Development and chemical characterization of Casparian strips in the roots of Chinese fir (*Cunninghamia lanceolata*)

Chengwei Song^{1,2} · Weiwei Shen^{1,2} · Liang Du^{1,2} · Jialong Wen³ · Jinxing Lin^{1,2} · Ruili Li^{1,2}

Received: 20 October 2018 / Accepted: 7 February 2019 / Published online: 15 February 2019
© The Author(s) 2019

Abstract

Key message Lignification and suberization of Casparian strips occurred simultaneously during the development of *Cunninghamia lanceolata* primary roots.

Abstract The Casparian strips in the cell walls of vascular plant root endodermis and exodermis cells are vital for the exclusion of salts and pathogens, selective nutrient uptake, and other processes related to the transport and assimilates of water. Despite the importance of Casparian strips, their chemical composition and its relevance to their diffusion barrier functions remain a matter of debate, especially in woody plants. Here, we describe the cytological features revealed by fluorescent staining of developing Casparian strips in the primary root endodermis of Chinese fir (*Cunninghamia lanceolata*), one of the most important timber species native to China. Using apoplastic permeability tests, we revealed that the Casparian strips provide their barrier function from the secondary stage of endodermis development, after lignin and suberin are deposited onto the surface of the protoplast next to tangential cell walls. Furthermore, we enzymatically isolated Casparian strips from the primary root endodermis and analyzed their chemical composition using Fourier transform infrared spectroscopy, revealing that they consist largely of lignin and suberin. Using confocal Raman microscopy for label-free, in situ, structural and biochemical analysis, we showed that lignin and suberin accumulated simultaneously in the developing Casparian strips. Taken together, these results elucidated Casparian strip development and characterized the lignification and suberization processes in situ in the primary roots of Chinese fir. Since this species shows broad adaptability to varying environmental conditions, our findings may facilitate work to understand the barrier function of Casparian strips in relationship to environmental stresses.

Keywords Casparian strips · Lignin · Suberin · In situ analysis · Confocal Raman microscopy · *Cunninghamia lanceolata*

Introduction

Plant roots grow in direct contact with the soil to facilitate their uptake of water and necessary solutes, but must simultaneously avoid the uptake of unwanted or toxic solutes. The roots of virtually all vascular plants have developed anatomical and physiological adaptations to exclude unwanted solutes and pathogens; these adaptations include the formation of Casparian strips in the cell walls of endodermal and exodermal cells in the primary roots (Enstone et al. 2003; Lehmann et al. 2000; Peterson et al. 1993). Casparian strips were first described by Robert Caspary (1865) as a single layer that very closely approximated cells, and were first isolated by Priestley and North (1922). They were more comprehensively illustrated by Schreiber et al. (1994), and have since been described in a wide range of plants.

Communicated by Y. Sano.

Chengwei Song and Weiwei Shen contributed equally to the manuscript.

✉ Ruili Li
liruili@bjfu.edu.cn

¹ Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, Beijing Forestry University, Beijing 100083, China

² College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China

³ College of Materials Science and Technology, Beijing Forestry University, Beijing 100083, China

Casparian strips are a uniseriate sclerenchymatous layer that acts as a physical barrier to apoplastic transport. Casparian strips are tissue-specific and involved in blocking the nonselective apoplastic bypass flow of water (Hose et al. 2001; Steudle 2000; Steudle and Peterson 1998), preventing the uptake of ions into the stele (Nagahashi et al. 1974; Perumalla et al. 1990), and facilitating the response to different environmental stress conditions (Degenhardt and Gimmler 2000; Karahara et al. 2004; Shen et al. 2015; Yang et al. 2015). The function of Casparian strips as apoplastic transport barriers in roots has been established in many different plant species.

