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*

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Abstract

Application of the dintroaniline compound, oryzalin, which inhibits microtubule formation, to the unicellular green alga *Penium margaritaceum* caused major perturbations to its cell morphology, such as swelling at the wall expansion zone in the central isthmus region. Cell wall structure was also notably altered, including a thinning of the inner cellulosic wall layer and a major disruption of the homogalacturonan (HG)-rich outer wall layer lattice. Polysaccharide microarray analysis indicated that the oryzalin treatment resulted in an increase in HG abundance in treated cells but a decrease in other cell wall components, specifically the pectin rhamnogalacturonan I (RG-I) and arabinogalactan proteins (AGPs). The ring of microtubules that characterizes the cortical area of the cell isthmus zone was significantly disrupted by oryzalin, as was the extensive peripheral network of actin microfilaments. It is proposed that the disruption of the microtubule network altered cellulose production, the main load-bearing component of the cell wall, which in turn affected the incorporation of HG in the two outer wall layers, suggesting coordinated mechanisms of wall polymer deposition.

Key words: Cellulose, homogalacturonan, microtubule, oryzalin, pectin, *Penium*.

Introduction

Plant cell walls are composites of polymers that are assembled and organized into intricate structures that surround the protoplast, where they serve multiple roles including defence, turgor resistance and controlled cell growth, water and mineral uptake, and communication (Baskin et al., 2004; Cosgrove, 2005; Sarkar et al., 2009; Keegstra, 2010; Fry, 2011). Cell wall architecture is highly dynamic, and synthesis, assembly, and any subsequent remodelling require precisely coordinated interactions between the cell endomembrane system, cytoskeletal network, plasma membrane, and multiple cross-talking signal transduction pathways. Cell wall production and maintenance therefore involve not just a substantial amount of the total photosynthate, but also a major portion of the genetic repertoire (Popper et al., 2011).

The structural and developmental characteristics and functional competency of the plant wall are also fundamentally...
affected by complex multipolymeric associations. The nature of these interactions, especially during development and in response to environmental stresses, is poorly understood and only recently has this been the focal point of detailed study. For example, cellulose microfibrils are generally described as being tethered by xyloglucan and other hemicellulosic (cross-linking glycan) polymers, and these have been proposed to influence microfibril slippage during wall and cell expansion (Popper and Fry, 2005, 2008; Fry, 2011); the nature, extent, and significance of this cross-linking have recently been discussed (Cosgrove and Jarvis, 2012; Park and Cosgrove, 2012). There is also recent evidence that the neutral sugar side chains (e.g. arabinans and galactans) of the pectin class rhamnogalacturonan-I (RG-I) may be directly bound to cellulose (Zykwnska et al., 2005, 2007). Yoneda et al. (2010) further suggested that pectin cross-bridges support and maintain the direction of cellulose microfibril orientation and slippage during cell expansion. However, there are doubtless many other interpolymeric associations that are critical for wall architecture and function, but that have yet to be recognized and characterized.

Evaluating such interactions within the context of multicellular plants is very challenging, and the extraction of cell wall polymeric complexes inevitably disrupts or abolishes a number of the molecular associations. Moreover, the physical restriction of specific polymer probes in dense tissues and the inability to use live material in many labelling and analytical protocols effectively further limit dissection of interpolymeric interactions. In contrast, the identification and use of a unicellular plant system, particularly one with clearly defined cell wall polymeric domains, would significantly enhance such studies.

A unicellular taxon of the Charophycean green algae (CGA or Streptophyta; i.e. the group of green algae most closely related to land plants; Lewis and McCourt, 2004; Wodniok et al., 2011), Penium maragaritaceum, has a number of characteristics that suggest it would provide a potentially valuable model system for the study of cell wall development, including interpolymeric associations. First, Penium only produces a permanent primary cell wall, comprising two prominent polymeric domains that are easily identified by microscopy: a pectin domain primarily consisting of homogalacturonan (HG) organized into a lattice-like network in the outer layer of the wall; and an inner domain consisting mostly of cellulose, together with smaller amounts of other glycan classes (Sorensen et al., 2010, 2011; Domozych et al., 2011). Secondly, the focal point of HG secretion, which in Penium appears to drive cell wall growth and cell development, is a clearly defined narrow band located at the cell centre or isthmus, or the isthmus band (Domozych et al., 2009b). This facilitates visualization of wall polymer secretion in a spatially well-defined area. Thirdly, Penium can be grown in large, fast-growing cultures, enabling extraction of substantial amounts of cell wall material for biochemical and immuno-based screening (Møller et al., 2007; Sorensen and Willats, 2011). Fourthly, wall polymer dynamics can be conveniently monitored by live cell labelling utilizing probes such as monoclonal antibodies (mAbs) directed against higher plant wall polymers or carbohydrate-binding modules (CBMs; Domozych et al., 2011). Finally, the cell cultures can be readily treated with agents that promote or disrupt cellular processes, including enzymes and pharmacological inhibitors, at precise concentrations and over controlled time periods.

