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ORIGINAL ARTICLE

The life of phi: the development of phi thickenings in roots of the orchids of the genus *Miltoniopsis*

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Abstract Phi thickenings, bands of secondary wall thickenings that reinforce the primary wall of root cortical cells in a wide range of species, are described for the first time in the epiphytic orchid *Miltoniopsis*. As with phi thickenings found in other plants, the phi thickenings in *Miltoniopsis* contain highly aligned cellulose running along the lengths of the thickenings, and are lignified but not suberized. Using a combination of histological and immunocytochemical techniques, thickening development can be categorized into three different stages. Microtubules align lengthwise along the thickening during early and intermediate stages of development, and callose is deposited within the thickening in a pattern similar to the microtubules. These developing thickenings also label with the fluorescently tagged lectin wheat germ agglutinin (WGA). These associations with microtubules and callose, and the WGA labeling, all disappear when the phi thickenings are mature. This pattern of callose and WGA deposition show changes in the thickened cell wall composition and may shed light on the function of phi thickenings in plant roots, a role for which has yet to be established.

Keywords Callose · Cellulose deposition · Orchids · Microtubule organization · *Miltoniopsis* · Mycorrhizae · Phi thickenings · Wheat germ agglutinin

Abbreviation
WGA Wheat germ agglutinin

Introduction

Phi thickenings were first described in plant roots by van Tieghem (1871) when he observed that a layer of root cortical cells lying immediately exterior to the endodermis and in which only a thin, primary cell wall might be expected, had large, band-like thickenings in their radial cell walls. In cross sections, these cell wall modifications resembled the Greek letter Φ, which lead to their name (Russow 1875; as cited in van Tieghem 1888 and others). Further scrutiny of tangential and radial sections revealed that the phi thickenings form a net-like structure surrounding the cell with branched and unbranched thickenings that align between adjacent cells. They form due to the highly localized deposition of cellulose and lignin into bands that are coordinated between multiple cells (Pratikakis et al. 1998).

Phi thickenings in the root plant cortex can be categorized into three types according to the cell layers of the cortex in which they occur. The most common pattern is type 1 in which the cell wall thickenings are located in the cell layer immediately external to the endodermis while in the second pattern, the thickenings occur in the cell immediately inside the epidermis. In the type 3 pattern, the phi thickenings are present in multiple

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cell layers throughout the root cortex (van Tieghem 1888). The growth of phi thickenings is not continuous, dissimilar to the stele in roots (Pratikakis et al. 1998), with early formation of the thickenings generally occurring within the first 1 cm from the root tip. It has also been observed that the cells with phi thickenings will eventually slough off during secondary growth (Weerdenburg and Peterson 1983). These factors may contribute to the various observations of phi thickenings in roots of different plants including broccoli (López-Pérez et al. 2007; Fernandez-Garcia et al. 2009), carob (Pratikakis et al. 1998), loquat (Pan et al. 2006), maize (Degenhardt and Gimmler 2000), cherry (Soukup et al. 2004), apple (Mackenzie 1979; Peterson et al. 1981; Weerdenburg and Peterson 1983) and numerous gymnosperms (Gerrath et al. 2002, 2005). Their widespread and consistent occurrences within a plant family have even been proposed as a useful character for gymnosperm systematics (Gerrath et al. 2002).

Despite their prominence in some important crop plants, the functions of phi thickenings remain obscure. Phi thickenings have often been compared to the Casparian band present in the endodermis because of their location and their apparently similar, thickened structure (Wilcox 1962; Mackenzie 1979; Pratikakis et al. 1998). A mechanical role in cell and root support has also been suggested for the phi thickenings, although this has never been physically demonstrated (Haas et al. 1976; Peterson et al. 1981; Degenhardt and Gimmler 2000). Studies using electron microscopy have demonstrated that cellulose microfibrils run parallel to the long axis of cell wall thickenings with the organization of cortical microtubules parallel and consistent with this pattern (Haas et al. 1976; Mackenzie 1979).

This study has investigated the cellular development and function of phi thickenings in the orchids of the genus *Miltoniopsis*, a tropical epiphytic orchid. As there have only minimal descriptions of phi thickenings in monocots (e.g., maize, Degenhardt and Gimmler 2000) and a single description of phi thickenings in orchids (Moreira and Isaias 2008), basic histological characterizations of the thickened cell walls were conducted along with analysis of their development. Microtubules aligned with the cellulose along the length of the thickening during development but disappeared as the phi thickening matured. However, no association was found in the development of phi thickenings with actin microfilaments. The phi thickenings also showed several unusual developmental properties, as antibodies against callose and fluorescently tagged wheat germ agglutinin (WGA) also labeled developing but not mature thickenings.

Materials and methods

Plant material

Miltoniopsis sp. plants of various horticultural varieties were obtained from Dr. John Clemens (Christchurch Botanical Gardens) and from John Clements and other members of the Canterbury Orchid Society, while some plants were also purchased from Woolf Orchid Culture (Drayton, QLD, Australia). Plants were maintained in the university greenhouse, growing under natural light and in standard orchid potting mix (Southern Horticultural Products, Rolleston, New Zealand). Fresh root samples were cut 1–2 cm from the root tips and washed under running tap water to remove surface debris and soil. For basic histological stains, transverse hand sections were cut with a double-edged blade from unfixed roots and placed on slides for staining.

Histological and fluorescence staining

Cell walls were stained with 0.1 % (w/v) toluidine blue (Electron Microscopy Sciences, Hatfield, PA, USA) in distilled water. To demonstrate lignification of the secondary cell walls, root sections were stained with either Maule reaction or the phloroglucinol–HCl to visualize lignin compounds according to standard procedures (Strivastava 1966). In the Maule reaction, root sections were incubated in potassium permanganate [1 % (w/v), 10 min], washed in distilled water, acidified with hydrochloric acid [37 % (v/v), 1 min], incubated in sodium bicarbonate [5 % (v/v), 5 min] and then mounted in glycerol. The phloroglucinol stain was prepared by saturating a solution of phloroglucinol (BDH, Poole, UK) in 20 % (v/v) hydrochloric acid. Root sections were stained with several drops of this solution for 1 min before being rinsed and mounted in water.

For the fluorescent stains berberine hemisulfate and fluorol yellow, roots were first hand-sectioned, then cleared with potassium hydroxide [10 % (w/v), 1 h at 70 °C] and rinsed in distilled water. Sections were stained with berberine hemisulfate [0.1 % (w/v), 1 h] (Edward Gurr, London, UK) or with 0.01 % (w/v) fluorol yellow 088 (Setareh Biotech, Eugene, OR, USA) dissolved in 90 % (v/v) lactic acid (1 h). Sections were then counter-stained with 0.1 % (w/v) aniline blue in distilled water for 30 min to quench non-specific staining and background fluorescence, and finally mounted in glycerol (Lux et al. 2005).

For other fluorescent cell wall stains, Vibratome sections were used (see following section). Sections were stained with pontamine fast scarlet 4B (Sigma, St Louis, MO,

Table 1 Antibodies

Antigen	Supplier	Host	Dilution
Primary antibodies			
α-tubulin (clone B512)	Sigma (St Louis, MO, USA)	Mouse	1:1,000
Maize actin	Dr. Chris Staiger (Purdue University, West Lafayette, IN, USA)	Rabbit	1:200
Clathrin	Sigma	Goat	1:20
Callose [anti (1–3)-β D-glucan]	BioSupplies (Bundoora, Victoria, Australia)	Mouse	1:200

Target species	Supplier	Host	Dilution	Fluorochrome
Secondary antibodies				
Mouse	Silenus-Amrad (Boronia, Victoria, Australia)	Sheep	1:200	Fluorescein
Mouse	Jackson (West Grove, PA, USA)	Goat	1:200	Cy5
Rabbit	Jackson	Goat	1:200	Alexa fluor 647
Goat	Jackson	Cow	1:200	Texas red

USA) by incubation in pontamine stain (0.1 % (w/v) in distilled water) for 5 min, and then mounted in glycerol. For calcofluor white (Sigma), sections were stained [0.01 % (w/v) in distilled water that had been de-acidified with several drops of 1 N, NaOH] (5 min), rinsed in water and mounted in glycerol. Slides were sealed with nail polish to prevent drying.

Immunolabeling

Whole roots were fixed in PME solution (50 mM Pipes, pH 7.2 (K^+), 2 mM EGTA, 2 mM $MgSO_4$) supplemented with 0.1 % (v/v) Triton X-100), 3.7 % (v/v) formaldehyde, 0.5 % (v/v) glutaraldehyde, and 0.1 % (v/v) dimethyl sulfoxide for 1–2 h in vacuum. After fixing, roots were washed several times for 5 min in PME solution before extraction in PME solution with 1 % (v/v) Triton X-100 for 1 h. After washing in PME solution, the roots were permeabilized in methanol at −20 °C for 15 min, and then rehydrated in phosphate-buffered saline (PBS; 131 mM NaCl, 5.1 mM Na_2HPO_4, 1.56 mM KH_2PO_4, pH 7.2).