In most species, root endodermal cell development occurs continuously and involves three stages (Peterson 1988; Ranathunge et al. 2004; Van Fleet 1961). In the primary stage of endodermis development, lipophilic and aromatic substances are deposited in the radial and transverse walls to form the Casparian strips. In the secondary stage, suberin lamellae are deposited onto the surface of the protoplast next to the anticlinal and tangential cell walls. In the tertiary stage, cellulose is deposited on the radial walls, inner tangential walls, and transverse walls, often accompanied by lignification. Various environmental factors, such as light (Karahara and Shibaoka 1994), water (Stasovski and Peterson 1993; Enstone and Peterson 1998), and salinity (Karahara et al. 2004), as well as phytohormones (Chen et al. 2011; Nakayama et al. 2017), have been shown to influence the formation and development of the Casparian strips.

Elucidating the chemical composition of the developing Casparian strips is vital for providing a better understanding of their structure and function. It is currently believed that Casparian bands are deposited at the intermicrofibrillar spaces of the primary cell walls, which are largely comprised of suberin, an aliphatic polyester and the main component of cork (Schreiber et al. 1994; Schreiber 1996; Zeier and Schreiber 1997), whereas other works found evidence that Casparian strips largely consist of a lignin-like polymer (Karahara et al. 2004; Schreiber et al. 1999; Yokoyama and Karahara 2001). The relative abundance of lignin and suberin in the Casparian strips varies throughout endodermal cell development, and between dicotyledonous and monocotyledonous species (Zeier et al. 1999a; Zeier and Schreiber 1998). Nevertheless, the chemical composition of the Casparian strips has remained a contentious issue. Several approaches have been used to analyze the structure and chemical composition of the Casparian strips in many plant species (Schreiber et al. 1994; Zeier and Schreiber 1999; Hartmann et al. 2002); however, traditional histological and histochemical techniques could not accurately separate the depositions of lignin and suberin from the endodermal cells. The chemical nature of the Casparian strip polymers, therefore, requires further elucidation.

In the present study, we used a combination of techniques to examine the Casparian strips of the Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook. (Cupressaceae)), which is native to China. Chinese fir is a dominant tree species in southern China because of its adaptability to diverse environmental conditions, and is widely planted commercially for its rapid growth and the properties of its highly durable, scented wood. This species is currently grown on over 11 million hectares (Shi et al. 2010), but its many applications mean that this area is expanding in 15 provinces across China (State Forest Administration 2014).

Here, we used fluorescent staining to observe the development of the Casparian strips under laser scanning confocal microscopy. Fourier transform infrared (FTIR) spectroscopy was used to examine the chemical composition of the Casparian strips, while confocal Raman microscopy enabled the label-free, in situ structural, and nondestructive analysis of these barriers. Confocal Raman microscopy, a combination of Raman spectroscopy and confocal microscopy, has emerged as a powerful biomedical imaging modality (Das and Agrawal 2011; Dieing and Hollricher 2008). The use of this technique has provided new insights into the chemical composition of plant cell walls in the context of their structure, making it a convenient method of quantitative chemical imaging (Agarwal 2006; Ma et al. 2011).

The purpose of this study is to examine the developmental stages and barrier functions of the endodermal Casparian strips in Chinese fir primary roots, with a special emphasis on the deposition of lignin and suberin. The results of this study should further elucidate the composition and function of Casparian strips in the primary roots of this and other tree species.

Materials and methods

Plant materials and growth conditions

Newly matured seeds were collected from Chinese fir trees in Minhou County, Fujian Province, China (N26°35', E119°24', 1020 m above sea level), on December 20, 2013 and air-dried at room temperature for 10 days. The seeds were sterilized with 0.2% NaClO for 15 min, rinsed three times with sterile water, and incubated for 1 week under continuous light at 22 °C on pledgets moistened with distilled water. The germinating seedlings were transferred to a growth chamber under a 16-h light:8-h dark photoperiod, with 500–600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation, 22 °C, and 70% relative humidity during the day, and 18 °C and 90% relative humidity at night. The seedlings were watered with sterilized distilled water every 2 or 3 days, and their primary roots reached up to 11–13 cm in 6 weeks under the above growth conditions.