In this study, the structural and developmental dynamics of the pectin and cellulose domains during Penium cell wall expansion and cell morphogenesis following treatment with the dinitroanilone herbicide, oryzalin, were analysed. This compound blocks microtubule polymerization and consequently inhibits cell wall development and anisotropic growth (Hugdhall and Morejohn, 1993). A combination of high resolution microscopy, polysaccharide microarray analysis, and experimental manipulation was used to study oryzalin-induced changes to the cell wall. Distinct effects of oryzalin on the pectin and cellulose domains of the cell wall and concurrent alterations to the cytoskeletal system are described, and the implications of the results for the control and coordination of cell wall disassembly are discussed.

Materials and methods

General

Penium maragaritaceum (‘Skd-8’ clone, Skidmore College Algal Culture Collection) was grown in liquid Woods Hole medium (WHM; Domozych et al., 2007) under the following conditions: 5400 lux of cool white fluorescent light, 15±1°C; 16 h light/8 h dark photocyte. Subcultures were made every 2 weeks and cells used for experiments were collected after 5–7 d in culture. Cells were harvested and washed as previously described (Domozych et al., 2007).

Oryzalin was obtained from AccuStandard (New Haven, CT, USA) and the final concentration chosen for experimental procedures was 280 nM as this concentration provided the most evident phenotypes. Specific experiments were conducted in 5 ml aliquots of culture medium, each containing 500 cells ml⁻¹. After the addition of oryzalin (from a stock solubilized in methanol), the cells were cultured under the conditions described above. Control experiments included growing cells in 0.01% methanol. Reversibility experiments entailed harvesting oryzalin-treated cells at various time intervals, washing five times in fresh WHM, and culturing in fresh WHM. Washed cells were then monitored via microscopy over the next 72 h. Total reversibility of effects could be visualized in cells incubated in oryzalin for ≤96 h. Cells were also treated with isoxaben (10 µM) or dichloronitrobenzene (DCB; 0.2 µM; Sigma Chemical, St Louis, MO, USA) and monitored after 24 h.

Live cell labelling

Treated and untreated cells were harvested, washed with WHM, and labelled with the following mAbs, as previously described (Domozych et al., 2007): JIM5 [specificity for HG with a relatively low degree of esterification (DE); Clausen et al., 2003]; JIM7 (specificity for relatively high DE HG; Clausen et al., 2003), and INRA-RU2 [specificity for (1→2)-α-L-rhamnose (Rha) (1→4)-α-D-galacturonic acid (GaIA) p-(1→7) with at least two Rha-GaIA acid repeats; Ralet et al., 2010]. All primary antibodies were obtained from Plant Probes (Leeds, UK), with the exception of INRA-RU2, which was a generous gift from Dr M.-C. Ralet (INRA Nantes, France). Secondary antibodies for immunofluorescence studies included anti-rat or anti-mouse antibodies conjugated with tetramethylrhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) (Sigma). For labelling with CBM3a (specificity for crystalline cellulose), the protocols recommended by the supplier (Plant Probes; also see Blake et al., 2006) were employed with the modification that WHM was used as the labelling buffer. Labelled cells were either viewed via light microscopy (LM) or washed with WHM and placed back into culture. Aliquots of cells were subsequently removed at...
Microtubule network disruption and the *Penium* cell wall

**Supernatants of extracted cell wall material**

Aliquots of washed cells were treated for 24h or 48h with pectate lyase (PL) (Megazyme, IR; E-PECL Y , final concentration 1.2 μg ml⁻¹). The cells were then collected and resuspended in 280mM oryzalin in either the PL or cellulase solutions for 24–48h. Cells were then collected and viewed with DIC-LM, or labelled with JIM5 and observed with FLM or CLSM. Microtubule network disruption and the *Penium* cell wall

**Enzyme pre-treatment**

**Microtubule and actin labelling**

Immunolocalization of microtubules was performed using the freeze shatter technique of Wasteneys *et al.* (1997). Rhodamine–phalloidin labelling was performed using the method described by Holzinger *et al.* (2002).