To section the roots, tissue were embedded in acrylamide in a method modified from Germroth et al. (1995). Samples were embedded in polyacrylamide [21 % (v/v) acrylamide (Biorad, Hercules, CA, USA), 0.01 % (v/v) TEMED (tetramethylethylenediamine) and 0.1 % (w/v) ammonium persulfate in PBS] and sectioned with a Vibratome 3000 Plus sectioning system (Vibratome, St. Louis, MO, USA) through the root tip. Acrylamide embedding allowed for better Vibratome sectioning of the orchid roots than the more commonly used embedding matrix of 3 % agar that we have previously used (Collings and Harper 2008). We suggest that this may be due to the acrylamide solution being able to enter the velamen layer before it polymerizes, thus giving the supporting matrix a better grip on the root.

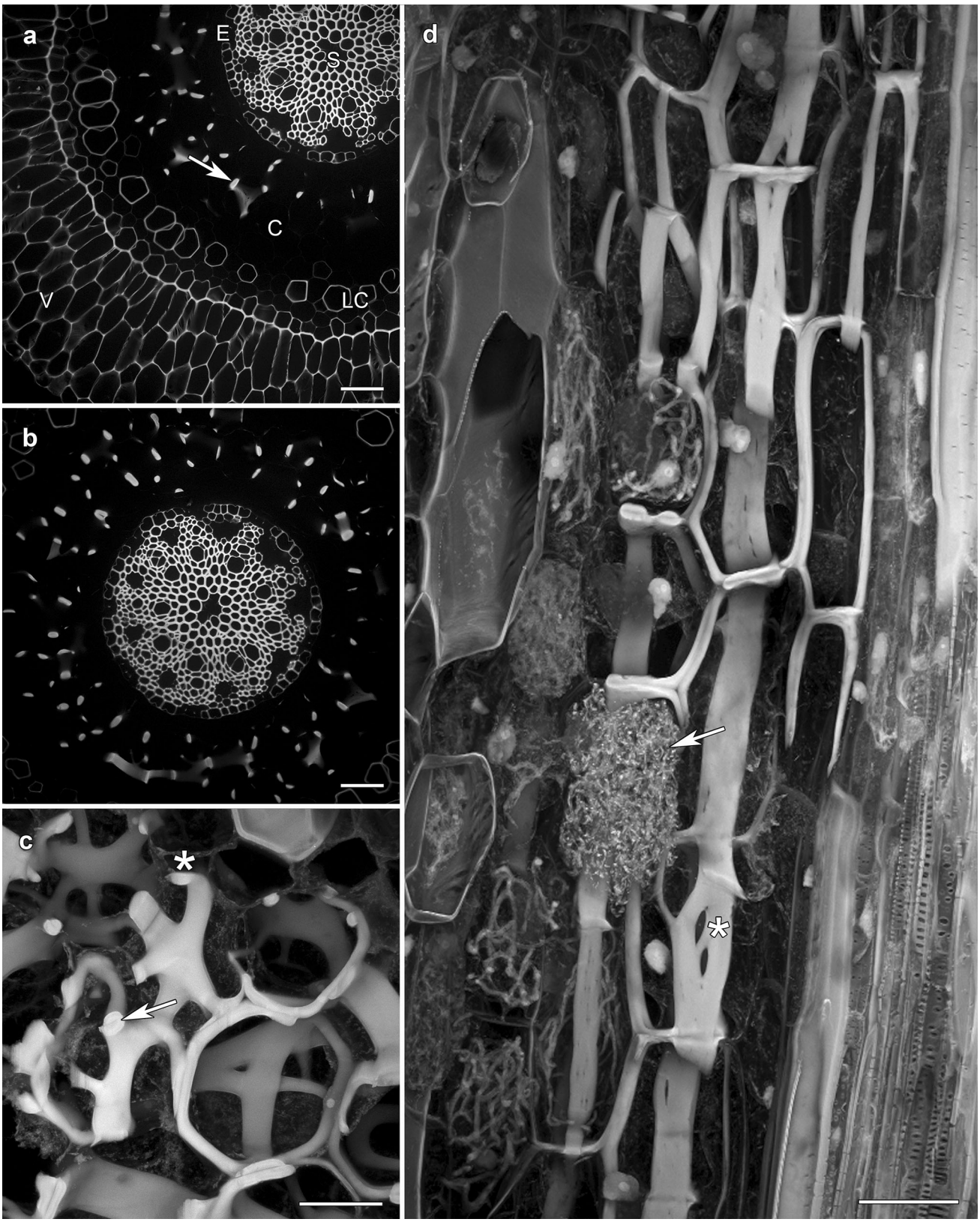
Sections were then added to 1 % (w/v) sodium borohydride in PBS for 15 min to reduce free aldehyde groups, and to eliminate aldehyde-induced autofluorescence. After washing in PBS, the sections were immersed in incubation buffer [PBS containing 1 % (w/v) bovine serum albumin (BSA) and 0.5 % (v/v) Tween-20] for 15 min. Primary antibodies (Table 1) were diluted in incubation buffer and incubated for 1 h at room temperature or overnight at 4 °C. After extensive washing with PBS for up to 30 min, samples were incubated in the secondary antibodies (Table 1) diluted in incubation buffer for 1 h at room temperature or overnight at 4 °C. After several washes in PBS, the sections were mounted on a glass slide with a drop of antifade agent AF1 (Citiflour, London, UK), then covered with a cover slip and sealed with nail polish.

Wheat germ agglutinin labeling

Whole roots were fixed and sectioned as described above, but with the methanol extraction and borohydride step was eliminated. Fixed and Triton-extracted roots were acrylamide embedded and sectioned. Sections were stained in 0.01 % (v/v) rhodamine-conjugated WGA (Vectorlab, Burlingame, CA, USA) for 30 min, rinsed and mounted in water.

Microscopy

Sections were observed with a confocal microscope (model Leica SP5, Leica, Wetzlar, Germany) with 20× NA 0.7 and 63× NA 1.3 glycerol-immersion lenses with images collected using multiple fluorescence and transmitted light modes. High-resolution images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◀**Fig. 1** Phi thickenings in *Miltoniopsis* acrylamide-embedded root sections stained with berberine hemisulfate. Images are either single confocal optical sections (**a**, **b**) or maximum projections of confocal stacks (**c**, **d**). **a** Phi thickenings (*arrow*) were present in the root cortex (C) inside a layer of lignified cortical cells (LC). Other tissues included the stele (S), velamen (V) and endodermis (E). **b** Phi thickenings could encircle the vascular tissue. **c** Phi thickenings encasing cells in a root cross section. Sections through phi thickenings typically showed similarities to the Greek letter Φ (*arrow*) although when only one cell developed wall thickenings, a half phi thickening similar to the Saxon/Icelandic letter Þ was present (*asterisk*). **d** In longitudinal sections, bands of phi thickenings were in alignment between adjacent cells, although gaps (*asterisk*) were present. Pelotons containing mycorrhizae could be present in cells with thickenings (*arrow*). *Bars* = 100 μm (**a**, **b**) and 50 μm (**c**, **d**)

(1,024 × 1,024 pixels) were collected with threefold line averaging, and for *Z*-stacks of optical sections, a 1.00 μm step-size was used. Calcofluor white fluorescence was collected from 420 to 480 nm using 405 nm excitation while berberine hemisulfate and fluorol yellow were both excited with the 405 nm laser and emission collected from 480 to 520 nm. Fluorescein was excited with the 488 nm laser, and fluorescence collected from 500 to 600 nm, Texas Red, pontamine fast scarlet 4B and rhodamine were excited with the 561 nm laser with emission from 580 to 680 nm, while Cy5 and Alexa Fluor 647 were excited with 633 nm laser with emission from 650 to 760 nm. Autofluorescence images from lignified cell walls were collected at 400 to 480 nm using the 405 nm laser. In multiple labeling experiments, the different fluorophores were excited sequentially to prevent cross-talk between the lasers and fluorophores. Transmitted light images were collected concurrently to the confocal images, either as brightfield images or in polarized light mode through the manual addition of a polarizing filter to the light path after the sample. Computer generated 3D reconstruction of images were produced using Leica LAS AF software and all images were processed with Adobe Photoshop version CS4 (version 11.0.1, Adobe Systems, San Jose, CA USA).

For observations of pontamine bifluorescence, samples were imaged using the same confocal microscope setup, but with the normal galvo stage replaced with a polarized light microscope stage which allowed free rotation of the sample that generated different excitation polarizations (Thomas et al. 2013). Fluorescence and polarized light images were recorded at different orientations, with these orientations then corrected in Photoshop.

For the histological stains, color transmitted light and polarized light images were collected along with fluorescence images using UV excitation. A color camera system (model DFC310FX, Leica) was attached to the confocal microscope, and images collected and viewed with Leica LAS imaging software.

Results

Phi thickenings in *Miltoniopsis*

Miltoniopsis roots are thick (diameter ~12 mm) and, as found in many epiphytic orchids, have a velamen layer external to the exodermis. This layer is three to four cells wide, consists of dead cells that have special thickenings in the cell walls, and aids in rapid water absorption (Zotz and Winkler 2013). The cortical region inside the exodermis is usually eight to nine cells wide, and contains some lignified cortical cells that are arranged peripherally, and idioblast cells containing raphides subjacent to the exodermis. Phi thickenings distribute throughout the cortical layer of the root, either singularly, in patches or even encircle the entire vascular cylinder. As they occur in multiple cell layers, they are type 3 phi thickenings (Fig. 1a, b).

