Casparian bands occur in the periderm of *Pelargonium hortorum* stem and root

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INTRODUCTION

Casparian bands are impregnations of suberin, a polymer with poly(aromatic) and poly(aliphatic) domains, in the intermicrofibrillar spaces of primary cell walls (Schreiber et al., 1994; Schreiber, 1996; Zeier and Schreiber, 1997). Due to their location, the bands essentially prevent movement of ions and possibly also water through the cell walls (see Enstone et al., 2003). Casparian bands are a well-known feature of roots, where they occur in both the endodermis and the exodermis (Evert, 2006). They have been less frequently observed in stems and leaves (Lersten, 1997).

Casparian bands are often, but not always, associated with suberin lamellae. These lamellae are structures that occur on the inner face of the primary walls. In some species, the root endodermis does not develop lamellae so that only the bands are present. By contrast, the mestome sheaths in leaves of the endodermis does not develop lamellae so that only the bands are present. By contrast, the mestome sheaths in leaves of *Pelargonium hortorum* have suberin lamellae but not Casparian bands (Eastman et al., 1988a, b). In the periderm, mature phellem cells characteristically possess several layers of suberin lamellae (Sitte, 1955). To the best of our knowledge, Casparian bands have never been detected in this tissue in any species. It is possible that their presence is masked by the suberin lamellae.

In the current study, the presence or absence of a Casparian band in the phellem of *Pelargonium hortorum* stems and roots was determined. We used a well-established fluorescence staining procedure that previously allowed observation of Casparian bands in the endodermis and exodermis of roots when suberin lamellae were also present (Brundrett et al., 1988). In addition to other histochemical tests, berberine, a fluorescent apoplastic tracer, was used to probe the permeability of the phellem primary walls. Development of the periderm was followed in both normal and wounded stem tissue.

MATERIALS AND METHODS

Plant material and growth conditions

Plants of *Pelargonium hortorum* L.H. Bailey ‘Maime Red’, started from stem cuttings, were grown for 2.5 years in a greenhouse with natural light and humidity, and temperatures ranging from 18 to 27°C throughout the year. Plants were watered with tap water as needed to maintain moist to slightly dry soil. They were fertilized weekly with 20–20–20 NPK, and monthly with 21–7–7 NPK amended with 7% Fe chelate (Plant Products, Brampton, ON, Canada). Experiments were conducted from August to October 2010, by which time the older parts of both stems and roots were invested with a well-developed periderm.

Wound periderm was induced in stems using the technique of Biggs (1986). A 5-mm-diameter cork borer was forced into the internodes for 1 mm, a depth sufficient to penetrate the periderm without injuring the vascular cambium. In stems,
each internode was wounded on opposite sides so that the areas were never axially aligned with wounds made on adjacent internodes. The outermost layers (epidermis and mature phellem) within the injured area were removed with fine-tipped forceps. After 0–17 d, the wounded regions were removed using a 7-mm-diameter cork borer.

Anatomy, histochemistry and permeability

The course of normal periderm development in stems was established by sampling regions along their lengths. The development and anatomical features of both periderm types (and associated tissues) was ascertained from freehand cross- and longitudinal sections on which a variety of tests were performed. These were (1) staining with berberine hemisulphate and counterstaining with aniline blue to identify Casparian bands (Brundrett et al., 1988); (2) staining with Sudan red 7B (fat red) to identify lipids, including suberin lamellae (Brundrett et al., 1991); (3) staining with phloroglucinol-HCl to detect lignin (Jensen, 1962); and (4) resistance to digestion by concentrated sulphuric acid to locate suberin polymers (Johansen, 1940). Sections were also observed with UV light for autofluorescence, indicative of phenolics. Results of all staining procedures were compared with control, unstained sections using white or UV light as appropriate.

The apoplastic permeability of stem periderms during their development was probed with berberine, a fluorescent tracer dye that was precipitated in place by adding potassium thiocyanate (Enstone and Peterson, 1992). Internode zones of interest were excised and the cut ends were sealed with molten sticky wax (Kerr Manufacturing, Mississauga, ON, Canada). The internodes were either left intact, abraded with a razor blade to disrupt the cuticle, cut longitudinally through the periderm or partially peeled to remove the outermost mature cell layers (epidermis and phellem cells), leaving the immature phellem, phellogen and phelloderm intact. The prepared internodes were immersed in 0-1 % berberine hemisulphate for 1.5 h, rinsed briefly with water and then treated with 0.05 M potassium thiocyanate (KCN) for another 1.5 h. The internodes were rinsed with KCN prior to sectioning. Specimens were mounted in KCN and viewed with UV light. Results were compared with both positive and negative controls, i.e. sections stained with berberine or left unstained, respectively.

