Malaysian Journal of Microscopy 10: 20-26 (2014) ISSN: 1823-7010

CELL WALL DEVELOPMENT IN THE VELAMEN OF THE ORCHID *MILTONIOPSIS* INVESTIGATED BY CONFOCAL MICROSCOPY

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The velamen is a multicelled epidermal layer found in orchid roots. This layer consists of dead cells that have helical secondary wall thickenings and is usually thicker in epiphytic orchids with aerial roots than terrestrial orchids, consistent with the velamen's role in water capture. While velamen structure has been widely studied as a taxonomic character, few studies have looked at its development. We used confocal microscopy to investigate cell wall formation in the epiphytic orchid Miltoniopsis. Development of the helical thickenings begins with microtubule-dependant cellulose deposition but as the secondary wall ridges develop, parallel microtubules bind either side of the ridge. Cellulose organization was also investigated using pontamine staining which showed bifluorescence of the cellulose strands. These data may provide insight into the mechanical efficiency of the velamen layer in orchid roots.

Keywords: bifluorescence, confocal microscopy, microtubules, *Miltoniopsis*, orchid, velamen

INTRODUCTION

The velamen is a prominent characteristic of orchid roots. This unique layer of dead cells forms outside the root's exodermis, and occurs as a single-layered rhizodermis in terrestrial orchids but as a multi-cellular layer in epiphytic orchids. The velamen has been suggested to function in both the capture of water by the root [1] and in its retention in dry conditions [2], roles thought to be aided by the unusual nature of the cell walls within the velamen. These walls typically contain secondary thickenings that are made of cellulose and lignin that form a wide range of different patterns. The diversity found in the velamen structures across different orchid genera, categorized by [3], have allowed taxonomic studies to utilise velamen organization to define relationships between various orchid taxa [4, 5].

Despite numerous studies investigatingthe functions of the velamen and its use in taxonomy, the development of this important structure remains poorly documented. The velamen develops through periclinal divisions (parallel to the root surface) in the primary root meristem, with the cells formed by these divisions developing a secondary cell wall and then undergoing programmed cell death [4]. However, the development of the cell wall in the velamen layer, from primary cell wall of the living cell through to the cellulose-thickened secondary cell wall of the dead cell layer, has not been documented. Our preliminary investigations have used confocal microscopy to study velamen

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development in roots of the epiphytic orchid, *Miltoniopsis*. We focussed on the organization of cellulose within the cell wall, the timing of secondary cell wall lignification, and investigated microtubule organization as it is known that microtubules align cellulose deposition [6, 7].

MATERIALS AND METHODS

Plant Material and Preparation

Horticultural varieties of *Miltoniopsis* sp. were obtained from John Clemens (Christchurch Botanical Gardens) and grown in the university greenhouse in standard orchid potting mix (Southern Horticultural Products, Rolleston, New Zealand) with natural light. Fresh root samples were cut 1-2 cm from the root tips and washed under running tap water to remove surface debris.

Immunolabelling

Whole roots were fixed (1-2 h in vacuum) in PME solution (50 mM Pipes, pH 7.2 (K^+), 2 mM EGTA, 2 mM $MgSO_4$) containing 0.1% Triton X-100), 3.7% formaldehyde, 0.5% glutaraldehyde and 0.1% dimethyl sulfoxide. Roots were washed in PME solution, extracted in PME solution with 1% Triton X-100 (1 h) and permeabilized in methanol (–20°C, 15 min) before being rehydrated in phosphate-buffered saline (PBS; 131 mM NaCl, 5.1 mM Na_2HPO_4, 1.56 mM KH_2PO_4, pH 7.2). For sectioning, roots were embedded in PBS solution containing polyacrylamide (21% acrylamide, 0.01% TEMED (tetramethylethylene-diamine) and 0.1% ammonium persulfate, and sectioned using a Vibratome 3000 Plus sectioning system. Using multi-well tissue culture trays, sections were incubated in 1% sodium borohydride in PBS (15 min) to reduce free aldehyde groups, and after washing in PBS, were blocked in incubation buffer (PBS with 1% bovine serum albumin and 0.5% Tween-20) (15 min). Sections were incubated (1 h) in monoclonal anti-α-tubulin (clone B512, Sigma) diluted 1/1000 in incubation buffer. After multiple washes with PBS (30 min), samples were incubated with a fluorescein-tagged goat anti-mouse secondary (Jackson) diluted 1/200 in incubation buffer (1 h). After several washes in PBS, sections were mounted in a drop of antifade agent AF1 (Citiflour).