To better understand phi thickening organization, we generated 3D reconstructions of acrylamide-embedded cross and longitudinal root sections that contained phi thickenings that had been fluorescently labeled with berberine hemisulfate (supplementary movies 1 and 2). Although the cell wall thickenings were often irregular, with the formation of the branched and unbranched modifications around cells, in most cases the wall thickenings were perfectly aligned between adjacent cells so that they formed the Greek letter phi (Φ) in cross section (arrow, Fig. 1c). However, in some cases, the cell wall thickenings did not align, with a thickening present in one cell but not the adjacent one [which generated patterns similar to the Old English/Icelandic letter thorn (Þ)] (asterisk, Fig. 1c). A longitudinal view of the root showed that the thickenings were also aligned in long bands between cells running through the root length and parallel to the vascular tissue (Fig. 1d). The formation of phi-thickened cell walls did not inhibit the colonization of orchid mycorrhiza, shown by the presence of pelotons or specialized hyphal coils, in various stages of development in the vicinity of the thickenings (arrow, Fig. 1d). More intriguing were the gaps and uneven textures present in the phi thickening surface (asterisk, Fig. 1d).

Histological characterizations show lignification but not suberization

We used several common lignin stains to characterize phi thickenings in orchids in the genus *Miltoniopsis* (Fig. 2). The Maule reaction differentiates guaiacyl (G)-rich lignin from syringyl (S) lignin by staining them red and brown, respectively. In *Miltoniopsis*, the surface of the phi thickenings stained red with phloroglucinol, confirming the presence of lignin, although the stain was only observed at the cut surface suggesting a lack of infiltration through the

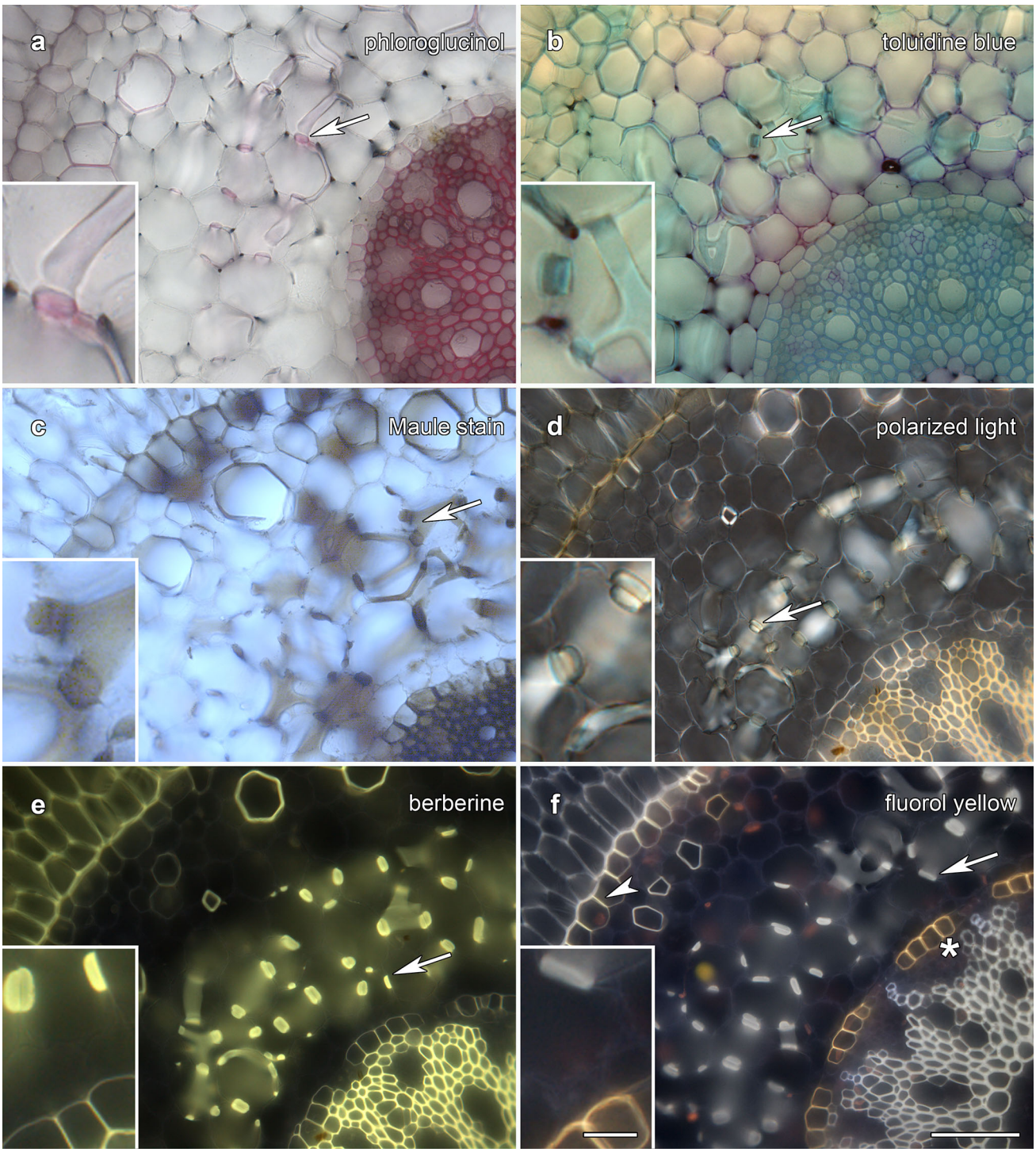


Fig. 2 Histological observations of phi thickenings. Images of hand-sectioned roots were collected using a conventional, non-confocal microscope in either transmitted light (**a**–**c**), polarized light (**d**) or fluorescence modes (**e**, **f**) with phi thickenings (*arrows*) shown at higher magnification in *insets*. **a** Phloroglucinol stained the surface of phi thickenings red consistent with the presence of lignin. **b** *Toluidine blue* stained phi thickenings *blue* indicating a secondary cell wall. **c** *Browning* of phi thickenings with Maule stain showed the presence of G lignin. **d** Phi thickenings under polarized light showed strong birefringence. **e** Berberine hemisulfate fluoresced *yellow*, consistent with the presence of both lignin and suberin. **f** *fluorol yellow* differentiated phi thickenings from the *brightly yellow* suberized endodermis (*asterisk*), and the less strongly suberized exodermis (*arrowhead*). *Bars* = 100 μm for *main image*, 10 μm for *inset*

section (Fig. 2a). Toluidine blue is a metachromatic stain that is commonly used to differentiate lignin or secondary cell wall formation (stained a greenish blue color) from primary cell walls containing pectin (stained reddish purple). The blue phi thickenings confirmed the presence of a lignified secondary cell wall in the cortical cells (Fig. 2b). To further verify the lignification of the phi thickenings, Maule staining showed brown staining of both the phi thickenings and the vascular cylinder, indicating a higher composition of G lignin, which is more commonly found in gymnosperms as they lack S lignin (Fig. 2c). Formed from highly ordered arrays of cellulose, the phi thickenings were also readily observable with polarized light (Fig. 2d).

To test for suberization of phi thickenings in *Miltoniopsis*, two fluorescent stains (berberine hemisulfate and fluorol yellow 088) were used to stain root sections (Fig. 2e, f). With ultraviolet excitation, berberine hemisulfate generated yellow fluorescence in both the phi thickenings and the endodermis, a structure which should contain suberin. This is consistent with studies that have shown that berberine hemisulfate do not bind specifically to suberin but rather to lignin (Brundrett et al. 1988). Fluorol yellow 088, however, is more specific to suberin, and fluoresces yellow when excited with ultraviolet light. Compared to the suberized endodermis which fluoresced yellow, and the weakly suberized exodermis which had some yellow fluorescence, the phi thickenings fluoresced the same blue to white color as the central stele. This indicated that they lacked suberin. These observations of lignified but non-suberized phi thickenings are consistent with phi thickening composition in other plants such as apple (Mackenzie 1979), *Pelargonium* (Haas et al. 1976), *Ceratonia* (Pratikakis et al. 1998) and loquat (Pan et al. 2006).

Phi thickening development

Phi thickenings were present in the apical 3–5 mm of the root tip, and could be identified via observations under transmitted light, polarized light, and autofluorescence under ultraviolet light. These different observational modes showed subtle differences during the development of phi thickenings (Fig. 3). Polarized light provides the best method of detecting phi thickenings, with the polarized effect most intense in mature phi thickenings, although these mature thickenings were also clearly visible with transmitted light and fluorescence (Fig. 3c). Developing thickenings, however, which were visible with polarized light and weakly visible by transmitted light could completely lack lignin autofluorescence. This suggested that lignin deposition begins sometime after the initial formation of the thickening (Fig. 3a). Lignin autofluorescence can also be used to measure the depth of the thickenings. Mature thickenings averaged 9.7 ± 0.1 μm ($n = 20$) from one side of the thickening in one cell to the edge in the adjacent cell thickenings (±standard error). Developing thickenings averaged only 4.8 ± 0.1 μm ($n = 20$). However, the developing thickenings used for these measurements already had some autofluorescence indicating that they were already at an intermediate stage of development (Fig. 3b).