A similar study of wound periderm development and permeability in stems was made by sampling at various times up to 17 d post-wounding. To observe the wound reaction, stem cores were cross-sectioned by hand and stained as described above. Apoplastic permeability was determined with excised internodes with sealed ends as described above.

For roots, cross-sections were cut and stained as described above for stems. Roots were prepared for permeability tests by grouping 10–20 individuals together, cutting their ends and blotting them prior to sealing with sticky wax as described above.

Microscopy

Sections were observed with a Zeiss Axiophot epifluorescence microscope using either white or UV light (filter set: exciter filter G 365, dichroic mirror FT 395, and barrier filter LP 420; Carl Zeiss, Inc.). Photomicrographs were taken with a Q-Imaging digital camera (Retiga 2000R, Fast 1394 Cooled Mono, 12-bit; Quorum Technologies Inc., Guelph, ON, Canada).

RESULTS

Native periderm of the stem – structure and permeability

When cross-sections of P. hortorum stem periderm were observed with UV light, the walls of the mature phellem produced a dark blue autofluorescence (Fig. 1A). When similar sections were stained with berberine hemisulphate and counterstained with aniline blue, yellow–green fluorescence characteristic of Casparian bands was evident in the radial walls of these cells (Fig. 1B). At higher magnification, small air spaces could be seen between individual cells (Fig. 1C). The Casparian bands extended throughout most of the radial wall (Fig. 1B, C). Longitudinal sections through the mature phellem were prepared to investigate the transverse (end) walls. In these, the transverse as well as the radial walls stained positively for Casparian bands with berberine–aniline blue (Fig. 1D). Further evidence for the presence of Casparian bands was obtained by testing for acid digestion. The cells in a mature phellem remained attached to each other after this treatment, indicating that a Casparian band was present within the primary walls and also extended across the middle lamellae between the cells (Fig. 1E). In addition to the phellem, the cuticle originally covering the epidermis also resisted acid digestion (Fig. 1E, inset). This was a thin layer to which remnants of cuticle covering the trichomes remained attached. Walls of the mature phellem cells stained red with Sudan red 7B, indicating the presence of lipids (Fig. 1F). Such staining was absent in walls of the immature phellem, the phellogen and phelloderm (Fig. 1F). Similarly, lignin (stained with phloroglucinol-HCl) was detected in cells of mature but not immature phellem (Fig. 1G).

The apoplastic permeability of the phellem and its neighbouring cells was tested with external applications of berberine, a tracer that fluoresces bright yellow under UV light. In a young stem where the cuticle was still intact, no dye entered (Fig. 2A). In an older stem, in which mature phellem had developed under the epidermis, the cuticle was disrupted prior to treatment with berberine. In this case, the tracer entered the remaining walls of the epidermis and some adjacent walls of the outermost phellem layer that had also sustained some damage (Fig. 2B). However, further penetration of the tracer was blocked by the anticlinal walls of the intact phellem cells (Fig. 2B). The extent to which berberine can penetrate tissue at various depths within an organ can be observed by making a slit along the length of the organ, thus opening up the interior tissues to lateral application of the tracer. When a young stem without mature periderm was treated with berberine, the tracer moved through the walls of the cells adjacent to the cut (Fig. 2C). These cells with permeable walls were those of the epidermis, immature phellem, phellogen, phelloderm and cortex (Fig. 2C). When the slit was made in an older region of the stem, one with two layers of mature phellem cells, berberine movement
through the other cell walls proceeded as usual (compare Fig. 2C and Fig. 2D). It also moved into the tangential walls of the mature phellem cells but did not diffuse further through the radial walls (Fig. 2D). The same pattern of berberine permeation was seen in stems in which different parts of the periderm had been ripped off prior to the tracer application (Fig. 2E, F).

Wound periderm of the stem – structure and permeability

The first apparent reaction of tissue adjacent to the crushed cells of the wound was the production of a boundary layer. Wall modifications were first noted 2 d after wounding. On the third day, the boundary layer was complete. After staining with berberine–aniline blue, the walls of this first layer became brightly fluorescent (Fig. 3A). This staining is typical of walls with phenolics. When the same specimen was viewed with white light, the boundary layer could be recognized by its highly vacuolated and fairly translucent cells (Fig. 3B). Staining with Sudan red 7B indicated the presence of lipids in the walls of this layer (Fig. 3C). At this time, 3 d after wounding, the boundary layer was impermeable to the passage of berberine (Fig. 3D). At 7 d post-wounding, further changes had occurred near the wound. The boundary zone had remained intact (Fig. 3E) but a wound periderm had begun to form centripetally and adjacent to the boundary zone (Fig. 3F). A phellogen, phelloderm and a few immature phellem cells were present at this time (Fig. 3E, F). By 17 d post-wounding, some of the phellem cells had matured (Fig. 3G). These cells had Casparian bands in their radial walls, like the cells of the native periderm (Fig. 3G).