Histochemistry of Casparian strips

Primary roots of 6-week-old Chinese fir plants, which were approximately 8–10 cm long, were divided into three zones: zone I (0–1 cm), zone II (1–4 cm) and zone III (4–8 cm), and 0.3-cm segments were cut at 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 cm from the root tip. The samples were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 24 h at 4 °C. The samples were then rinsed in 0.1 M cacodylate buffer, dehydrated in a graded ethanol and acetone series, then infiltrated and embedded in Spurr resin (Electron Microscopy Sciences). The resin blocks were polymerized for 24 h at 70 °C, then cut into 10- μ m slices using an ultramicrotome (Leica Biosystems). The Casparian bands were stained with 0.1% (w/v) berberine hydrochloride for 1 h and counter-stained with 0.5% (w/v) aniline blue for an additional hour, as reported previously (Brundrett et al. 1988; Man et al. 2018). The stained sections were viewed under a Leica TCS SP8 confocal laser microscope (Leica Microsystems) with an excitation wavelength of 488 nm and a detection wavelength of 520 nm.

Permeability tests

The apoplastic fluorescent dye calcofluor was used to test the permeability of the root endodermis cells using a previously described method (Perumalla et al. 1990; Weis et al. 2014), with some modifications. Using a razor blade, the fresh primary roots were cut into 0.5-cm segments starting 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 cm from the root tip, after which the cut edges were sealed with molten paraffin. The root segments were carefully wounded by scratching the exodermis, which is otherwise impermeable to dyes. Care was taken to ensure that only exodermis tissue was affected by this wounding, with no damage to the endodermal cell layer. The root segments were incubated in 0.01% calcofluor (w/v) for 24 h, after which they were rinsed in phosphate buffer and sectioned using a double-edged razor blade. The sections were mounted in glycerin and directly viewed under a Leica DM2500 fluorescence microscope (Leica Microsystems). For the calcofluor-treated sections, a blue fluorescence filter was chosen, which included an exciter filter BP 365 nm, chromatic beam filter FT 395 nm, and barrier filter LP 420 nm.

Isolation of Casparian strips from roots

The primary roots of 6-week-old Chinese fir seedlings were divided into two zones, zone II (1–4 cm) and zone III (4–8 cm), which were dissected into 1-cm pieces using a razor blade. The Casparian strips were enzymatically isolated and purified following the method previously described by Schreiber et al. (1994), with some modifications. Root segments were

incubated for 1 h at 34 °C in a separating solution containing an equal volume of 30% hydrogen peroxide and acetic acid, rinsed three times with sterile water, and then incubated at 46 °C in an enzymatic solution containing 2.5% (w/v) cellulase (R-10; Yakult, Japan) and 1.5% (w/v) pectinase (Y-23; Yakult) dissolved in 0.01 M citric buffer adjusted to pH 4.8. After about 5 h, the root segments were rinsed three times with sterile water. The endodermal and exodermal cell walls were separated using two dissecting needles under stereomicroscopy, and the Casparian strips of the endodermis cells from zones II and III were thoroughly washed and stored in sterilized distilled water at 4 °C. The isolated Casparian strips were viewed under a Leica DM2500 fluorescence microscope using a blue fluorescence filter, which contained the exciter filter BP 365 nm, chromatic beam filter FT 395 nm, and barrier filter LP 420 nm.

FTIR spectroscopy

The Casparian strips isolated from the primary roots of Chinese fir were analyzed using an FTIR spectrometer (Vertex 70; Bruker, Germany). The FTIR spectra were recorded with a resolution of 2 cm^{-1} in the spectral range 4000–500 cm^{-1} . The samples were scanned three times, and all data were analyzed using Origin 6.0 software.