Cellulose organization in phi thickenings

Cellulose microfibril orientation can also be determined through optical microscopy by the use of polarized light microscopy, as cellulose microfibrils are birefringent having the ability to rotate the polarization plane of light (Abraham and Elbaum 2013), and through the polarization-dependent fluorescence (bifluorescence) of the cellulose-specific dye pontamine fast scarlet 4B which only fluoresces when bound to cellulose that is ordered parallel to the polarization orientation of the excitation light (Thomas et al. 2013). We generated different polarizations of the excitation lasers using a rotating stage on the confocal microscope. Polarized light images demonstrated changes in birefringence associated with the phi thickenings in cross sections that were consistent with cellulose aligned lengthwise, but the presence of multiple cell wall layers within the sections did confound the images (data not shown). Pontamine fluorescence, however, confirmed the longitudinal cellulose orientation. Images showed fluorescent bands at the edges of the thickenings that ran only in the horizontal direction. When the sample was rotated through 90°, mimicking a rotation in laser polarization, only the vertical bands fluoresced, an effect that was strikingly demonstrated in color overlays (Fig. 4). Thus, the pontamine bands running along the edges of the phi thickenings are consistent with cellulose aligned lengthwise within the edging band and the thickening. Pontamine labeling within the thickening proper was, however, blocked by the lignification of the cell wall, as seen in pine (Thomas et al. 2013). Lignin autofluorescence, which was not birefringent, remained unchanged when the sample was rotated (data not shown).

Microtubules during phi thickening development

Organization of microtubule arrays plays an important role in the development of a plant cell wall. As such, it is interesting to see the organization of microtubules in the formation of a secondary wall such as the phi thickenings. We used indirect immunofluorescence, with monoclonal anti-α-tubulin and confocal microscopy, to track microtubule organization during phi thickening development. The changes in cell wall autofluorescence and thickness

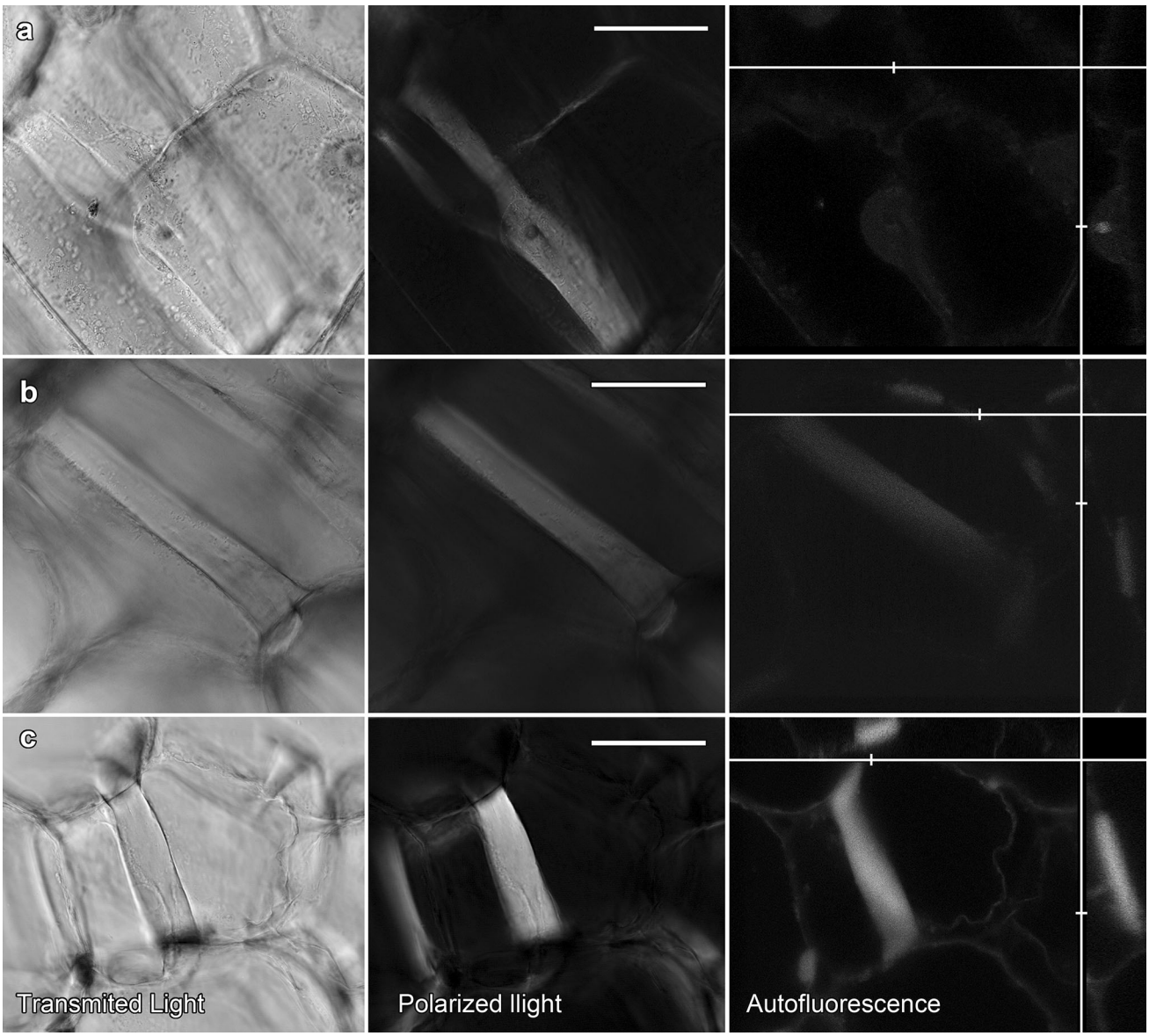


Fig. 3 Different stages of phi thickening development. Sections were imaged with transmitted light (*left column*), polarized light (*middle column*) and lignin autofluorescence (*right column*, single confocal section), with optical cross sections reconstructed from confocal stacks (*location marked*). **a** Young developing phi thickenings were faint with transmitted light and autofluorescence but visible with polarized light. **b** Intermediary phi thickenings were more visible with transmitted light and autofluorescence. **c** Mature phi thickenings were clearly observed by transmitted light, polarized light and autofluorescence. These were also thicker, as seen in cross section. *Bars* = 50 µm

correspond to changes in microtubule organization in cells with phi thickenings, with the same three stages being visible during thickening development (Fig. 5). Microtubule organization in cells developing phi thickenings differed greatly from surrounding cells where oblique microtubules were spaced evenly through the cell. Instead, microtubules associated with developing phi thickenings ran parallel to the length of the thickening, as would be expected if cellulose ran lengthwise. In the early developing stage, dense bands of microtubules ran parallel along developing thickenings, where formation could be determined with polarized light but with exhibited only faint autofluorescence (Fig. 5a). The intermediary stage of development had fewer microtubules present, although these still ran parallel along the length of the thickening that had increased autofluorescence (Fig. 5b). Finally, mature phi thickenings that had strong autofluorescence lacked any association with microtubules, suggesting that cellulose deposition may have ceased (Fig. 5c).

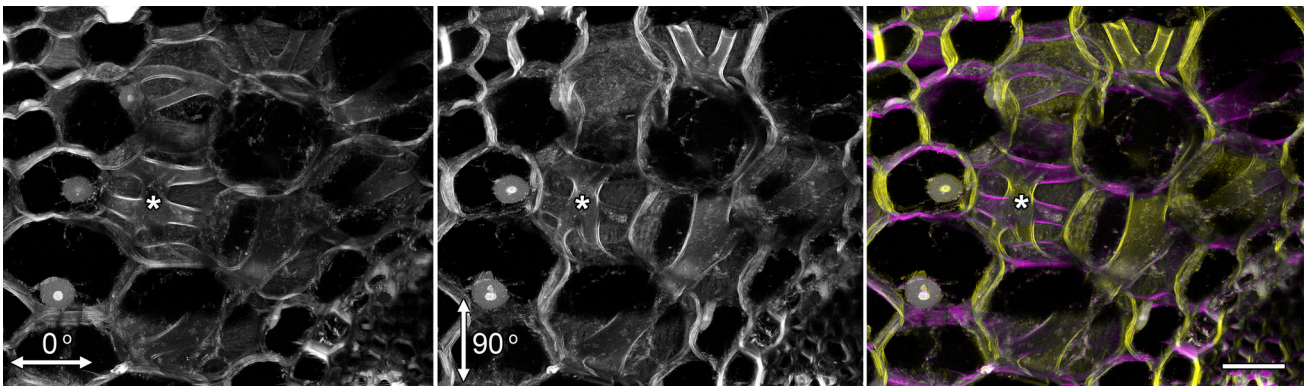


Fig. 4 Determination of cellulose orientation in phi thickenings by pontamine bifluorescence. Pontamine fluorescence only occurs when the excited, cellulose-bound dye is parallel to the direction of laser polarity (*arrows*). Images are maximum projections of confocal stacks. When the sample was rotated through 90°, fluorescence from the phi thickenings (*asterisks*) changed showing that cellulose ran lengthwise through the thickening edges, as explained by the overlay image. *Bar* = 50 µm

Actin microfilaments were also investigated in cells containing phi thickenings using polyclonal anti-maize actin. There were no associations observed between the microfilament bundles and either developing or mature phi thickenings (Fig. 6a, b). This is not altogether surprising, as the immunofluorescence protocols used may not have preserved finer, cortical actin microfilaments associated with the plasma membrane and cell wall formation, and because any signaling and alignment might have occurred before any formation can be detected. No association was established either between phi thickenings and clathrin immunolabeling. Minute dots or immunolabeled vesicles were observed scattered throughout the cell with no association with the phi thickenings (Fig. 6c).