**Quantitative measurements**

The surface area (SA) of a cell covered by new cell wall, as recognized by new HG in relation to whole cell SA, was calculated for JIM5-labelled cells incubated in oryzalin for 48 h or 72 h, or in control cultures. The cylindrical morphology of *Penium* and the constant cell width (17 μm) of each cell allows for SA measurements to be obtained using the standard formula for determining the SA of a cylinder: SA=2πr²+2πrL, where r=radius of the cell, L=length of the designated area (i.e. length of the cell or length of the cell area with newly deposited HG). For L, the length of specific areas with new cell wall was calculated as the non-fluorescent zones produced post-initial JIM5 labelling. Measurements were made using standard Cell B software (Olympus). Triplicate samples of 100 cells each were measured and a 0.98 (SA) curvature factor employed to account for the blunt rounding of the cells at the poles. For calculating SA of the swollen, spherical isthmus regions of oryzalin-treated cells, the diameter of the central, spherical, swollen zones was measured, in addition to the adjacent cylindrical polar regions. The SA of the spherical regions was determined using the standard formula for a sphere: SA=4πr², where, r=the radius of the sphere. This SA was added to the surface areas of the cylindrical regions at the poles to determine whole cell SA of treated cells.

**Polysaccharide microarray analysis**

Polysaccharide microarray analysis was performed as described by Møller *et al.* (2007). Supernatants of extracted cell wall material were spotted in three replicates and three dilutions, and three independent analyses were carried out. Mean spot signals from the three experiments are presented as a heatmap created using the online tool available at http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper.cgi, with the values normalized to the highest value (set to equal 100). A cut-off of 5% of the highest mean signal value was imposed and values below this are represented as 0. Antibodies and CBM3a were obtained from PlantProbes, CCRC (University of Georgia, Athens, GA, USA), Dr M.-C. Ralet (INRA, Nantes, France), or BioSupplies (Parkville, Australia).

**Results**

**Morphology and immunolabelling patterns of cultured Penium**

Under normal growth conditions, *P. margaritaceum* is an elongate cylinder with rounded poles. Each cell is ~17 μm wide and cell length varies from 150 μm to 220 μm (Figs 1A, 2A). Live cells may be labelled with mAbs with specificity for epitopes present in land plant cell wall polymers (see also Domozych *et al.*, 2011) or CBMs, and placed back into growth medium where they continue division/expansion and retain the label for 10 d or more, depending on the polymer in question. JIM5, an mAb that recognizes HGs with a relatively low DE, labels a lattice-like structure over most of the cell surface (Fig. 1A; Domozych *et al.*, 2011) except for a narrow, non-labelled band in the isthmus region (Fig. 1B). This band represents the major HG secretion zone during pre-division (i.e. the isthmus band; or ‘HGSB’, Domozych *et al.*, 2009b) and is labelled by the mAb JIM7, which recognizes HG with relatively high DE (Fig. 1C, D). RG-I was also identified in the cell wall using INRA-RU2 (Fig. 1E) but, unlike JIM5, INRA-RU2 localized in a layer
below the outer wall lattice and in a more homogenous labelling that was interrupted by dark puncta. This was determined by CLSM optical sectioning. CBM3a, a CBM with specificity toward crystalline cellulose, also labelled most of the cell wall except for the isthmus band (Fig. 1F). CLSM-based optical sectioning through the wall layers revealed CBM3a labelling at the innermost region of the wall that is generally uniform, but interrupted by unlabelled puncta (Fig. 1F). Approximately 10–12 of these puncta were found per square micrometre, matching the number and pattern of the outer wall layer lattice projections observed in the JIM5-labelled cell walls. These unlabelled puncta were interpreted as the shadows of HG of the outer layer. Scale bar=3.0 μm. (H) CBM3a control where the CBM3a was left out of the labelling process. Scale bar=12 μm. All images were taken using CLSM.