Root periderm structure and apoplastic permeability

The primary root of P. hortorum was composed of an outermost epidermal layer, followed by a hypodermis and central...
Fig. 2. Berberine apoplastic permeability tests on native stem periderm of *P. hortorum*. The photomicrographs show cross-sections of the stems. (A) Intact stem with epidermal cuticle (white arrowheads) but lacking mature phellem cells. The cuticle prevented permeation of berberine. (B) The stem was superficially scraped to remove cuticle. Berberine, which fluoresced yellow, permeated epidermal cell walls, and the outer tangential and radial walls of the outermost damaged phellem cell layer (yellow arrowheads). Berberine permeation was blocked by the Casparian band in the intact anticlinal walls of underlying phellem layers. (C) Stem that lacked mature phellem cells was incised through the cortex. Berberine permeated walls of the epidermis, immature phellem cells, phellogen, phelloderm and cortex cells (yellow arrowheads). (D) Stem with two mature phellem layers was incised through the cortex. Berberine permeation was blocked only by Casparian bands in the anticlinal walls of mature phellem cells from both the epidermal side and the cortex side (between yellow arrowheads). (E) Stem partially stripped exposing immature phellem cells, the radial and tangential walls of some mature phellem cells, and epidermal cells. Berberine permeated all walls of epidermal, immature phellem, phellogen, phelloderm and cortex cells (yellow arrowheads). Additionally, the dye was detected in exposed inner and outer tangential walls of mature phellem cells (light blue arrowheads). However, berberine did not penetrate radial walls of mature phellem cells – the location of Casparian bands. (F) Panoramic view of a stem that was partially stripped of its mature phellem layers. As in D and E, berberine transport was prevented by Casparian bands in mature phellem cells from both the epidermal and cortex sides. The extent of lateral flow of berberine through the epidermis is evident. Abbreviations: epi, epidermis; mph, mature phellem cell; iph, immature phellem cell; pg, phellogen; pd, phelloderm; co, cortex. Note that the clarity of the figure when viewed electronically may be affected by the brightness/contrast settings of the computer screen. In addition, images obtained from fluorescence microscopy are best viewed under low ambient light. Scale bars: (A, C–F) = 100 μm; (B) = 50 μm.
cortex that both contained phi thickenings in their anticlinal walls (Fig. 4A, D). These cellulosic, lignified thickenings are known to occur in *P. hortorum* roots (Haas *et al.*, 1976). Interior to the central cortex was a single-layered endodermis, followed by a typical eudicot stele with a pericycle, and poles of primary phloem and xylem in a diarch arrangement (not shown).

After development of the periderm from the pericycle, all primary cell layers from the epidermis to the endodermis inclusive were sloughed off. When the periderm was stained with berberine–aniline blue, Casparian bands were detected in the radial walls of mature phellem cells (Fig. 4B). At higher magnification, fluorescent material was also observed in the corners of these cells (Fig. 4C). This trait differed from the stem periderm in which mature phellem cells had definite intercellular air spaces at cell corners (see Fig. 1C). When roots were stained with phloroglucinol-HCl, lignin was detected in the phi thickenings and vessel secondary walls (Fig. 4D). However, with this staining test, lignification of the mature phellem cell walls was seldom seen. Roots were also stained with Sudan red 7B, upon which mature phellem cells stained positively for lipids (Fig. 4E, F). In older roots, a tannin-rich region in the outermost half of the periderm was evident. The tannins were brown in colour, and prevented the lipids from being detected with Sudan red 7B (Fig. 4F).

Roots were also subjected to berberine apoplastic permeability tests. Tracer transport was not restricted by the wall-modifying structures of primary cell layers. In other words, the phi thickenings in the hypodermis and primary cortex do not inhibit apoplastic flow (Fig. 4G). In contrast, berberine did not permeate the radial walls of the outermost mature phellem layer (Fig. 4G, H). Interestingly, the tannin-rich region in older periderms masks the UV autofluorescence of phenolics (Fig. 4H). In ruptured areas of the periderm, berberine permeates the tangential walls of damaged phellem cells, but the dye is still blocked by their intact radial walls (Fig. 4I).
DISCUSSION

In the past, Casparian bands have been difficult to detect when the cells of the tissue also have suberin lamellae, as is the case in phellem cells. The autofluorescence of the lamellae and their positive staining for phenolics and lipids overwhelms the staining of the less conspicuous Casparian bands in adjacent, primary walls. For this reason, Casparian bands in the exodermis were overlooked for many decades. However, they can be visualized by two methods, i.e. clearing followed by staining with Chelidonium majus extract (Peterson et al., 1982), and staining with berberine followed by counterstaining with aniline blue (Brundrett et al., 1988).

