biosynthetic machinery to reinforce CW architecture at the EZ by forming a septum. Likewise, the septum may also physically define two cellular units during the stress period that could quickly develop into daughter cells upon removal of the stress agents (e.g. recovery). This possibility is also strengthened by the appearance of polar swellings that quickly form during PL/APM stress, indicating that two future EZs are in place for post-stress recovery. The evolution of CWI monitoring and DAMP-induced stress mechanisms in zygnematophytes will require further studies but both may have been key "toolbox" items available to their ancestors that were required during the colonization of land.

Production of Reactive Oxygen Species (ROS)

The production of ROS is a widely noted mechanism produced in plants when subjected to biotic or abiotic stressors (Mittler et al, 2022; Ravi et al., 2023). ROS serves as a key signaling molecule that initiates rapid responses to stress. In this study, ROS was identified by the label 2,7 CFDA (Kim and Xue, 2020). This agent labeled the altered zones of the cells including the swollen isthmus of APM- and polar zone (PL/APM) as well as the isthmus zone of PL/APM-treated cells where the septum is often found and the zone with clusters of Golgi bodies and vesicles. These observations indicate that ROS is produced in localized cellular zones where corresponding changes to cell shape and the production of the septum.

Other subcellular changes

While our focus was on changes to the ECM and endomembrane system, changes to other subcellular systems following treatment with PL, APM, or PL-APM were also detected. Previously it has been shown that mitochondria in streptophyte algae, *Micrasterias*, exhibit distinct ultrastructural changes under stress (Steiner et al., 2020, 2018). Typically, mitochondria in *Penium* are 10-20µm long, and lie at the bottom of the valley between the lobes of the chloroplast (Suppl. Fig. 6A). Treatment with PL led mitochondria to become shorter and rounder (Suppl. Fig. 6B). In the swollen region of cells treated with APM, branched mitochondria structures are seen (Suppl. Fig. 6E). Cells treated with PL-APM exhibit both round and branched structures, and the mitochondria are no long confined within the valley of the chloroplast (Suppl. Fig. 6F).

Plants often exhibit changes to starch storage in response to stress, biotic stress often resulting in starch accumulation (Ribeiro et al. 2022), and abiotic stress leading to either mobilization or accumulation starch depending on the species/stress (Thalmann and Santelia 2017). In *Penium*, starch is typically found in the center of the chloroplast, directly ensheathing

the pyrenoid (Suppl. Fig. 7A, E-F). Under PL, APM, and PL-APM stress conditions, *Penium* was seen to accumulate starch throughout the chloroplast (Suppl. Fig. 7B-D, G-H). Interestingly, in the PL-APM treated cells, it was also noted that there was an increase in number and decrease in size of pyrenoid structures found in the chloroplast. The pyrenoids were not confined to the center of the chloroplast, but were also found distributed throughout the lobes of the chloroplast. A similar pyrenoid phenotype was seen in a *Chlamydomonas reinhardtii* mutant with abnormal starch (Itakura et al., 2019).

Conclusion

Penium provides an efficacious system for studying phenotypic plasticity in plant cells. Moreover, it may offer a way to elucidate the origins of CWI monitoring and plant immunity that ultimately became key mechanisms in the plant kingdom.

Supplementary Data

Fig. S1 – Effect of PL, APM, and PL-APM treatment on EPS secretion

Fig. S2 – Effect of PL, APM, and PL-APM treatment on the Golgi Apparatus

Fig. S3 – TEM images of stacked endoplasmic reticulum tubules

Fig. S4 – Recovery of *Penium* following PL, APM, and PL-APM treatment

Fig. S5 – Additional ultrastructural changes seen following PL-APM treatment

Fig. S6 – Effect of PL, APM, and PL-APM treatment on mitochondrial structure

Fig. S7 – Effect of PL-APM treatment on starch and pyrenoid structures

Movie S1 – Timelapse imaging of CW expansion in Penium

Movie S2 – Timelapse imaging of PL treatment

Movie S3 – Timelapse imaging of APM treatment

Movie S4 – Timelapse imaging of recovery from APM treatment

Movie S5 – Timelapse imaging of PL-APM treatment

Movie S6 – Timelapse imaging of recovery from PL-APM treatment

Movie S7 – Animation of 3D model of PL-APM cell

Acknowledgements

Erin Cocks, Massimo Micaroni, Julia Fernandez-Rodriguez and the Centre for Cellular Imaging at the University of Gothenburg (Sweden) for training in serial array tomography.