Callose deposition during phi thickening development

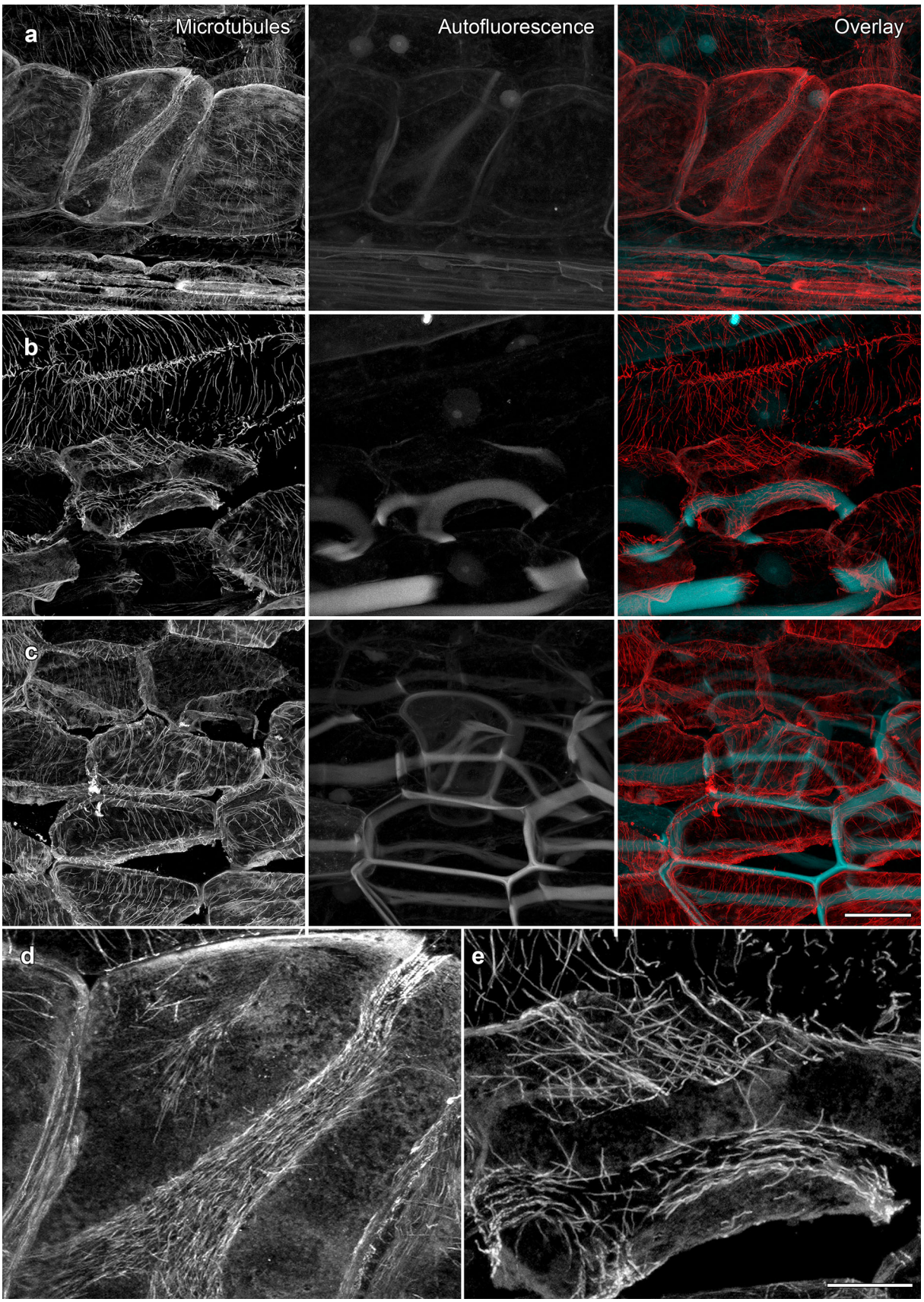
Immunolabeling with antibodies against callose [anti (1-3)-β D-glucan] showed consistent deposition of callose in cell wall pit-fields (Fig. 7) and cell plates and the cross walls of newly divided cells within the meristem (data not shown). Callose labeling was also present in developing phi thickenings that were characterized by less-intense autofluorescence and a thinner thickening diameter across the two adjacent cells (Fig. 7a, c). This textured deposition eventually vanished as thickenings matured and increased in diameter (Fig. 7b). In some cases, the textured deposition was fibrillar- or strand-like, clearly reminiscent of microtubule organization (arrow, Fig. 7d). Counterstaining with the cellulose stain calcofluor white increased the fluorescence of the cell walls and dramatically aided the visualization of callose localization. New, developing 'branches' of phi thickenings were distinguished by callose deposition, even though no indication of the developing thickening could be observed by calcofluor cell wall fluorescence (asterisk, Fig. 7c).

Other cell wall components during phi thickening development

Fluorescently tagged WGA is a stain that has been extensively used to label chitin in the cell walls of fungi (Deshmukh et al. 2006; Hogekamp et al. 2011; Wang et al. 2013). During investigations of the orchid mycorrhizae in orchids of the genus *Miltoniopsis*, we observed that the stain also labeled the developing phi thickenings. WGA labeling of the thickenings behaved in a similar way to callose labeling being present in early development (Fig. 8a) and absent when the thickenings were autofluorescent and mature (Fig. 8b). However, WGA labeling was found to wash away with either of the methanol or sodium borohydride washes used during the regular immunolabeling procedure (data not shown). WGA-labeled idioblast cells that contained raphides (crystals) (arrow, Fig. 8c) as well as degrading orchid mycorrhizal pelotons (asterisks, Fig. 8d) were used as positive controls to ensure correct labeling of phi thickenings. Interestingly, mycorrhizae in undegraded pelotons did not label with WGA (Fig. 8d).

Phi thickenings can form throughout the root system

Cells with developing thickenings were usually found scattered towards the root tip. It was initially thought that phi thickenings only developed in cells near the root tip, as it was difficult to differentiate developing phi thickenings from mature ones using polarized light. However, with the use of tubulin and callose immunolabeling, as well as


a
Microtubules
Autofluorescence
Overlay
b
c
d
e

◀**Fig. 5** Different stages of microtubule organization during development of phi thickenings observed with indirect immunofluorescence using anti-α-tubulin. **a** Dense alignment of microtubules (*left column*) along the length of the young, developing phi thickenings that were detected by their faint autofluorescence (*middle column*). **b** Increased autofluorescence, but with microtubules still aligned, marked an intermediary stage in phi thickening development. **c** In the mature stage, microtubules did not associate with the intensely autofluorescent phi thickenings. Higher magnification of the microtubule association in developing (**d**) and intermediate stages (**e**) of phi thickening development. *Bar* in **c** = 50 μm for **a**–**c**, *bar* in **e** = 20 μm for **d**, **e**

WGA staining, we demonstrated the development of new phi thickenings in cells and regions that already contained mature phi thickenings (Fig. 9). A mature phi thickening indicated by strong fluorescence (arrow) but no callose deposition was easily detected among young developing phi thickenings (Fig. 9a) while a strongly WGA-labeled young, developing phi thickening (arrow) was present among networks of mature phi thickenings (Fig. 9b). Similarly, aligned microtubules were also present on