**Morphology and growth dynamics of oryzalin-treated *Penium***

*Penium* has a uniform cylindrical morphology consisting of two ‘equal’ sized semi-cells attached at the central isthmus. During most of the cell cycle, the nucleus resides at the isthmus and is flanked by two chloroplasts housed within each semi-cell (Fig. 2A). After 24h of treatment with 280nM oryzalin, noticeable swelling occurred at the isthmus zone (Fig. 2B), which increased further after 36h (Fig. 2C). The nucleus remained in the swollen isthmus and became ensheathed by the chloroplast filling this zone. After 48h, the swelling increased dramatically (Fig. 2D), creating a large spherical central zone within the cell that was sandwiched between the two cylindrical polar zones, suggesting that oryzalin affects the new but not the pre-existing cell wall. Cells did not divide when treated with oryzalin but remained alive for up to 96h, after which the protoplast and cell wall often ruptured at the isthmus. Recovery experiments, involving removal of oryzalin and transfer of the cells to fresh WHM, resulted in a return to the cylindrical morphology. The time taken for recovery was dependent on the time taken for elimination of already internalized oryzalin (Sampathkumar et al., 2011) and, after 12h of recovery, new expansion yielded a narrow, cylindrical morphology arising at the isthmus flanked by the swollen regions that arose during incubation in oryzalin (Fig. 2E). Cells were also able to divide during recovery, yielding products that have narrow cylindrical morphology at the poles (i.e. formed during pre- and post-oryzalin-treatments) and a swollen central region (i.e. formed while incubated in oryzalin; Fig. 2F).
VPSEM was used to analyze the severe morphological changes to the cell and alterations to the cell wall surface resulting from oryzalin treatment (Fig. 3A–B). More specifically, isthmus-based swelling occurred from 6 h to 24 h after the treatment (Fig. 3C–E), during which time the HG lattice of the outer wall became disrupted, before ultimately disappearing at ~48 h treatment (Fig. 3F). During the recovery experiments, a narrowing of the isthmus region became apparent (Fig. 3G), similar to that observed by LM (Fig. 2E). Cell division reinitiated during recovery (Fig. 3H) and the outer HG lattice reappeared in the growing zone at the polar tip of the expanding daughter cell (Fig. 3I).

Wall compositional modifications induced by oryzalin treatment

Polysaccharide microarray analysis was performed in order to compare the relative amounts of epitopes of wall polymers in untreated cells with those treated with oryzalin for 48 h (Fig. 4). This semi-quantitative technique has been used successfully with several CGA species (Sorensen et al., 2011) and involves sequential extraction of cell wall polysaccharides using CDTA, followed by sodium hydroxide, and cadoxen (diaminoethane and cadmium oxide), prior to spotting onto a nitrocellulose membrane and probing with mAbs or CBMs with specificity for a range of cell wall epitopes. Differences in the mean spot signal intensities were observed after probing with several mAbs, but one particularly striking change was an increase in oryzalin-treated cells of the relative levels of HG epitopes, as recognized by the mAbs LM18 and LM19 which represent new mAbs that label HG epitopes in a similar fashion to JIM5 (Verhertbruggen et al., 2009). Conversely, the relative levels of the RG-I backbone epitope, recognized by the mAbs INRA-RU1 and INRA-RU2, AGP epitopes, recognized by the mAb JIM8, and LM16 and extensin epitopes, recognized by mAbs JIM20 and LM1 all decreased in oryzalin-treated cells. Another notable finding was that the RG-I epitopes were almost as abundant in material extracted with NaOH as in material extracted with CDTA. It should be noted that equal volumes of extract are used for each spot, which allows comparisons between treatments (i.e. with or without oryzalin) but not between extracts (i.e. CDTA, NaOH, and cadoxen) and so the extractability of the epitope-bearing polymers is not considered here. The results of this microarray study were used as a guide for choosing specific targeted polymers for subsequent labelling.

Immunocytochemical examination of alterations to the HG lattice in oryzalin-treated cells