Author Contributions

JGL, SM, AB, NX, KB, AE, GS, AW, BG, LB, RE, DSD planned and executed the experiments described in this study. JGL and DSD composed the manuscript that was reviewed by all authors.

Conflicts of Interest

No conflict of interest declared.

Funding Statement

This work was supported by the National Science Foundation (NSF) [MCB grant number 2129443 to DD].

Data Availability

The data used in the paper is available at the image banks of the Skidmore Microscopy Imaging Center and are available on request (DSD).

References

Aniento F, Sánchez de Medina Hernández V, Dagdas Y, Rojas-Pierce M, Russinova E. 2022. Molecular mechanisms of endomembrane trafficking in plants. Plant Cell 34, 146-173. doi: 10.1093/plcell/koab235

Baez LA, Bacete L. 2023. Cell wall dynamics: novel tools and research questions. Journal of Experimental Botany 74, 6448-6467. doi: 10.1093/jxb/erad310

Baez LA, Tichá T, Hamann T. 2022. Cell wall integrity regulation across plant species. Plant Molecular Biology 109, 483-504. doi: 10.1007/s11103-022-01284-7

Bacete L, Hamann T. 2020. The Role of Mechanoperception in Plant Cell Wall Integrity Maintenance. Plants (Basel) 9, 574. doi: 10.3390/plants9050574

Bascom CS Jr, Hepler PK, Bezanilla M. 2017. Interplay between Ions, the Cytoskeleton, and Cell Wall Properties during Tip Growth. Plant Physiology 176, 28-40. doi: 10.1104/pp.17.01466

Bashline L, Lei L, Li S, Gu Y. 2014. Cell wall, cytoskeleton, and cell expansion in higher plants. Molecular Plant 7, 586-600. doi: 10.1093/mp/ssu018

Bethke G, Thao A, Xiong G, *et al*. 2016. Pectin Biosynthesis Is Critical for Cell Wall Integrity and Immunity in Arabidopsis thaliana. The Plant Cell 28, 537-556. doi: 10.1105/tpc.15.00404

Bidhendi AJ, Geitmann A. 2019. Methods to quantify primary plant cell wall mechanics. Journal of Experimental Botany 70, 3615–3648. doi: 10.1093/jxb/erz281

Bidhendi AJ, Geitmann A. 2018. Finite Element Modeling of Shape Changes in Plant Cells. Plant Physiology 176, 41-56. doi: 10.1104/pp.17.01684

Bradshaw AD. 2006. Unravelling phenotypic plasticity -- why should we bother? New Phytologist 170, 644-648. doi: 10.1111/j.1469-8137.2006.01761.x.

Brandizzi F. 2021. Maintaining the structural and functional homeostasis of the plant endoplasmic reticulum. Developmental Cell 56, 919-932. doi: 10.1016/j.devcel.2021.02.008.

Buschmann H. 2020. Into another dimension: how streptophyte algae gained morphological complexity. Journal of Experimental Botany 71, 3279-3286. doi: 10.1093/jxb/eraa181

Buschmann H, Zachgo S. 2016. The Evolution of Cell Division: From Streptophyte Algae to Land Plants. Trends in Plant Science 21, 872-883. doi: 10.1016/j.tplants.2016.07.004

Carpita NC, McCann MC. 2015. Characterizing visible and invisible cell wall mutant phenotypes. Journal of Experimental Botany 66, 4145-4163. doi: 10.1093/jxb/erv090

Chebli Y, Bidhendi AJ, Kapoor K, Geitmann A. 2021. Cytoskeletal regulation of primary plant cell wall assembly. Current Biology 31, R681-R695. doi: 10.1016/j.cub.2021.03.092

Chowdhury J, Henderson M, Schweizer P, Burton RA, Fincher GB, Little A. 2014. Differential accumulation of callose, arabinoxylan and cellulose in nonpenetrated versus penetrated papillae on leaves of barley infected with *Blumeria graminis* f. sp. *hordei*. New Phytologist 204, 650-660. doi: 10.1111/nph.12974

Codjoe JM, Miller K, Haswell ES. 2022. Plant cell mechanobiology: Greater than the sum of its parts. Plant Cell 34,129-145. doi: 10.1093/plcell/koab230

Cosgrove DJ. 2022. Building an extensible cell wall. Plant Physiology 189, 1246-1277. doi: 10.1093/plphys/kiac184

Dahiya P, Bürstenbinder K. 2023. The making of a ring: Assembly and regulation of microtubule-associated proteins during preprophase band formation and division plane set-up. Current Opinion in Plant Biology 73, 102366. doi: 10.1016/j.pbi.2023.102366.