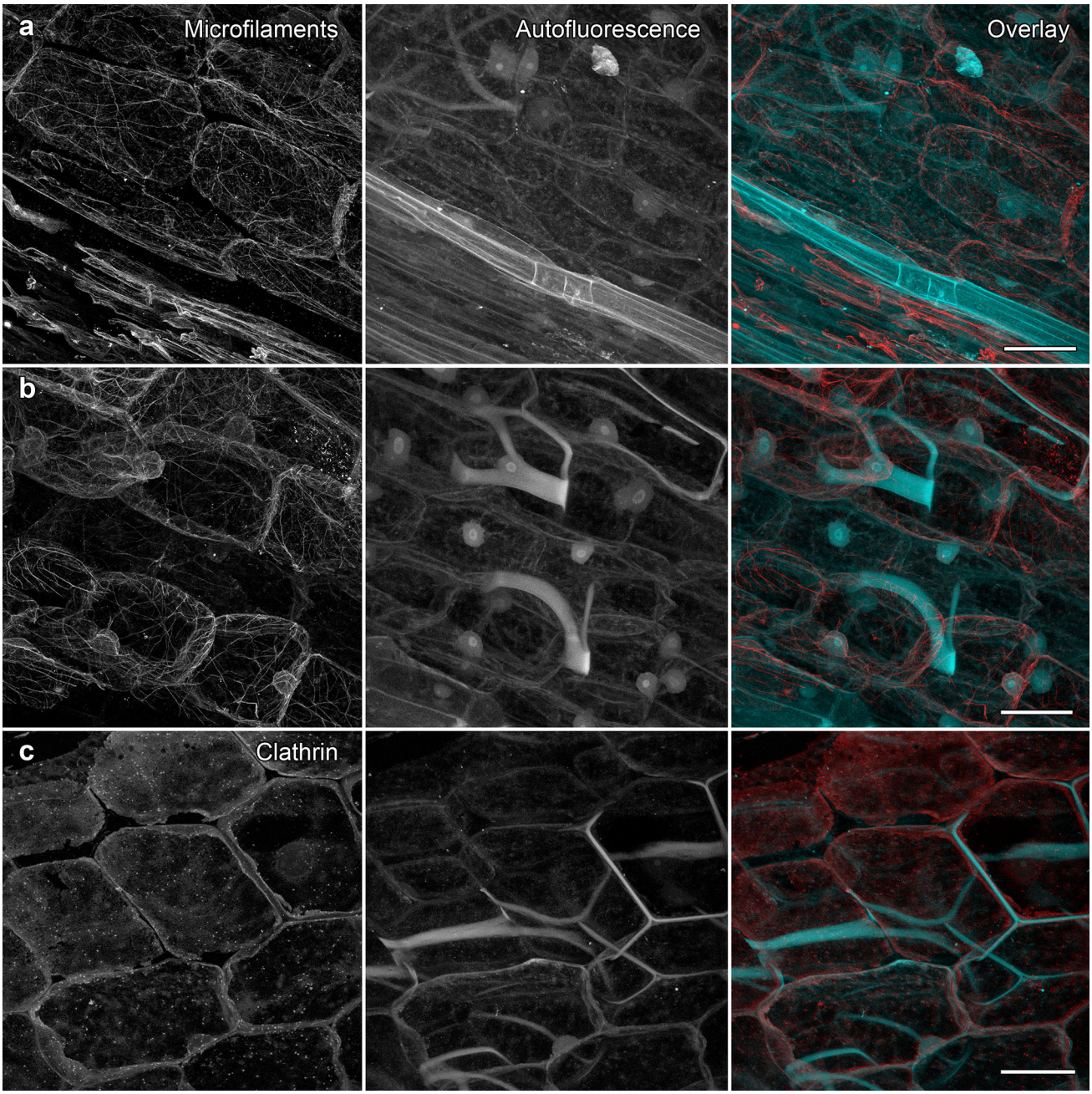


Fig. 6 Actin microfilaments do not associate with developing phi thickenings either in developing (**a**) or mature (**b**) phi thickenings. **c** there was no association of clathrin with phi thickenings. *Bars* = 50 μm

occasions in areas of dense phi thickening networks (arrow, Fig. 9c).

Discussion

Phi thickenings in *Miltoniopsis* are typical of those found in a wide range of angiosperms and gymnosperms, being lignified and non-suberized secondary cell walls present in cells that normally have only a primary wall. Although this was previously known, our use of confocal microscopy has allowed the formation and development of the thickening network to be examined more clearly. Being type 3 phi thickenings, they can form dense networks that radiate and propagate between cells through the cortical region. In comparison, phi thickenings found in broccoli are only formed in the innermost cells of the cortical layer next to the endodermis (Fernandez-Garcia et al. 2009) while in maize, phi thickenings occur only in the rhizodermis or outermost root cell layer (Degenhardt and Gimmler 2000). Within the *Miltoniopsis* phi thickening networks, the majority of thickenings are coordinated so that adjacent cells form a full phi (Φ) in cross section, although a minority of cases only forms a thorn (Þ). This raises the obvious puzzle as to how the phi thickenings are coordinated spatially, and there is also the thorny question (excusing the pun) as to why some cells fail to generate their half-phi. This problem then expands to ask how and why the thickenings are coordinated through long stretches of cells, as shown in Fig. 1d.

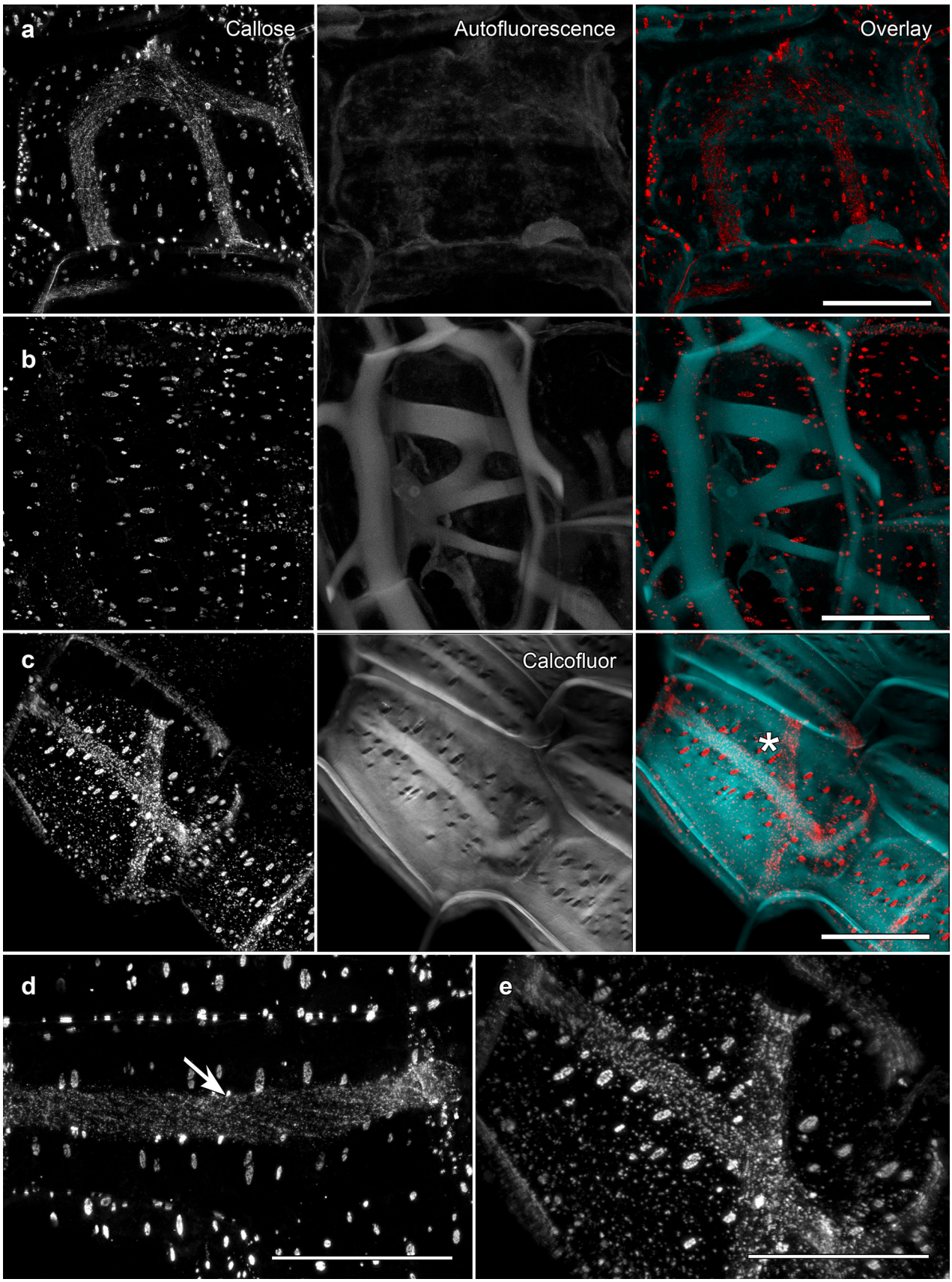
The development of phi thickenings in *Miltoniopsis* is consistent with longitudinally oriented cellulose in phi thickenings in Pelargonium, as observed by electron microscopy (Haas et al. 1976) and with formation of other secondary cell wall thickenings where cellulose runs lengthwise within the thickenings and parallel to microtubules. Cytoskeletal studies of secondary wall development in differentiating xylem have shown the dynamics of microtubule organization, with the dynamics of microtubules and microtubule-associated proteins regulating secondary wall patterning (Oda and Hasezawa 2006; Oda and Fukuda 2012). However, our work is the first in which microtubules have been linked to the alignment and deposition of cellulose during the development of phi thickenings. Upon maturation, the microtubules cease to align with the thickened wall as expected. Nevertheless, individual microtubules were associated with phi thickenings in apple roots (Mackenzie 1979), but whether this represents stray microtubules associated with developing of mature thickenings cannot be determined. We also observed microtubule alignment with the developing helical wall striations of the velamen, another secondary wall-like structure present in orchids (data not shown).