Berberine and Pontamine Staining

Berberine was used to visualize lignified structures in potassium hydroxide-cleared (10%, 1 h at 70˚C) hand sections of roots. Sections were stained with berberine hemisulfate (0.1%, 1 h) (Edward Gurr) then counter-stained with 0.1% aniline blue in distilled water (30 min). Pontamine was used to visualize cellulose in roots. Cleared hand sections were stained with pontamine fast scarlet 4B (Sigma) (0.1% in distilled water) (5 min), then mounted in glycerol.

Microscopy

Sections were observed by confocal microscopy (Leica SP5). High resolution images (1024 x 1024 pixels) were collected with 3-fold line averaging with 1.0 μm steps used for optical sectioning. Fluorescein and berberine were excited with the 488 nm laser, and fluorescence collected from 500–600 nm, while pontamine was excited with either the 405 or 561 nm lasers, with emission from 580-680 nm. Lignin autofluorescence was excited at 405 nm and collected at 420-480 nm. Transmitted light images in either brightfield or polarized light modes were collected. All images were processed with standard tools in Adobe Photoshop.

For confocal observations of bifluorescence (fluorescence dependent on incident light polarization), two approaches were tested. In the first [8], samples were imaged after rotation through 90° on a rotating stage, generating different excitation polarizations. In the second approach, the polarization of the 405 nm laser was rotated by 90° by rotating the imaging of the sample through 90° with the confocal microscope system's scanfield rotation setting. This effect did not occur with the other lasers. For both approaches, image orientations were corrected in Photoshop.

RESULTS

The Velamen in Miltoniopsis

Miltoniopsis orchids are tropical, South American epiphytes. The horticultural cultivars used for these experiments have thick roots (diameter ~2 mm) which, typical of epiphytic orchids, have a multi-celled velamen covering the exodermis (Fig. 1). The velamen consists of 4-8 layers of dead cells with bigger, radially-extended inner velamen cells and smaller, outer cells. The velamen cell walls are thickened by long strands of cellulose forming a mesh-like appearance visible in confocal images of berberine-stained sections (Fig. 1). Optical sectioning, however, confirmed that strands oriented in the same direction on one side of the wall can be oriented differently on the other side of the wall, creating a criss-cross pattern [3]. The strands surround pores which are areas of cell wall that are not thickened (Fig. 1b; arrow). The cortex contains thin-walled parenchyma cells, some of which have partial secondary cell walls referred to phi thickenings (Phi) [9]. Fungal hyphae were observed running through the velamen strands (not shown), while coils of orchid mycorrhizae called pelotons were often present within the living cortical cells (P).

Microtubules and Velamen Development

We observed the formation and lignification of secondary wall-like striations, and immunolocalized microtubules in these cells, during velamen development in *Miltoniopsis* roots (Fig. 2). Cells developing into velamen had loosely organized cortical microtubules, but there was no alignment of the microtubules between adjacent cells, and no evidence for cell wall thickenings by visible light or lignin auto-fluorescence (Fig. 2a). Microtubules became heavily bundled and regularly-spaced at the onset of cell differentiation, and unlignified wall striations became visible (Fig. 2b). The nuclei showed that these cells were still alive (arrow). At maturity, the microtubules and nuclei disappeared, leaving