Davis DJ, Wang M, Sørensen I, Rose JKC, Domozych DS, Drakakaki G. 2020. Callose deposition is essential for the completion of cytokinesis in the unicellular alga *Penium margaritaceum*. Journal of Cell Science 133, jcs249599. doi: 10.1242/jcs.249599.

de Vries J, Archibald JM. 2018. Plant evolution: landmarks on the path to terrestrial life. New Phytologist 217, 1428-1434. doi: 10.1111/nph.14975

Domozych DS. 2014. Penium margaritaceum: A Unicellular Model Organism for Studying Plant Cell Wall Architecture and Dynamics. Plants (Basel) 3, 543-558. doi: 10.3390/plants3040543

Domozych DS, Bagdan K. 2022. The cell biology of charophytes: Exploring the past and models for the future. Plant Physiology 190, 1588-1608. doi: 10.1093/plphys/kiac390

Domozych DS, Kozel L, Palacio-Lopez K. 2021. The effects of osmotic stress on the cell wall-plasma membrane domains of the unicellular streptophyte, *Penium margaritaceum*. Protoplasma. 258, 1231-1249. doi: 0.1007/s00709-021-01644-y

Domozych DS, Sørensen I, Popper ZA, *et al*. 2014. Pectin metabolism and assembly in the cell wall of the charophyte green alga *Penium margaritaceum*. Plant Physiology 165, 105-118. doi: 0.1104/pp.114.236257

Domozych DS, Sørensen I, Sacks C, Brechka H, Andreas A, Fangel JU, Rose JK, Willats WG, Popper ZA. 2013. Disruption of the microtubule network alters cellulose deposition and causes major changes in pectin distribution in the cell wall of the green alga, *Penium margaritaceum*. Journal of Experimental Botany 65, 465-79. doi: 10.1093/jxb/ert390

Domozych DS, Lambiasse L, Kiemle S, Gretz MR. 2009. Cell Wall Development and Bipolar Growth in The Desmid Penium margaritaceum (Zygnematophyceae, Streptophyta). Asymmetry

In an Asymmetric World. Journal of Phycology 45, 879–893. doi: 10.1111/j.1529-8817.2009.00713.x

Ferrari S, Savatin DV, Sicilia F, Gramegna G, Cervone F, De Lorenzo G. 2013. Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. Frontiers in Plant Science 4, 49. doi: 10.3389/fpls.2013.00049

Fürst-Jansen JMR, de Vries S, de Vries J. 2020. Evo-physio: on stress responses and the earliest land plants. Journal of Experimental Botany 71, 3254-3269. doi: 10.1093/jxb/eraa007.

Grebnev G, Ntefidou M, Kost B. 2017. Secretion and Endocytosis in Pollen Tubes: Models of Tip Growth in the Spot Light. Frontiers in Plant Science 8, 154. doi: 10.3389/fpls.2017.00154.

Gu Y, Rasmussen CG. 2022. Cell biology of primary cell wall synthesis in plants. The Plant Cell 34, 103-128. doi: 10.1093/plcell/koab249

Han GZ. 2019. Origin and evolution of the plant immune system. New Phytologist 222, 70-83. doi: 10.1111/nph.15596

Hoffmann N, King S, Samuels AL, McFarlane HE. 2021. Subcellular coordination of plant cell wall synthesis. Developmental Cell 56, 933-948. doi: 10.1016/j.devcel.2021.03.004

Howell SH. 2013. Endoplasmic reticulum stress responses in plants. Annual Review of Plant Biology 64, 477-499. doi: 10.1146/annurev-arplant-050312-120053.