Confocal Raman microscopy

The primary roots of 6-week-old plants were cut into 0.3-cm segments starting 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 cm from the root tip, which were then infiltrated and embedded in Spurr resin (Electron Microscopy Sciences). An ultramicrotome (Leica Biosystems) was used to cut the samples into 10- μ m slices, after which the Spurr resin was dissolved in a saturated solution of sodium hydroxide in ethanol. Raman spectra were acquired using a LabRam Xplora exquisite full-automatic confocal Raman microscope (Horiba Jobin Yvon) equipped with a confocal microscope (Olympus BX51) with a motorized x and y axis, and a high numerical aperture (NA) microscopy objective from Olympus (MPlan 100 $\times$ objective, NA = 1.40). The cross-sections were focused using a linear-polarized green laser (532-nm wavelength) providing 8 mW of power to the sample. For mapping, an integration time of 4 s and steps of 0.6 μ m were applied. Every pixel corresponded to one scan, and each location was obtained by averaging 2-s cycles. The wave number ranged from 3200 to 600 cm^{-1} .

Labspect 5 software was used for the image processing and spectral analyses (Ji et al. 2013). The lignin distribution was demonstrated by integrating over the 1600 cm^{-1} band (1550–1640 cm^{-1}), which is attributed to aromatic C=C bond vibrations (Gierlinger and Schwanninger 2006). The suberin distribution was demonstrated by integrating over the

2850 cm^{-1} band (2830–2890 cm^{-1}), which is dominated by the CH_2 stretching vibration (Bernards 2002).

Results

Casparian strip development in the endodermis

To characterize the different stages of Casparian strip development in the endodermis of Chinese fir, we took cross-sections at six different positions (0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 cm from the tip) along the primary root. Berberine-aniline blue was then used to stain the Casparian strip and the resulting green fluorescence was visualized under blue light excitation (Fig. 1). The Casparian strips were observed in the radial cell walls of endodermal cells 0.5 cm from the root tip (Fig. 1a), where the primary stage of endodermal cell development occurred. This signal increased 1 cm from the root tip, indicating that the deposition and fluorescence of Casparian strips was much greater in this region (Fig. 1b). The endodermal cells showed a thick deposition of radial walls in the region approximately 1–4 cm from the root tip, alongside an increase in Casparian strip signal strength (Fig. 1b–d). In the region approximately 4–8 cm from the root apex, the Casparian strip signal on the radial cell walls of the endodermal cells further increased in strength (Fig. 1d–f).

Apoplastic permeability of Casparian strips

We used the apoplastic tracer calcofluor, which binds to cellulose, to detect the apoplastic permeability of the root endodermis. Calcofluor could diffuse into the cortex tissue of roots with an experimentally damaged exodermis, as indicated by the blue fluorescence of their calcofluor-stained cell walls. At 0.5 cm from the root tip, this fluorescence was observed in the exodermis, cortex tissue, and stele (Fig. 2a). At 1 cm from the root tip, the blue fluorescence was only observed in the exodermis and cortex tissue below the wound and was hardly detectable in the vascular tissue (Fig. 2b).

Casparian strips during endodermis development

The cytological features of the enzymatically isolated endodermal Casparian strips from zones II (1–4 cm) and III (4–8 cm) of the primary roots were observed using fluorescence microscopy. The Casparian strips formed continuous nets, revealed by their bright green fluorescence under blue light (Fig. 3). The cytological features of the isolated Casparian strips from primary root zones II (Fig. 3a) were similar to those of zone III (Fig. 3b), with no pit fields detected in the Casparian strips in the radial or transverse cell walls.

Fig. 1 Fluorescence images of cross-sections taken at different distances from the tip of Chinese fir primary roots. **a–f** Cross-sections were obtained from 7-week-old roots at 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 cm from the root tip and stained with berberine hemisulfate and aniline blue. *Cs* Casparian strips, *en* endodermis, *Pe* pericycle. Scale bars 25 μm

Fig. 2 Fluorescence micrographs of Chinese fir primary root cross-sections treated with calcofluor, an apoplastic tracer. **a** Cross-section 0.5 cm from the root tip treated with calcofluor. **b** Cross-section 1.0 cm from the root tip treated with calcofluor. *Co* cortex, *En* endodermis, *St* stele. Scale bars 100 μ m

Fig. 3 Fluorescence micrographs of isolated Casparian strips from the primary roots of Chinese fir. **a** The isolated endodermal Casparian strips from zones II. **b** The isolated endodermal Casparian strips from zones III. *Tw* transverse walls of endodermal cells, *Rw* radial walls of endodermal cells. Scale bars 50 μ m