JIM5 was used as a marker to monitor lower DE HG during wall development. This antibody has previously been successfully employed for live cell immunolabelling of Penium in the past (Domozych et al., 2009b, 2011). Oryzalin treatment (Fig. 5A) initially (~2 h) resulted in a narrow region of disruption to the HG lattice of the outer wall layer at the isthmus band (Fig. 5A). After 24 h of treatment (Fig. 5B), distinct breaks in the HG lattice were visible and these progressively...
Fig. 4. Polysaccharide microarray analysis of the alcohol-insoluble residue (AIR) from untreated *Penium* cells and *Penium* cells treated for 48 h with oryzalin. Cell wall polysaccharides were sequentially extracted from the AIR with 50 mM CDTA, 4 M NaOH, and cadoxen. The solubilized polysaccharides were printed as microarrays and probed with a range of monoclonal antibodies (listed in the top row). The highest mean signal value in the entire data set was set to 100 and all other signals adjusted accordingly. HG, homogalacturonan; RGI, rhamnogalacturonan I; AGP, arabinogalactan protein; CBM, carbohydrate-binding module.
expanding over 36 h and 48 h of treatment (Fig. 5C, D), at which point the lattice was severely disrupted. When cells were allowed to recover for 12 h, the HG lattice and cylindrical shape reappeared at the isthmus band (Fig. 5E) and at the expanding polar zone of recently divided daughter cells (Fig. 5F). Quantitative analysis of SA coverage by the HG lattice showed that the percentages of cell SA covered by new cell wall in cells treated for 48 h and 72 h and untreated cells were approximately equal (Table 1). However, in untreated cells, new wall material was found primarily in new cylindrical growth at the isthmus zone whereas in treated cells the majority of new wall material was present in the spherically swollen isthmus. Experiments were carried out to determine if cellulose alteration caused by treatment with the known cellulose synthesis-disrupting agents, isoxaben and DCB, affected the expansion zone and cell shape. When treated with isoxaben (10 μM; Fig. 5G) or DCB (0.2 μM: Fig. 5H), similar swelling of the isthmus was observed. These effects were also reversible by extensive washing of the cells and removal of the disrupting agent.

**FESEM imaging of HG lattice alteration**

FESEM was employed to provide detailed surface imaging of the cells. During cell swelling at the isthmus band upon 24 h treatment with oryzalin, the HG lattice began to tear apart (Fig. 6A). The lattice of the pre-existing cell wall consisted of an inner fibre-based network and outward-extending projections (Fig. 6B). The wall of the swollen isthmus was highlighted by irregular patterns of the HG fibres interspersed with wall regions possessing no lattice (Fig. 6C). These observations corresponded well with JIM5-labelled cells displaying lattice alterations (Fig. 5B–D).
the distribution of pectins with a higher DE. After 12 h of treatment, the typical distribution of label in the narrow isthmus band was observed, even in the swollen isthmus zone (Fig. 8A) but, after 36 h, the signal became more diffuse and was irregularly displaced over the central part of the swollen zone (Fig. 8B). Other aspects of the pectin network were disrupted by oryzalin, as evidenced by INRA-RU2 labelling of the RG-I backbone. This showed intensely labelled striations after 12 h (Fig. 8C) and then a highly irregular pattern in the swollen isthmus after 36 h (Fig. 8D). The spatial distribution of crystalline cellulose, as detected using CBM3a, was similarly perturbed by the oryzalin treatment (Fig. 8E), resulting in a shredded appearance at the swollen isthmus region.

Cytoskeletal changes induced by oryzalin treatment

Oryzalin has previously been demonstrated to be a potent microtubule-affecting agent in land plants (Hugdahl and Morejohn, 1993; Morrissette et al., 2004). Likewise, cortical microtubule and actin microfilament networks have been shown to be closely associated with cell wall synthesis, secretion, and development. (Mutwil et al., 2008; Paradez et al., 2008). In this study, tubulin immunolabelling and rhodamine-phalloidin labelling of actin cables were used to observe the two cytoskeletal networks in order to elucidate any changes to these cytoskeletal components upon treatment with oryzalin. In untreated cells, the cortical microtubule network was highlighted by distinct rings of microtubules aligned perpendicular to the long axis of the cell (Fig. 9A). The isthmus region contained the largest ring, consisting of a network of 10–20 parallel-aligned microtubules (Fig. 9B). After 36 h of oryzalin treatment, the microtubular network became disorganized and no ring was apparent in the swollen isthmus region (Fig. 9C). Upon recovery, the microtubule band of the isthmus region reappeared within 4 h (data not shown). The actin microfilament network of *Penium* consists of parallel arrays of microfilament bundles in the subplasma membrane cortical region running parallel to the longitudinal axis (Fig. 9D). At the isthmus zone, parts of these microfilament bundles converged inward to form a ring at the same location as the microtubular band and corresponding to the JIM7-labeled region (Fig. 9E). After 36 h of oryzalin treatment, the isthmus-based microfilament band became highly disorganized (Fig. 9F) but the parallel alignment of microfilament bundles in the unaltered regions of the cell remained. After 12 h of recovery, the normal distribution of microfilaments returned (Fig. 9G).