Ichikawa S, Sakata M, Oba T, Kodama Y. 2024. Plant Physiology 194, 662-672. doi: 10.1093/plphys/kiad528

Itakura AK, Chan KX, Atkinson N, Jonikas MC. 2019. Proceedings of the National Academy of Sciences 116, 18445-18454. doi: 10.1073/pnas.190458711

Ito Y, Uemura T. 2022. Super resolution live imaging: The key for unveiling the true dynamics of membrane traffic around the Golgi apparatus in plant cells. Frontiers in Plant Science 13, 1100757. doi: 10.3389/fpls.2022.1100757

Jiao C, Sørensen I, Sun X, *et al*. 2020. The *Penium margaritaceum* Genome: Hallmarks of the Origins of Land Plants. Cell 181, 1097-1111. doi: 10.1016/j.cell.2020.04.019

Jawaid MZ, Sinclair R, Bulone V, Cox DL, Drakakaki G. 2022. A biophysical model for plant cell plate maturation based on the contribution of a spreading force. Plant Physiology 188, 795-806. doi: 10.1093/plphys/kiab552

Khoso MA, Zhang H, Khoso MH, *et al*. 2023. Synergism of vesicle trafficking and cytoskeleton during regulation of plant growth and development: A mechanistic outlook. Heliyon 9, e21976. doi: 10.1016/j.heliyon.2023.e21976.

Kim H, Xue X. 2020. Detection of Total Reactive Oxygen Species in Adherent Cells by 2',7'-Dichlorodihydrofluorescein Diacetate Staining. Journal of Visualized Experiments 160, 60682. doi: 10.3791/60682.

Kim SJ, Brandizzi F. 2014. The plant secretory pathway: an essential factory for building the plant cell wall. Plant and Cell Physiology 55, 687-93. doi: 10.1093/pcp/pct197

Kremer JR, Mastronarde DN and McIntosh JR. 1996. Computer visualization of three-dimensional image data using IMOD. Journal of Structural Biology 116, 71-76. doi: 10.1006/jsbi.1996.0013

Li R, Zhao R, Yang M, Zhang X, Lin J. 2023. Membrane microdomains: Structural and signaling platforms for establishing membrane polarity. Plant Physiology 193, 2260-2277. doi: 10.1093/plphys/kiad444

Liu L, Wang T, Bai Y, Yan P, Dai L, Du P, Persson S, Zhang Y. 2023. Actomyosin and CSI1/POM2 cooperate to deliver cellulose synthase from Golgi to cortical microtubules in Arabidopsis. Nature Communications 14, 7442. doi: 10.1038/s41467-023-43325-9.

LoRicco JG, Kozel L, Bagdan K, Epstein R, Domozych DS. 2023. Endosidin 5 disruption of the Golgi apparatus and extracellular matrix secretion in the unicellular charophyte Penium margaritaceum. Annals of Botany 131, 967-983. doi: 10.1093/aob/mcad054

Lütz-Meindl U. 2016. *Micrasterias* as a model system in plant cell biology. Frontiers in Plant Science 7, 999. doi: 10.3389/fpls.2016.00999

Mendrinna A, Persson S. 2015. Root hair growth: it's a one way street. F1000Prime Reports 7, 23. doi: 10.12703/P7-23.

Mittler R, Zandalinas SI, Fichman Y, Van Breusegem F. 2022. Reactive oxygen species signalling in plant stress responses. Nature Reviews Molecular Cell Biology 23, 663-679. doi: 10.1038/s41580-022-00499-2

Nebenführ A, Dixit R. 2018. Kinesins and Myosins: Molecular Motors that Coordinate Cellular Functions in Plants. Annual Review of Plant Biology 69, 329-361. doi: 10.1146/annurev-arplant-042817-040024

Ochs J, LaRue T, Tinaz B, Yongue C, Domozych DS. 2014. The cortical cytoskeletal network and cell-wall dynamics in the unicellular charophycean green alga Penium margaritaceum. Annals of Botany 114,1237-49. doi: 10.1093/aob/mcu013

Onishi M, Umen JG, Cross FR, Pringle JR. 2020. Cleavage-furrow formation without F-actin in *Chlamydomonas*. Proceedings of the National Academy of Sciences USA 117, 18511-18520. doi: 10.1073/pnas.192033711

Palacio-Lopez K, Sun L, Reed R, Kang E, Sørensen I, Rose JKC, Domozych DS. 2020. Experimental Manipulation of Pectin Architecture in the Cell Wall of the Unicellular Charophyte, *Penium margaritaceum*. Frontiers in Plant Science 11, 1032. doi: 10.3389/fpls.2020.01032.