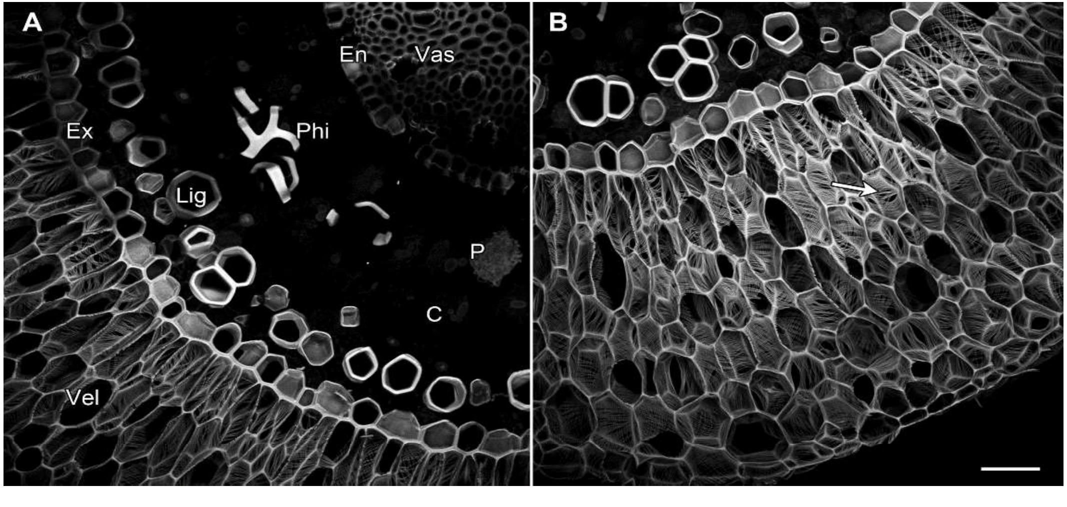

Fig. 1: The *Miltoniopsis* velamen. Berberine-stained root cross sections imaged by confocal microscopy (maximum projections). **a** The velamen layer (Vel) is a sheath external to the exodermis (Ex) and cortex (C) of the root. Lignified cortical cells (Lig), phi thickenings (Phi), peloton (P), endodermis (En) and vascular tissue (Vas). **b** The multi-celled velamen layer showing secondary wall striations of different directions with pores (*arrow*). Bar = 50 µm.

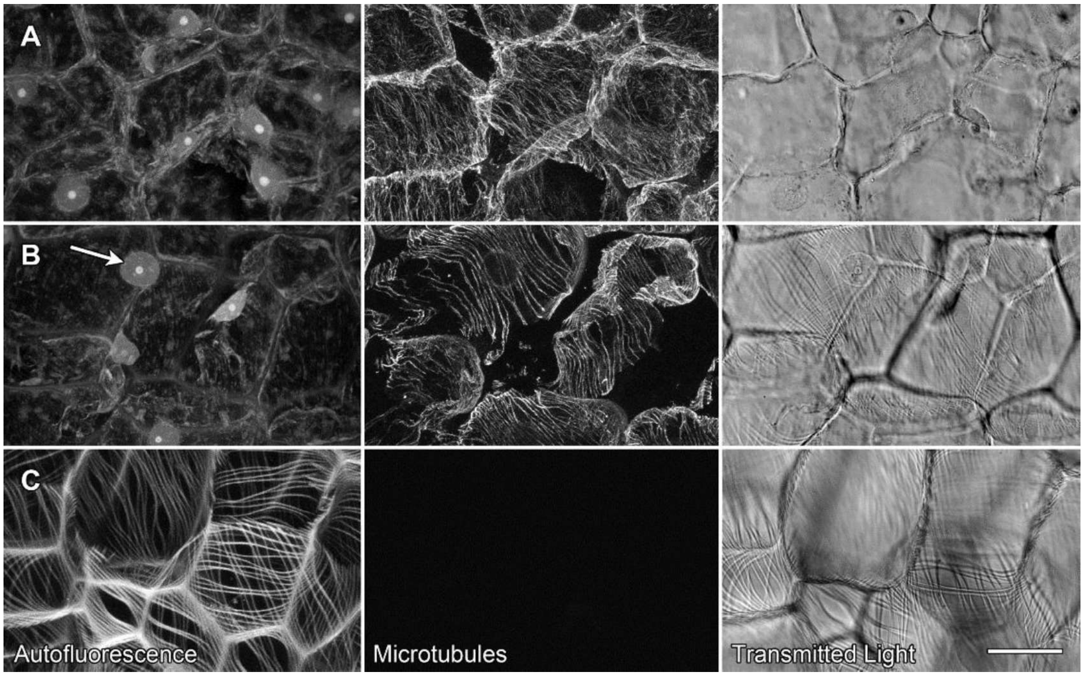

Fig. 2: *Microtubule organization and wall development in the velamen. Maximum projections of tubulin immunolabelling (central column) and autofluorescence (left column), compared to single transmitted light images. Arrow indicates living nucleus. a Young cells before velamen differentiation. b Developing secondary thickenings. c Mature velamen. Bar = 20 µm.*