Fig. 7 Callose deposition during the different stages of phi thickening development. **a** Faint autofluorescence indicated a developing phi thickening and was associated with callose deposition. **b** No callose was detected in mature phi thickenings. **c** Deposition of callose in young, developing phi thickenings (*asterisk*) counter-stained with *calcofluor white* to demonstrate that the cell wall surface *dotted* was with pit-fields. Newly developing phi thickenings can be distinguished by their callose deposition and only weak calcofluor staining. High magnification of the fibril-like (**d**) and textured (**e**) callose deposition in developing phi thickenings (*arrow*). *Bars* = 50 μm

The formation of matched phi thickenings in adjacent cells means that microtubule organization must be coordinated between subdomains within adjacent cells. While microtubule arrays are coordinated in elongating cells in transverse arrays, with coordination reduced in specific *Arabidopsis thaliana* mutants such as *rsw6* (Bannigan et al. 2006) and also organized between adjacent epidermal cells in *Tradescantia* (Barton and Overall 2010), the precise coordination of microtubules in specific and localized subdomains such as the development of the phi thickenings may be novel. The discontinuous nature of the phi thickening network is intriguing, as cell-to-cell signaling must happen for the precise alignment of the thickenings in adjacent cells. Endocytosis is a known regulator of signaling receptors on the cell surface into the cell with the clathrin-mediated pathway one of the most studied mechanisms of endocytosis (Chen et al. 2011; Kitakura et al. 2011). Clathrin was immunolocalized with the possibility of finding some association between endocytotic vesicles and the developing thickening but none could be observed, although this does not mean that signaling might not have occurred earlier during thickening development.

Orchid root phi thickenings

Studies on phi thickenings in orchid roots have been limited to a single description Moreira and Isaias (2008). However, several earlier studies investigating the orchid velamen, the spongy layer of dead cells that lies external to the root cortex, also described the presence of velamen-like cells in the root cortex. These cells, termed a pseudovelamen (Porembski and Barthlott 1988; Burr and Barthlott 1991), can have reticulate secondary cell walls that are eerily similar to phi thickenings. In their description of the pseudovelamen, Burr and Barthlott (1991) noted that van Tieghem (1871) has observed 'cortical tissues with lignified, annular thickenings in the direction of the long axis of the root' in numerous gymnosperms and angiosperms which he had described as a support network. These structures were subsequently described as phi thickenings (Russow 1875). In our view, it is probable that the pseudovelamen is actually a specialized version of the phi thickenings in which the pattern of the wall thickenings can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become more complex, and can be used for taxonomical purposes (Figueroa et al. 2008; Pedroso-de-Moraes et al. 2012). These pseudovelamen cells within the living cortex are, however, fundamentally different to the velamen in which the cells have died.

The constituents of the phi thickening cell wall

We report three novel features about the composition of the phi thickening cell wall. These are that the developing wall is initially non-lignified, with lignification beginning only some time after secondary cell wall deposition begins, that callose is associated with the developing wall, and also that WGA can bind to this developing wall.

Callose is a plant polysaccharide regularly found at the plasmodesmata and cell plate during cytokinesis but is also known to be deposited in the cell wall as a response to wounding or pathogen infection. Callose synthases produces callose and it is degraded by β-1,3-glucanases (Luna et al. 2011). Callose is also observed in transfer cells at sites of the wall ingrowths (McCurdy et al. 2008) and it is deposited in channel-like structures at the base of newly forming wall ingrowths in pea nodule transfer cells. When mature, callose deposition disappears and can only be found at the main body of the wall invaginations (Dahiya and Brewin 2000). They proposed that the channel-like structures with callose deposition may actually be 'multiply branched plasmodesmata' and increase open channels to reduce the diffusion resistance in the apoplast. However, in the phi thickenings, callose deposition was quite distinct from the plasmodesmata pit-fields.

The textured deposition of callose in developing phi thickenings, with patterns running parallel to the orientation of microtubules, is consistent with observations of callose deposition being linked to microtubules in three other systems. This association with microtubules was first reported by Scherp et al. (2002), who proposed a model where callose and cellulose biosynthesis are both regulated by the binding of callose and cellulose synthase complex to cortical microtubules. In tobacco pollen tubes, callose and cellulose biosynthesis associate differently, but callose synthase remains dependent on cortical microtubule organization (Cai et al. 2011). Fibrillar arrays of callose running parallel to radial microtubules have also been observed in fern stomatal cells. Because microtubule depolymerization with oryzalin disrupted callose deposition, this must be linked to microtubule organization (Apostolakos et al. 2009). We have yet to explore this link between callose deposition and microtubule organization in phi thickening development, as controlled application of microtubule disrupting drugs such as oryzalin to *Miltoniopsis* roots growing in soil may be difficult.

Fig. 8 WGA labeling during phi thickening development. Overlay of ▶ WGA-labeled phi thickenings (*red*) and autofluorescence (*cyan*) show young (**a**) and mature (**b**) stages of phi thickening development. **c** WGA-labeled idioblast cells (*arrow*) and (**d**) degrading mycorrhizal pelotons (*asterisk*) as positive controls. *Bars* = 100 μm

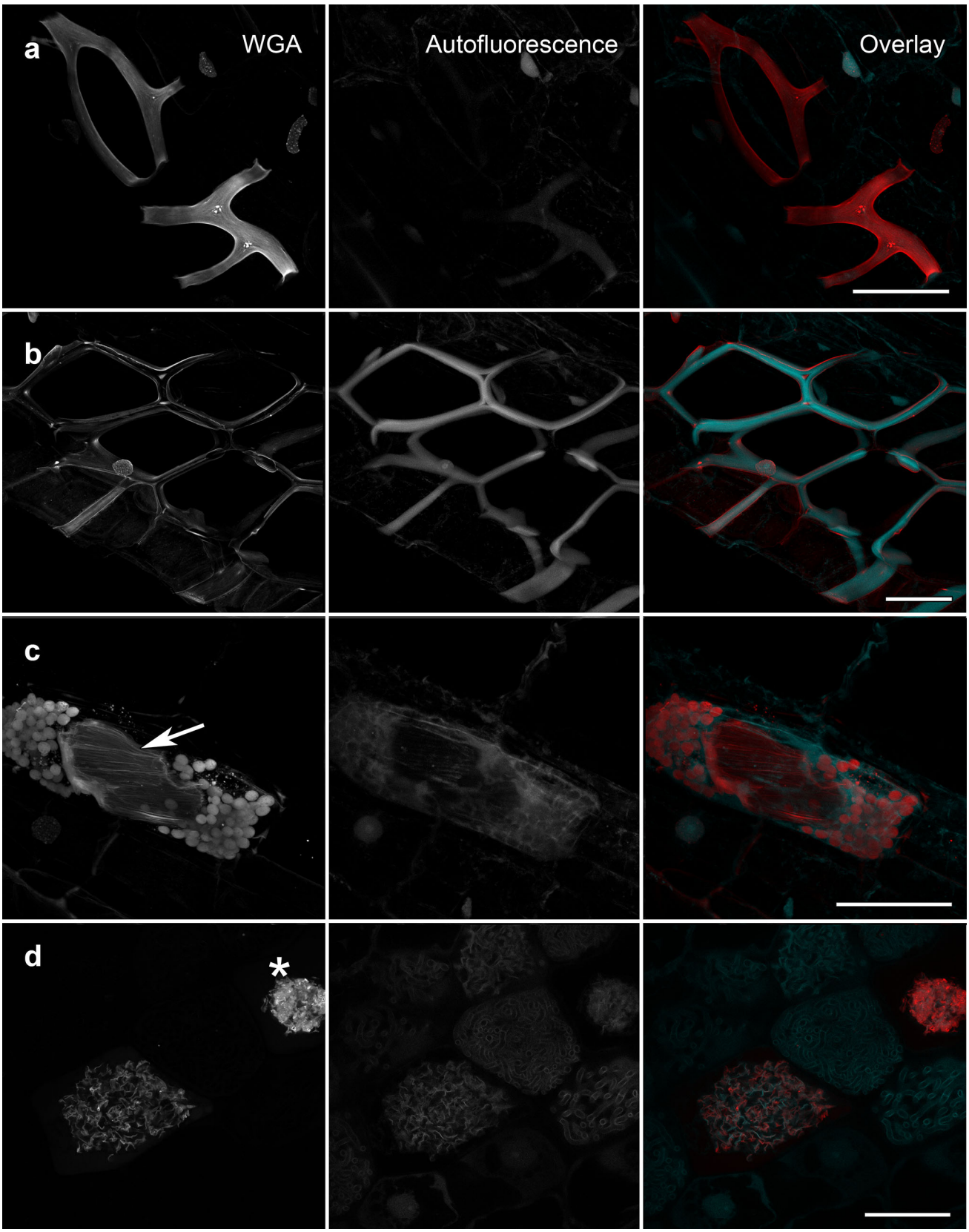
Changes in cell wall components during phi thickening development were also documented using rhodamine conjugated to the lectin WGA. WGA bind specifically to *N*-acetyl-D-glucosamine and sialic acid (Mansfield et al. 1988) with these commonly found in cell wall peptidoglycans, chitin or glycolipids. Thus, WGA has been used extensively to label fungi within plant roots (Deshmukh et al. 2006, Hogekamp et al. 2011, Wang et al. 2013) although somewhat surprisingly, we only observed WGA labeling of mycorrhizae in *Miltoniopsis* as pelotons undergo degradation. However, WGA has also been shown to bind specifically to plant tissues notably during the development of the secondary wall in xylem (Hogetsu 1990; Wojtaszek and Bolwell 1995; Oda and Hasezawa 2006). Interestingly, in one of these studies, WGA labeling was only observed during the differentiation of vascular cells with a decrease in binding upon maturity (Hogetsu 1990) which is consistent with this study. The nature of this secondary wall labeling remains unclear, as the WGA targets (*N*-acetyl-D-glucosamine and sialic acid) are not normal components of the plant cell wall. While Hogetsu (1990) suggested that WGA might be binding to hemicellulose, Wojtaszek and Bolwell (1995) confirmed that WGA bound to specific secondary cell wall glycoproteins present in French bean hypocotyls.