Pickett-Heaps JD. 1975. Green Algae: Structure, Reproduction and Evolution in Selected Genera.Sinauer Associates, Inc., Sunderland, MA

Pollard TD. 2020. Myosins in Cytokinesis. Advances in Experimental Medicine and Biology 1239, 233-244. doi: 10.1007/978-3-030-38062-5_11

Pontiggia D, Benedetti M, Costantini S, De Lorenzo G, Cervone F. 2020. Dampening the DAMPs: How Plants Maintain the Homeostasis of Cell Wall Molecular Patterns and Avoid Hyper-Immunity. Frontiers in Plant Science 11, 613259. doi: 10.3389/fpls.2020.613259

Quinn O, Kumar M, Turner S. (2023) The role of lipid-modified proteins in cell wall synthesis and signaling. Plant Physiology 194, 51-66. doi: 10.1093/plphys/kiad491

Ravi B, Foyer CH, Pandey GK. 2023. The integration of reactive oxygen species (ROS) and calcium signalling in abiotic stress responses. Plant Cell and Environment 46, 1985-2006. doi: 10.1111/pce.14596

Ribeiro C, Stitt M, Hotta CT. 2022. How Stress Affects Your Budget – Stress Impacts on Starch Metabolism. Frontiers in Plant Science 13, 774060. doi:10.3389/fpls.2022.774060

Rui Y, Dinneny JR. 2019. A wall with integrity: surveillance and maintenance of the plant cell wall under stress. New Phytologist 225, 1428-1439. doi: 10.1111/nph.16166

Rydhal MG, Fangel JU, Mikkelsen MD, Johansen IE, Andreas A, Harhold J, Ulvskov P, Jørgensen B, Domozych DS, Willats WGT. 2015. Penium margaritaceum as a model organism for cell wall analysis of expanding plant cells. Methods in Molecular Biology 1242, 1-21. doi: 10.1007/978-1-4939-1902-4_1

Steiner P, Buchner O, Andosch A, Wanner G, Neuner G, Lütz-Meindl U. 2020. Fusion of Mitochondria to 3-D Networks, Autophagy and Increased Organelle Contacts are Important Subcellular Hallmarks during Cold Stress in Plants. Internation Journal of Molecular Sciences 21: 8753. doi: 10.3390/ijms21228753

Steiner P, Luckner M, Kerschbaum H, Wanner G, Lütz-Meindl U. 2018. Ionic stress induces fusion of mitochondria to 3-D networks: An electron tomography study. Journal of Structural Biology 204, 52-63. doi: 10.1016/j.jsb.2018.06.010

Ruan J, Lai L, Ou H, Yi P. 2023. Two subtypes of GTPase-activating proteins coordinate tip growth and cell size regulation in Physcomitrium patens. Nature Communication 14, 7084. doi: 10.1038/s41467-023-42879-y

Shi Y, Luo C, Xiang Y, Qian D. 2023. Rab GTPases, tethers, and SNAREs work together to regulate *Arabidopsis* cell plate formation. Frontiers in Plant Science 14, 1120841. doi: 10.3389/fpls.2023.1120841.

Sinclair R, Hsu G, Davis D, Chang M, Rosquete M, Iwasa JH, Drakakaki G. 2022. Plant cytokinesis and the construction of new cell wall. FEBS Letters 596, 2243-2255. doi: 10.1002/1873-3468.14426

Smertenko A. 2018. Phragmoplast expansion: the four-stroke engine that powers plant cytokinesis. Current Opinion in Plant Biology 46, 130-137. doi: 10.1016/j.pbi.2018.07.011

Smertenko A, Hewitt SL, Jacques CN, *et al*. 2018. Phragmoplast microtubule dynamics - a game of zones. Journal of Cell Science 131, jcs203331. doi: 10.1242/jcs.203331.

Srivastava R, Li Z, Russo G, Tang J, *et al*. 2018. Response to Persistent ER Stress in Plants: A Multiphasic Process That Transitions Cells from Prosurvival Activities to Cell Death. The Plant Cell 30, 1220-1242. doi: 10.1105/tpc.18.00153

Thalmann M, Santelia D. 2017. Starch as a determinant of plant fitness under abiotic stress. New Phytologist 214, 943-951. doi:10.1111/nph.14491

Terasaki M, Shemesh T, Kasthuri N, *et al*. 2013. Stacked endoplasmic reticulum sheets are connected by helicoidal membrane motifs. Cell 154, 285-296. doi: 10.1016/j.cell.2013.06.031.

Wan J, He M, Hou Q, Zou L, Yang Y, Wei Y, Chen X. 2021. Cell wall associated immunity in plants. Stress Biology 1, 3. doi: 10.1007/s44154-021-00003-4.