Ultrastructural effects of oryzalin treatment

The effects of oryzalin on cell wall ultrastructure were assessed using TEM. After 48 h of treatment, noticeable alterations to the wall were observed (Fig. 10A). In addition to alterations in the HG lattice, the wall was thinner, and little, if any, lattice was apparent. The *Penium* cell wall consists of three layers, an outer layer containing the HG lattice, an inner fibrous layer of cellulose, and a middle ‘interface’ layer where the HG of the outer layer embeds in the cellulose (Fig. 10B). Treatment
of cells with oryzalin for 24 h (Fig. 10C) resulted in a notable disruption of the wall architecture with a sharp interface between altered and unaltered regions of the wall. All three wall layers were present in the region formed before oryzalin treatment. However, in wall formed during oryzalin treatment (i.e. the swollen zone), little of the HG lattice remained. After longer treatments (36 h), the medial layer appeared as multiple linear ‘streaks’ positioned nearly perpendicular to the long axis of the wall (Fig. 10D). These micrographs were taken from sections of cells embedded in plastic sheets to enable observation of their longitudinal wall profiles. After 48 h (Fig. 10E), the cell wall of the swollen region became notably thinner and contained remnants of the medial layer components located at the outermost region of the inner layer. In a comparison of 50 micrographs of the cell walls of treated and untreated cells, oryzalin treatment resulted in a decrease of 25% (±4%) of the inner/medial wall layer thickness.

Discussion

Oryzalin induces a loss of wall biomechanical strength at the primary site of wall deposition

The cell wall of *Penium* consists of two major domains that are arranged in three recognizable layers. One domain consists of HG (Domozych et al., 2007; Sørensen et al., 2011) that binds with Ca$^{2+}$ to form the distinctive lattice that constitutes the outer layer of the wall. The second domain is cellulose based (Domozych et al., 2011) and makes up the inner cell wall layer. Aggregates of HG fibrils emerging from the base of the outer layer embed in the microfibrillar infrastructure of the inner cellulose-rich layer and form the medial layer. This layer contains both HG and RG-I, and represents the zone where pectin and cellulose physically intersect. This architectural design of the cell wall supports the elongate cylindrical shape of the cell and resists the pressures of internal turgor. Treatment of *Penium* with oryzalin compromises this cylindrical design and causes distinct swelling at the isthmus zone. This swelling is accompanied by significant alterations to both wall domains and the overall structural architecture of the wall. The isthmus is the site of the isthmus band during pre-division expansion where HG is secreted and incorporated into the wall, where cellulose microfibrils are synthesized, and where the pectic and cellulosic domains most probably become interconnected (also see Domozych et al., 2009b). Consequently, the developing cell wall at the isthmus band is more elastic than at other parts of the cell and is more susceptible to the pressure of internal turgor if its structural integrity is compromised. This would explain why oryzalin-induced swelling occurs here. Alternatively, oryzalin does not affect pre-existing wall, suggesting that the mature wall is not significantly remodelled after it forms, or that any post-synthesis remodelling is not affected by application of oryzalin.

The mechanism of highly focused wall expansion and the oryzalin-induced swelling at the isthmus band in *Penium* have some notable similarities to that observed in other anisotropically growing plant cells. For example, in expanding pollen tubes, the focal point of wall expansion is also a narrow band, specifically the apical zone located at the tube tip (Geitmann and Steer, 2006; Geitmann and Ortega, 2009; Aouer et al., 2010; Dardelle et al., 2010; Fayant et al., 2010;
Oryzalin treatment also causes swelling at this apical tip (Anderhag et al., 2000). In the pollen tube apex, high DE HG secretion and callose/cellulose synthesis produce an elastic wall zone capable of regulating turgor-driven expansion. Immediately beyond the apex, pectin methylesterase (PME) remodelling of the HG followed by Ca\(^{2+}\) cross-linking creates a rigid gel which strengthens the wall that will surround the long tube shank. In *Penium*, the isthmus band is functionally equivalent to the expanding apical tip of the pollen tube; that is, where HG secretion/modelling and cellulose microfibril synthesis actively occur. However, the *Penium* wall synthesis mechanism differs from that of pollen tubes in that although there is a single wall expansion zone (the isthmus band), wall expansion is bi-directional. This predicates the presence of a currently described mechanism that allows for both PME processing of secreted HG and displacement of this HG toward both poles of the cell.