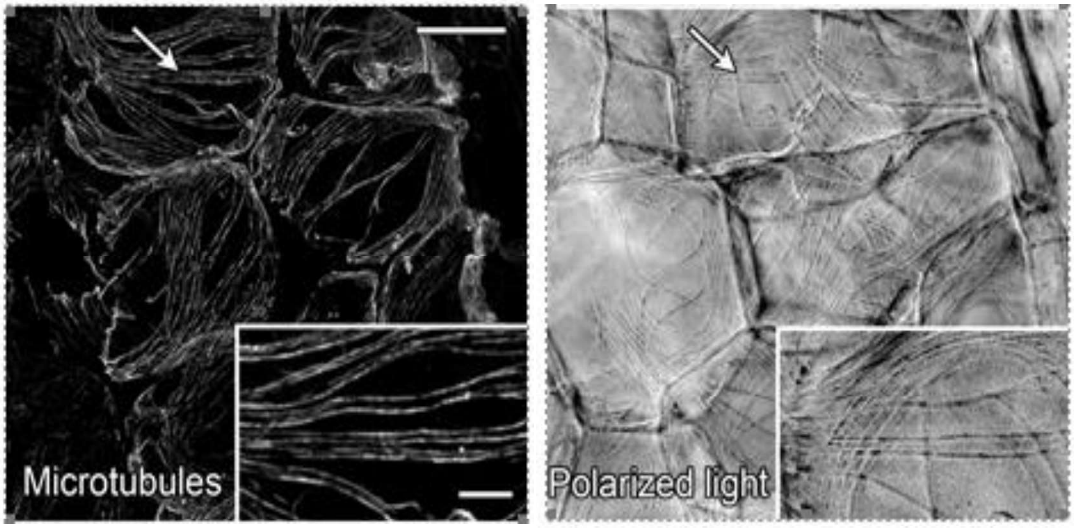

Fig. 3: *Microtubule bundles adjacent to developing wall striations, late in velamen developing (arrow). Images are maximum projections of fluorescence and inverted polarized light. Bar = 20 µm, 5 µm for inset.*

only the distinctive patterns of the lignified cellulose striations (Fig. 2c). In more mature but still alive cells, microtubule bundles were typically observed running on either side of the developing wall striation, rather than directly underneath it (Fig. 3; arrow).

Cellulose Orientation

We have previously shown that cellulose orientation can be determined through bifluorescence, the polarization-dependent fluorescence of pontamine-stained cellulose [8]. Bifluorescence occurs because the pontamine only absorbs light, and thus is only fluorescent, when it is oriented parallel to the orientation of the excitation light. Because pontamine binds specifically to cellulose [10], pontamine bifluorescence can be used to confirm cellulose orientation. We previously generated different polarizations of the excitation lasers with a rotating stage attached to the confocal microscope. When the sample was rotated through 90°, mimicking a rotation in laser polarization [8], the pontamine fluorescence image changed dramatically with only the velamen wall striations that ran parallel to the excitation laser being fluorescent (data not shown). This approach is not ideal as the rotations are technically very challenging.

We have also demonstrated velamen bifluorescence through a new approach that is technically more simple. The scanfield rotation function in the Leica confocal system was used to rotate the scanned image through 90°. This scanfield rotation rotated the polarization of the 405 nm laser through 90°, although it did not rotate the polarization of the other lasers within the confocal system (data not shown). This means that the scanfield rotation function could be used to modulate pontamine excitation because the dye is excited at 405 nm (Fig. 4). When a pontamine-labelled velamen sample was rotated through 90°, mimicking a rotation in laser polarization, the pontamine fluorescence image changed dramatically with only the velamen wall striations that ran parallel to the excitation laser being fluorescent. However, striations that ran at 45° to the excitation were fluorescent in both images, and appeared white in the colour overlay (*asterisk*).

DISCUSSION

Miltoniopsis orchid roots provide an exceptional system to showcase links between intracellular functions such as the cytoskeleton and cell wall production, and the cell wall and whole plant physiology. Within roots, the velamen is a unique tissue whose helical cell wall thickenings are an adaptation that optimizes water and nutrient intake [1] and water retention [2], important factors for epiphytic orchids whose roots are not typically under moist soil.