Wasteneys GO, Willingale-Theune J, Menzel D. 1997. Freeze shattering: a simple and effective method for permeabilizing higher plant cell walls. Journal of Microscopy 188, 51–61. doi: 10.1046/j.1365-2818.1977.2390796.x

Wymer CL, Fisher DD, Moore RC, Cyr RJ. 1996. Elucidating the mechanism of cortical microtubule reorientation in plant cells. Cell Motility and the Cytoskeleton 35, 162-173. doi: 10.1002/(SICI)1097-0169(1996)35:2<162::AID-CM8>3.0.CO;2-C

van de Meene AM, Doblin MS, Bacic A. 2017.The plant secretory pathway seen through the lens of the cell wall. Protoplasma 254, 75-94. doi: 10.1007/s00709-016-0952-4

Zang J, Kriechbaumer V, Wang P. 2021. Plant cytoskeletons and the endoplasmic reticulum network organization. Journal of Plant Physiology 264, 153473. doi: 10.1016/j.jplph.2021.153473.

Zhang B, Gao Y, Zhang L, Zhou Y. 2021. The plant cell wall: Biosynthesis, construction, and functions. Journal of Integrative Plant Biology 63, 251-272. doi: 10.1111/jipb.13055.

Zhang R, Xu Y, Yi R, Shen J, Huang S. 2023. Actin cytoskeleton in the control of vesicle transport, cytoplasmic organization, and pollen tube tip growth. Plant Physiology 3, 9-25. doi: 10.1093/plphys/kiad203

Zhang W, Staiger CJ. 2021. Revising the Role of Cortical Cytoskeleton during Secretion: Actin and Myosin XI Function in Vesicle Tethering. International Journal of Molecular Science 23, 317. doi: 10.3390/ijms23010317.

Zhang Y, Nikolovski N, Sorieul M, *et al*. 2016. Golgi-localized STELLO proteins regulate the assembly and trafficking of cellulose synthase complexes in Arabidopsis. Nature Communications 7, 11656. doi: 10.1038/ncomms11656

Zheng L, Guo Z, Cao S, Zhu B. 2021. Elucidating the degradation pattern of a new cold-tolerant pectate lyase used for efficient preparation of pectin oligosaccharides. Bioresources and Bioprocessing 8, 121. doi: 10.1186/s40643-021-00475-2

Figure Legends

Fig. 1: *Penium* cell wall (CW) and Expansion Zone (EZ). A: *Penium* exhibits an elongate cylindrical phenotype. 2-4 chloroplasts (Cp) fill the opposite polar regions of the cell (e.g. semi-cells) and surround the central isthmus zone that contains the nucleus (N). Expansion of the cell occurs at the expansion zone (EZ) which is highlighted in brackets. B: Cryo-FESEM image of the CW of *Penium* which exhibits a distinct, lattice-like appearance. A narrow, lattice-free zone can be seen at the isthmus (white arrow), as well as two peripheral "edges" (black arrows). C: JIM5 (sp.: low esterified HG) labels the HG lattice of the outer layer of the CW except for a thin unlabeled band at the isthmus (white arrows). Two peripheral "edges" can also be seen (black arrows). D: CBM64-GFP (carbohydrate-binding module 64) labeling of cellulose shows labeling over the entire cell surface, including at the isthmus (white arrow). Two peripheral bands (black arrows) of lower intensity at the regions corresponding to the peripheral "edges" seen in the pectin lattice. E: Comparison of JIM5 and CBM64-GFP labeling through a single CLSM slice. At the isthmus zone (dashed line) we see CBM64-GFP (magenta), but no JIM5 (green). At regions outside the isthmus (solid line), JIM5 (green) can be seen to label the outer edge of the CW, while CBM-GFP (magenta) is found in the inner layer of the CW. F: INSERT G: JIM7 (sp.: high methyl esterified HG) labels the newly secreted HG at the EZ (white arrow). As the CW/cell expands outward, it displaces older CW toward the poles. H: Labeling of microtubule bands with anti-tubulin antibody. The isthmus microtubule band (IMB) is found at the center of the cell (arrow), and is surrounding by two satellite bands (SBs) of microtubules. The location of the SBs depends on the growth stage of the cell. I: Magnified view of the isthmus microtubule band (IMB) found within the EZ labeled with anti-tubulin (arrow). J: TEM micrograph showing the IMB (black arrows) within the EZ (white arrow). K: TEM micrograph showing IMB microtubules (white arrows) parallel to the inner layer of cellulose microfibrils (black arrow). L: Rhodamine phalloidin labeling reveals parallel bundles of microfilaments at the cell periphery, i.e., the site of cytoplasmic streaming. M: The actin microfilament band located at the isthmus (arrows). N: Ultrastructural collage of untreated *Penium* cell.