Oryzalin disrupts *Penium* microtubular dynamics and wall deposition but not cell expansion

In previous studies, oryzalin has been shown to affect microtubule dynamics in plants and some protists directly by sequestering tubulin dimers (Hugdahl and Morejohn, 1993; Morrissette et al., 2004). In land plants, this leads to changes in cell wall infrastructure and subsequent cell swelling (Nakamura et al., 2004; Bannigan et al., 2006; Paradez et al., 2006; Corson et al., 2009) similar to that observed in this study. In *Penium*, it was shown that parallel bands of cortical microtubules aligned perpendicular to the cell’s longitudinal axis are found in the central region of the cell, the largest and most prominent of which resides at the isthmus band. More importantly, this microtubule band was dramatically altered during oryzalin treatment, resulting in a random display of microtubules dispersed throughout the cytoplasm of the isthmus. This corresponded to alterations to the cell wall and the swelling at the isthmus region. What might be the link between the cortical microtubular cytoskeleton and wall expansion dynamics occurring at the isthmus band?

Throughout the past half-century of cell wall research, close associations of cortical microtubules with cellulose microfibril orientation have been noted in many plant cells (Smith and Oppenheimer, 2005; Mutwil et al., 2008; Lloyd and Chan, 2008; Anderson et al., 2010; Chan et al., 2010; Endler and Persson, 2011). Recently, live cell imaging using fluorescent protein fusions with cellulose-synthesizing enzymes (e.g. cellulose synthase, or CesA complexes) has further demonstrated the dynamic interaction between the cellulose synthetic machinery residing on the plasma membrane and the underlying layer of cortical microtubules (Paradez et al., 2006). It is widely believed that cortical microtubules serve as guides that direct the movement of cellulose synthase complexes on the plasma membrane and, in turn, the production of cellulose microfibrils in specific orientations in the cell wall. According to this model, perturbation of the cortical microtubular network by an agent such as oryzalin would affect the synthesis of the cellulose microfibrillar network.

**Fig. 8.** JIM7 (specificity toward relatively highly esterified HG), CBM3a (specificity toward crystalline cellulose), and INRA-RU2 (specificity toward the backbone of RG-I) labelling of oryzalin-treated cells. (A) The JIM7-labelled band of higher DE HG (arrow) in the swollen isthmus zone after 12 h of oryzalin treatment. Chloroplast autofluorescence is also visible. Scale bar=12 \(\mu\)m. (B) Diffuse pattern of JIM7 labelling in the swollen isthmus after 36 h (arrow). Scale bar=9.0 \(\mu\)m. (C) INRA-RU2 labelling after 12 h showing striations in the isthmus (arrow). Scale bar=10 \(\mu\)m. (D) INRA-RU2 labelling after 36 h. Highly irregular labelling occurs in the isthmus zone (arrows). Scale bar=5 \(\mu\)m. (E) CBM3a labelling after oryzalin treatment. The labelling at the isthmus has a shredded appearance (arrow) at 36 h. Scale bar=6 \(\mu\)m. All images were taken using CLSM.
The results of this study also suggest that alteration of the cortical microtubule network by oryzalin in the active wall expansion zone, the isthmus band, directly affects both the cellulose synthesis machinery and wall microarchitecture. For example, TEM imaging demonstrated that the cellulose-based inner wall layer was reduced in thickness by 25% at the oryzalin-induced swollen zones. It is possible that the microtubule disruption at the isthmus band slows or alters cellulose microfibril synthesis, yielding a thin cellulosic layer. Turgor pressure at this thin zone would then cause deformation of cell shape. The cellulosic framework here would still be sufficient to keep the cell from bursting but would be unable to maintain the narrow cylindrical shape at the isthmus (i.e. swelling occurs). It is also possible that oryzalin-induced alteration of the cellulose synthesis machinery causes an increased stretching in the cellulosic layer (i.e. increased sliding of microfibrils) which then contributes to the thinning of the inner layer and subsequent perturbation of the HG lattice. The link of oryzalin treatment to cellulose domain disruption is further strengthened by observations from this study whereby cellulose-affecting agents (e.g. isoxaben or DCB) also cause swelling at the isthmus. Interestingly, the present study also showed that in oryzalin-treated cells, the percentage of surface area covered by new wall material in relation to the whole cell was approximately the same as in untreated cells. This suggests that while structural changes occurred in the wall following oryzalin treatment, the rate of wall expansion is not noticeably altered. The geometry of expansion changes from linear (cylinder) to spherical (swollen isthmus) but not the amount of cellular expansion.
Evidence for coordinated deposition and interaction of the pectin and cellulose cell domains

The thinning of the cellulosic inner layer during oryzalin treatment also results in distinct alterations to the pectin domain of both the medial and outer layers. Proper formation of the cellulosic layer probably serves as the framework for the deposition and anchoring of the HG-based outer layer. When formation of this cellulosic layer becomes compromised by oryzalin, alteration of the HG lattice also occurs (Fig. 11). This result exemplifies a complex structural interaction between two polymer domains that must be developmentally coordinated and adds to the growing evidence supporting pectin–cellulose interactions (Zykwinska et al., 2005, 2007; Peaucelle et al., 2012). In Penium, the identification of RGI in the medial layer suggests that this polymer may also be involved in this interaction, although its relative abundance appears to be relatively low compared with that of land plants (Sørensen et al., 2011).