The labeling that we observed in the vacuole of raphide (crystal)-containing cells was exceptionally strong and, unlike the labeling of phi thickenings, not subject to being washed from the tissue. This labeling remains unexplained and, as far as we can see, there are no similar reports in the literature.

The possible functions of phi thickenings

Although phi thickenings have previously been reported in the roots of the terrestrial orchid *Prescottia montana* (Moreira and Isaias 2008), their function within orchid roots remains unexplored. Orchids contain numerous growth adaptations, including the presence of the velamen layer to aid in the uptake and retention of water (Zotz and Winkler 2013). It seems possible that the presences of phi thickenings are also associated with responses to water stress.

In other plants, one of the many speculated functions of the phi thickenings is to regulate nutrient uptake in a manner similar to the Casparian band in the suberized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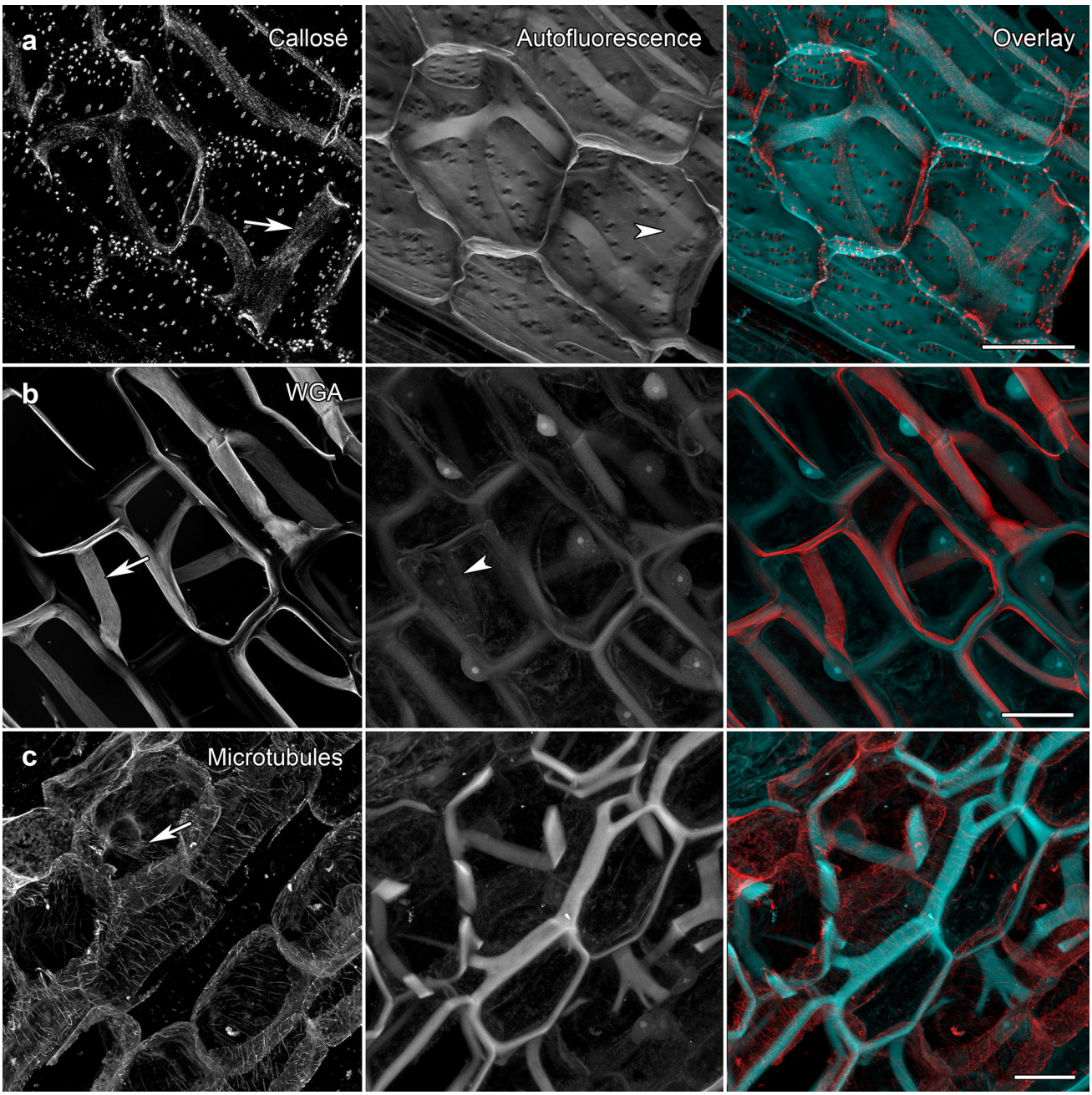

Fig. 9 Phi thickenings develop at different rates, so that immature Φ thickenings could be found amongst mature thickenings. **a** A developing phi thickening (*arrow*), marked by faint autofluorescence (*arrowhead*) but no callose deposition among developing phi thickenings. **b** WGA-labeling newly forming phi thickenings (*arrow*), yet undetected by autofluorescence (*arrowhead*). **c** microtubules align to a developing phi thickening (*arrow*) among a dense mature phi thickening network. *Bar* = 50 μm

endodermis. Despite not being composed of suberin (Mackenzie 1979; Brundrett et al. 1988) and not blocking apoplastic flow of calcofluor white (Peterson et al. 1981), the phi thickening's role in regulation of uptake should not be ruled out. Fernandez-Garcia et al. (2009) demonstrated that the phi cell layer can be a barrier for lanthanum ion movement when used as an apoplastic tracer in *Brassica oleracea* under salt stress. Furthermore, the importance of suberin as the main constituent of the Casparian band in regulating solute uptake is also under revision as Naseer et al. (2012) reported that mutant *Arabidopsis* plants that lack suberin still have a functioning, lignified Casparian band.

Phi thickenings have also been suggested to block the penetration of ectomycorrhizal fungi in the vascular cylinder. This concept is based on observations of coordinated

development of fungal invasion and the formation of type 1 phi thickenings in the cell layer immediately around the endodermis in both *Dryas* (family Rosaceae) (Melville et al. 1987) and yellow birch (Wilcox and Wang 1987). It would seem unlikely that this is the reason that phi thickenings develop in *Miltoniopsis*, as cells with the type 3 thickenings that we observed could contain pelotons (see Fig. 1d).

Phi thickenings may also develop and function as a response to environmental stress. This was also proposed by Degenhardt and Gimmler (2000), who reported an increased formation of phi thickenings in older portions of *Zea mays* roots grown in slag which is dense, and high in heavy metal concentration and salinity. In broccoli, phi thickenings are also rapidly formed as a response to salt stress (Fernandez-Garcia et al. 2009). In orchids of the genus *Miltoniopsis*, an epiphytic orchid, it will be interesting to determine whether substrate plays a role in the formation of the phi thickenings. Preliminary observations on *Miltoniopsis* plants propagated and grown in tissue culture show an absence of phi thickenings, but that these are induced when grown in potting mix (unpublished). Similar observations have been made in cherry (*Prunus avium*) and oak (*Quercus robur*) where phi thickenings were not found in roots grown in vitro but were present when the same plantlets were acclimatized and grown in soil (Soukup et al. 2004). This study and others in a variety of plant species (Degenhardt and Gimmler 2000; Gerrath et al. 2005; Pan et al. 2006; López-Pérez et al. 2007), suggest environmental factors and interactions are involved in the formation of phi thickenings, and that these structures function to provide mechanical support for the root cortex. Being a lignified secondary wall, the thickenings would be able to mechanically support the cortical cell during environmental stresses. These concepts are now being tested in *Miltoniopsis* using confocal microscopy and other techniques.

Conclusions

The development of phi thickenings is similar in terms of the deposition of cellulose in the formation of a secondary cell wall. However, the association of developing phi thickenings with callose and WGA warrants further investigation into the purpose of these peculiar root structures. From root solute uptake regulation, soil compaction or salinity response, to defense mechanism against pathogens, or in the case of *Miltoniopsis*, symbiotic mycorrhizal interaction, the life of phi (thickenings) remains a long journey to be explored.

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