Fig. 2: Effects of pectate lyase (PL) incubation. A: After 1 d incubation with PL, degradation of the pectin lattice can be seen most noticeably at the isthmus (white arrow) and two satellite bands (black arrows) with Cryo-FESEM. B: After 1d treatment with PL, the isthmus (white arrow) and two satellite bands (black arrows) are seen to be unlabeled with JIM5, as well as weakened labeling across the entire pectin lattice. C: Ultrastructure of the CW of cell incubated for 5 days in PL. Note the pre-existing HG lattice (white arrow) removed (black arrow) from the inner CW layers (IL). D: JIM5 immunogold labeling of the CW after 2 days incubation in PL. Note the outer HG lattice is removed from the inner wall zone. Labeling is primarily found in the HG lattice (black arrows) with some residual labeling in the inner layers (white arrow). E: After 5 days in PL, cells are smaller with a slightly swollen isthmus that tapers toward the poles. DIC image. F: JIM5 labeling after 5d treatment in PL. Note that very little labeling (arrows) remains on the CW surface. CLSM image. G: Ultrastructural collage of cell treated for 1 week with PL. Swelling is seen near the isthmus (black arrows). Some regions of the CW are completely devoid of pectin (white arrow). H: Ultrastructure of PL treated cell wall lacking pectin. CW consists of the inner cell wall layer (IL) and an outer layer of arabinogalactan-like fibrils (AL). I: Cell length histogram of cells treated with PL for up to 10 d.

Fig. 3: Effects of APM treatment. A: A large swelling (arrow) forms at the EZ at the isthmus after incubation for 24-48 h in 1 µg/mL APM. DIC image. B: A JIM5 labeled cell 48h after treatment with APM. The HG lattice (black arrows) is labeled on the CW surface of most the cell except for the central part of the swollen zone (white arrow) where expansion occurred. Only a limited amount of CW expansion is observed compared to control cells. C: JIM5 labeling of the swollen region of APM treated cells shows a disruption in the pectin lattice. D: The disruption in the pectin lattice in the swollen region of APM treated cell is also apparent with Cryo-FESEM. E: JIM7 labeling does not form the typical narrow band at the cell center but covers the swelling irregularly (arrow). F: TEM image of the swollen zone of an APM-treated cell. Note the disruption to the HG lattice (white arrow) at the swelling. The cytoplasm contains the lobed chloroplast (Cp) and many branched vacuoles (V). G: The CW of the swollen zone containing the inner (IL) and medial (ML) layers but the outer (OL) HG-lattice layer is reduced to fibrillar aggregates. TEM image. H: Ultrastructural collage of APM treated cell.

Fig. 4: 2,7-CFDA labeling of ROS production. Labeling was absent in controls cells (A) and PL-treated cells (B). C: The cytoplasm of the swollen isthmus of APM treated cells labels (arrow; 48h) while polar zones do not. D: The cytoplasm of the swollen polar zone and the central isthmus area label upon treatment with PL/APM (arrows; 4 days). All images are FLM.

Fig. 5: The nucleus and CW expansion. A: A JIM5 labeled cell which was allowed to expand for 1d. The nucleus was stained with Syto9 (white arrow) and can be found within the EZ (black arrows). B: A JIM5 labeled cell treated for 2d with APM. The nucleus was stained with Syto9 (white arrow) and is located in the swollen EZ (black arrows). C: JIM5 labeled cells were centrifuged at 12,000 x g for 20 min and then grown for 2d under the lights, followed by staining of the nucleus with Syto9. The nucleus was displaced to one side of the cell (white arrow) but expansion occurred at the isthmus (black arrow). D: Centrifuged cell allowed to recovery for 2d, followed by staining with JIM7 and Syto9. The nucleus was displaced to one side of the cell (white arrow) but high esterified HG was secreted at the isthmus (black arrow). E: JIM5 labeled cell centrifuged at 12,000 x g for 20 min, followed by 2d treatment with APM. The nucleus was stained with Syto9, and can be seen at the pole of the cell (white arrow). Swelling occured at the isthmus (black arrows). F: JIM5 labeled cell after 2d treatment with 30µM vinblastine. Expansion following cell division (black arrows) occurred at the central isthmus zone, despite the polar localization of the nucleus (white arrow). CLSM image. G: JIM7 labeling following 24h treatment with vinblastine indicates that newly secreted HG forms a narrow band at the cell isthmus (black arrow) despite the polar localization of the nucleus (black arrow). CLSM image. H: JIM5 labeled cell following 1d treatment with vinblastine shows a small amount of swelling and disruption of the pectin lattice at the pole near where cell division occurred. I: JIM5 labeled cell treated for 2d with vinblastine and cytochalasin E. The nucleus is replicated but the daughter nuclei are not separated into the two proto-daughter cells.