Previous research has shown that pectins and pectin-modulating enzymes such as PME (Mohnen, 2008; Bosch and Hepler, 2005; Tian et al., 2006), as well as the cellulose...
The pectin and cellulose domains: physically interacting but distinct functions?

This study has shown that oryzalin affects the architecture of both the cellulose and pectin domains of the cell wall and manifests in a major change to cell shape. These observations led to the question of which cell wall polymer and/or domain is primarily responsible for maintaining the structural integrity of the cell at the isthmus band and the cylindrical morphology of the cell. First, in untreated cells, the cell wall of the isthmus band consists primarily of the cellulose-rich inner layer (i.e. no HG lattice) and the typical cylindrical cell shape is maintained here. In oryzalin-treated cells, the cellulose layer at the isthmus band thinned, and swelling of the isthmus region occurred. Secondly, in cells pre-treated with cellulase and then treated with oryzalin and cellulase, the swelling at the isthmus zone became even more pronounced and, in some cases, leads to wall rupture. These observations indicate that the cellulose inner layer is most important in resisting inner turgor pressure driving expansion and in maintaining the cylindrical cell shape. If this cellulose-based infrastructure is compromised, as it is with oryzalin treatment, cell wall integrity and its tensile resistance to turgor-driven pressure are also compromised. This observation closely corresponds to other studies that show that if cellulose infrastructure is altered at an expansion site, the tensile resistance of the cell wall and/or cell shape may be severely altered (Auer et al., 2009).

What then is the role of the HG, and particularly the prominent HG lattice that covers most of the cell surface, in the structural mechanics of the cell wall? First, the present polysaccharide microarray analysis showed that levels of HG epitopes notably increased following oryzalin treatment. It may be the case that if, as suggested, HG is required for maintaining wall integrity in expanding Penium cells then the indirect disruption of cellulose synthesis and/or orientation caused by oryzalin treatment led to a compensatory increase in HG synthesis and/or deposition, as has been suggested to occur in land plants (Burton et al., 2000; Bischoff et al., 2008). If so, this is reminiscent of the effect of the cellulose inhibitor isoxaben on cell cultures, which causes a disruption of the cellulose crystallinity and a, presumably compensatory, increase in HG (Bernal et al., 2007). In this regard, it is probably significant that the polysaccharide microarray analysis showed a change in the binding of 2F4, which suggests that the effect of oryzalin is not just to induce production of HG per se, but rather the production of HG with sufficient contiguous non-methyl-esterified GalA residues to participate in the structurally important process of Ca^{2+} cross-linking. It should be noted that oryzalin may also exert a direct effect on the activity of PME, similar to its action on other wall enzymes (Vissenberg et al., 2005), which, in turn, affects its remodelling of secreted HG. However, while oryzalin treatment causes significant alteration to the HG production levels and the lattice infrastructure is disrupted at the swollen isthmus region, comparable experiments with PL pre-treatment followed by oryzalin treatment do not result in further cell swelling or rupture, as observed with cellulase pre-treatment (Fig. 7). This indicates that the HG is not primarily responsible for maintaining the structural integrity of the wall or cell shape at the isthmus band.

While further work is needed to resolve the role of the HG, it is suggested that the HG lattice may represent a network of reinforcing struts that are needed to support the large expanse of the elongate cylindrical shape of Penium. Struts are mechanical devices often organized in regular networks that are embedded in the external edifice of a structure, functioning to reinforce the integrity of structures that have large longitudinal axes (e.g. cylinders). While not serving as the main structural framework, they nonetheless help maintain elongate structures. Further biomechanical studies will be needed to confirm the role of the HG lattice and elucidate the tensile strength of the cellulose domain. It is also possible that the HG lattice does not affect wall rigidity but may function in cell adhesion. An interesting area of future research will
be to determine whether these domains, as well as other wall components such as RG-I, consistently show common organizations and functions in the walls of CGA and land plants.

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