The secondary thickenings of the velamen wall consist of cellulose microfibrils

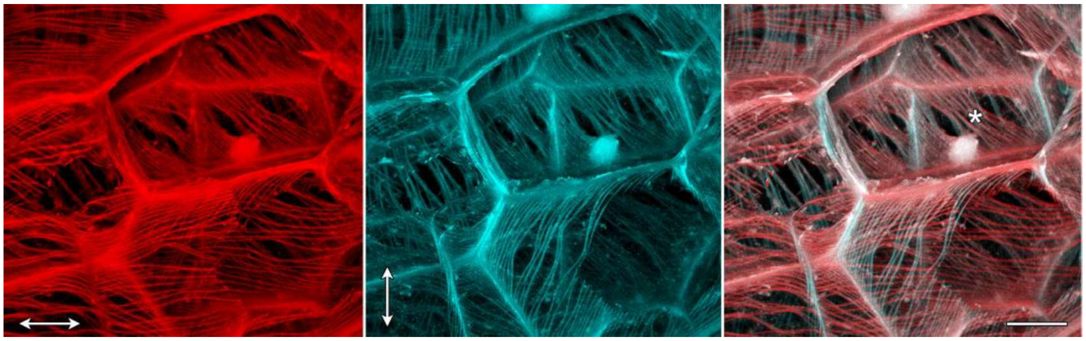

Fig. 4: *Pontamine bifluorescence in confocal maximum projections of the velamen. Pontamine fluorescence using 405 nm excitation in identical cells, with the laser polarity rotated through 90º using the scanfield rotation function. Excitation polarizations indicated with arrows. Striations that ran at 45° (asterisk) excite with both polarizations. Bar = 10 µm.*

orientated in striking patterns. The velamen develops from living, post-meristematic cells that undergo secondary wall deposition and programmed cell death to leave the shell of the cell wall. Co-alignment of cellulose deposition and microtubules is well-known and, according to the alignment hypothesis, cellulose synthase complexes that synthesize cellulose microfibrils coalign with and travel along microtubules [6, 7]. We observed bundled microtubules aligning to the ridges formed by deposition of cellulose microfibrils. Thus, cytoskeletal organization in velamen cells is consistent with other secondary cell wall thickenings where microtubules co-align with wall deposition, including the tracheary elements in various plants [11-14].

However, our observation of parallel microtubule bundles flanking ridges of secondary growth is intriguing. In several reports, microtubules do form borders along either side of a forming secondary wall and do not lie immediately underneath the plasma membrane adjacent to the wall itself. This has been reported by immunofluorescence for *Zinnia* tissue culture cells that develop into tracheary cells [11, 12], and with GFP-based probes in *Arabidopsis* tissue culture cells [15] and whole plants [13] undergoing xylem differentiation. However, other reports suggesting that *Arabidopsis* tissue cells that develop into tracheary cells do not show this pattern [14]. In the velamen, this patterning typically occurs at a later stage of secondary wall development, with the initial stages showing alignment of microtubules directly associated with the developing secondary wall rather than bordering it. It is then, perhaps, significant that the formation of the secondary wall associated with the phi thickenings present in the cortical cells of the roots of *Miltoniopsis* never show this edging pattern, remaining associated with the phi thickenings throughout their development [16]. Microtubule organization associated with the development of the secondary wall is not, however, the major difference nutrient uptake. Oecologia 171 (3), 733-741. between the phi thickenings and the velamen. Phi thickening-like wall modifications occur in the cortical cells of various orchid genera, and these have been described as a ‘pseudovelamen’ because of the apparent similarity in structure to the true velamen [3]. Unlike the true velamen in which dead cells regulate water uptake [1, 2], cells containing phi thickenings remain alive and likely play a structural role in stabilising the root [16].

In primary cell walls, the orientation of cellulose microfibrils is typically random with the exception of tissues undergoing cell elongation where cellulose is aligned transverse to the direction of cell expansion. In secondary cell walls, and especially in this case of the velamen, the orientation of cellulose microfibrils can be more variable. Using pontamine, a bifluorescent, of stain which is specific to cellulose [8], we have shown the orientation of the cellulose microfibrils is different in the inner face to the outer face of a velamen cell, causing a mesh-like appearance of the strands in the velamen when viewed from a regular light microscope and meaning that the thickenings form a helical pattern inside the cell. While the handedness of the helical striations has yet to be studied in the velamen, in the timber industry, the handedness of cellulose microfibrils is very important as it determines the mechanical properties of timber and processed fibre, and is therefore extensively studied [8].

ACKNOWLEDGEMENTS

We thank John Clemens (Christchurch Botanic Gardens) for providing orchids. NAI acknowledges the Malaysian Education Ministry for her PhD scholarship (SLAB) and funding from Biological Sciences at the University of Canterbury.

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