Fig. 6: Morphogenetic changes in cells treated with pectate lyase and APM. A: DIC image of a "dumbbell"-shaped phenotype formed after 4 days incubation. Two swollen zones (black arrows) are found at the polar zones but no swelling occurs at the cell center (white arrow) DIC image. B: FESEM image of the "dumbbell" phenotype showing the two polar swollen zones (black arrows) with no swelling in the cell center (white arrow). C: JIM5 labeling of a cell treated for 4 days. Note the alteration/removal of the HG lattice (black arrows). In this phenotype, swelling occurred asymmetrically at between the two poles. CLSM image. D: JIM5 (green) labeled cell treated for 5 d with PL-APM, followed by labeling with CBM64-GFP (magenta). Outer HG layer of cell wall (green) can be seen peeling away from inner cellulosic layer of the CW (magenta). The loss is most apparent at the swollen polar regions (white arrows). E: Calcofluor white labeling of the isthmus reveals the CW septum or ingrowth contains β-glucans (arrows). CLSM image. F: Rhodamine-phalloidin labeling shows the presence of an actin-depleted zone in the region surrounding the septum. G: DIC image of the cell center of a cell treated for 4 days. "Clear" zones of cytoplasm devoid of chloroplasts (Cp) are found here. Multiple needle-like components (black arrows) are found in this zone that is positioned next to the CW septum (white arrow). H: MDY-64 labeling of the cell center of a treated cell. Golgi bodies are found both in the valleys of cytoplasm (black arrow) between the lobes of the chloroplast and in the clear zone found in the cell center (white arrow). CLSM image. I: Magnified view of the dispersed Golgi bodies (black arrows) in the clear zone. The CW septum is also observed (black arrow). CLSM image. J: Syto9 labeling of the post-mitotic nuclei (black arrows) positioned at the isthmus in a cell treated for 4 days. The location of the septum is noted with the white arrow. CLSM image. K: Ultrastructural collage of a PL-APM treated cell which exhibited the "dumbbell" phenotype. Note the two swollen zones (black arrows) at the poles, the CW septum (white arrow, the nucleus (N) and clear zone (*) of cytoplasm.

Fig. 7: The CW septum and surrounding areas. A: After 3 days of treatment and during the latter stages of telophase, a CW septum (large black arrow) forms between daughter nuclei (N). Clusters of Golgi bodies (narrow black arrows) also appear near the nuclei. B: Magnified view of the isthmus after 4 days of treatment showing a Golgi body cluster (white arrows), the large number of vesicles (small black arrow) and the CW septum (large black arrows). C: The cell center of a cell treated for 4 days. Note the large clear zone (multi-arrow) that is filled with vesicles and the nucleus (N). A cluster of Golgi bodies (white arrows) is also located in this zone. A small CW septum (black arrows) is also apparent. D: Magnified view of the CW septum (black arrow) in a cell treated for 4 days. The zone situated at the growing tip and around the septum contain small branched compartments (white arrows). E: Cytoplasm of the isthmus of a cell treated for 6 days. Note that the cytoplasm (*) between the CW septum (black arrow) and nucleus (N) is filled both with vesicles and large clear vacuoles (V).

Fig. 8: CW septum- immunogold labeling. A: The CW septum (black arrow) in cell treated for 4 days. Note that the septum contains heterogenous components (i.e. mottled). The HG lattice is also reduced to irregulate aggregates (white arrow) in the zone outside the septum. B: Magnified view of the CW septum. Note the network of dark fibrils (black arrow) found in the center of the septum. The HG lattice is also no longer present (white arrow) on the outside of the septum. C: CCRC-M80 labeling of the CW septum. Labeling is highest at the interior dark zones of septum (white arrows) and at the interface of the septum and plasma membrane (dark arrows). D: JIM5 labeling of the septum. E: CCRC-M80 labeling of the plasma membrane and inner layer of the CW. F: JIM5 labeling of the outer CW layer (OL) of a control cell.