Casparian bands in the endodermis form a network in the primary walls of the cells. They were isolated and described by Priestley and North (1922), and beautifully illustrated by Schreiber et al. (1994). Proof of a Casparian band relies on responses to several tests (see Ma and Peterson, 2003). It should stain positively for phenolics and lipids, resist digestion with sulphuric acid, and provide resistance to the passage of apoplastically moving substances. Casparian bands are typically confined to the radial and transverse (end) walls of the cells, although exceptions have been noted where the band extends into the tangential walls (Seago et al., 1999; Meyer et al., 2009).

In the phellem, it is not possible to determine whether the primary walls contain phenolics and lipids because of the nearby suberin lamellae of the cells. However, the present
work demonstrates that, by all other criteria stated above, Casparian bands occur in mature phellem cells of *P. hortorum* root and shoot and also in the wound-induced periderm in the shoot. (Wound-induced periderm was not tested in roots due to their narrow diameters and soil-bound environment.) Using the berberine–aniline blue method, Casparian bands were seen in the radial and transverse walls of phellem cells in stems and roots. Their position correlates with blockage of an apoplastic tracer (berberine). The phellem cells did not separate from each other during treatment with strong acid. To the best of our knowledge, this is the first time that Casparian bands have been identified in stem and root periderm. There have been reports of bands in the related ‘polyderm’ tissue (Fahn, 1990), including six species of Lythraceae (Stevens et al., 1997; Lempe et al., 2001). In the case of a polyderm, a pericycle-derived phellogen generates a phellem composed of alternating layers of suberized cork cells and thin-walled, non-suberized cells (Evert, 2006). The apoplastic impermeability of the polyderm of *Eucalyptus pilularis* to berberine was observed by McKenzie and Peterson (1995), but there was no correlative positive staining of a Casparian band with berberine–aniline blue.

The Casparian bands of the phellem occupy almost the whole extent of the radial walls. This parallels the situation in the endodermis and exodermis of roots. In the former, the Casparian band typically occupies only a small fraction of the radial wall. However, if suberin lamellae form within the cells, the band extends to fill almost the whole of these walls (Barnabas and Peterson, 1992; McCully and Mallett, 1993). In the exodermis, Casparian bands and suberin lamellae form concurrently or nearly so, and here, too, the Casparian bands occupy most of the radial and transverse walls. Thus, the rather large Casparian bands in the phellem are to be expected.

In wounded tissue, a boundary layer with suberized walls forms early. Berberine tracer experiments showed that this layer substantially reduced the permeability of the apoplast. The early wall modifications in the boundary layer correlate with the observed blockage of infection by the fungal pathogen *Pythium ultimum* beginning 2 d after wounding *P. hortorum* stems (Cline and Neely, 1983).

A major function of the periderm is to prevent water loss from the plant to its surroundings. This is an obvious requirement in shoots but could also be important for older parts of roots near the soil surface. The suberin lamellae, located interior to the primary walls of the phellem, are in a position to reduce water flow through the transcellular path. As the cells are dead at maturity, the symplastic path of transport no longer exists. The remaining path is the apoplast located in the primary walls of the cells. Even in living tissue, the unmodified cell walls constitute the pathway of least resistance to water flow (Steudle and Peterson, 1998). Modifications to the primary walls of the phellem are therefore of importance in reducing water flow through the tissue. From previous work it is known that the primary walls of the phellem are lignified (Godkin et al., 1983; Biggs, 1986), and this was confirmed for *P. hortorum* shoots and roots in the present study. The addition of suberin, with its hydrophobic fatty acid component, to the lignin would reduce the permeability of the primary walls to water and improve the overall function of the periderm. Waxes associated with suberin are known to be important for the hydrophobicity of the walls (Schreiber et al., 2005). However, it would not be possible to quantify these from the Casparian bands of the phellem due to the large quantities of the suberin lamellae in the cells.

A second critical function of the periderm is to resist pathogen attack. According to Wood (1960), of all plant parts, suberized tissue is the most effective in this regard. The suberin component of the primary walls and middle lamellae in the phellem should be crucial in preventing invasion by pathogens entering by these routes.

This is the first report of Casparian bands in the phellem of any species, in this case *P. hortorum*. It would clearly be of interest to extend this work to other species. According to Lulai and Morgan (1992), Casparian bands are absent from *Solanum tuberosum* (potato) tuber periderm. Similarly, evidence from water permeability studies led Schönerr and Zeigler (1980) to conclude that a Casparian band is absent from *Betula pendula* phellem. We hope that any such future research on this topic will include results obtained with the basic methods used in the current work, i.e. staining with berberine–aniline blue, acid digestion and apoplastic permeability tests.

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### LITERATURE CITED