Fig. 4 FTIR spectra of Casparian strips isolated from the primary roots of Chinese fir. The FTIR spectra were generated for Casparian strips in the secondary developmental stage (Zone II, blue) and the tertiary developmental stage (Zone III, red) of the primary root endodermis of Chinese fir

Chemical composition of the Casparian strips revealed using FTIR spectroscopy

To further identify the chemical compositions of the Casparian strips, we isolated the endodermal Casparian strip network from zones II (1–4 cm) and III (4–8 cm) of the Chinese fir primary root and scanned them with a FTIR spectrometer (Fig. 4). The spectra of the endodermal Casparian strips isolated from zones II and III of the primary roots showed similar absorption bands; however, the relative intensity of the various absorption bands were lower for zone II than for zone III, indicating that the abundances of all endodermal Casparian strip components were slightly lower in zone II.

The spectra of the Casparian strips had a broad absorption band at around 3336 cm^{-1} , which is associated with the stretching vibrations of the hydrogen bonds in OH or NH groups. The two intense bands at 2918 cm^{-1} and 2856 cm^{-1} in these spectra could be assigned to the asymmetrical and symmetrical stretching vibrations of

methylene groups, which are present in suberin. The strong absorption bands detected around 1732 cm^{-1} in the spectra from both root zones could be attributed to the stretching vibrations of the C–O groups in suberin. The absorption bands around 1635 cm^{-1} and 1510 cm^{-1} were typical of the molecular vibrations in lignin and cellulose, respectively, while the bands around 1026 cm^{-1} were typical of carbohydrates such as cellulose.

In situ, label-free, chemical imaging of Casparian strips using confocal Raman microscopy

To further analyze the dynamic changes in the processes of suberification and lignification in the endodermal Casparian strips of Chinese fir, cross-sections were taken at six different positions along the primary root and imaged using confocal Raman microscopy. Two-dimensional chemical images were generated by integrating over the wavenumber ranges containing strong Raman bands. The output

Fig. 5 Confocal Raman images analysis of the endodermis from cross-sections of primary Chinese fir roots at different distances from the tip. **a–f** Lignin distribution in the Casparian strips of primary roots revealed by integrating over the 1600 cm^{-1} band ($1550\text{--}1640\text{ cm}^{-1}$). **a'–f'** Suberin distribution in the Casparian strips of primary roots revealed by integrating over the 2850 cm^{-1} band ($2830\text{--}2890\text{ cm}^{-1}$). Cross-sections were obtained at 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 cm from the root tip. *Cs* Casparian strips, *En* endodermis. Scale bars $20\text{ }\mu\text{m}$. Colour code, an increase of the signal intensity of lignin and suberin from black via blue, green, yellow to red

wavelengths of the optical parametric oscillators were set to about 2850 cm^{-1} for the suberin CH_2 stretching vibrations and about 1600 cm^{-1} for the aromatic $\text{C}=\text{C}$ vibrations in lignin. When compared with the images of the tissues stained using berberine-aniline blue, the confocal Raman images of the $\text{C}=\text{C}$ and CH_2 stretching vibrations showed a higher specificity for the lignin and suberin components of the Casparian strips, respectively (Fig. 5).

As with the berberine-aniline blue staining results, the use of confocal Raman microscopy also detected Casparian strips in the endodermal cell walls as little as 0.5 cm from the root tip, as illustrated by the presence of $\text{C}=\text{C}$ and CH_2 stretching vibrations. Moreover, the confocal Raman signals of lignin and suberin became stronger in the radial cell walls of the endodermal cells 1–8 cm away from the root tip (Fig. 5b–f, b'–f'). The highest intensities of lignin and suberin were observed 8 cm from the root apex, indicating the maturation of the Casparian strips in the endodermis. Figure 5 shows that the confocal signal was greater in the compound middle lamella (comprising the middle lamella and adjacent primary walls) in the endodermal radial cell walls.

Discussion

The Casparian strips in the walls of root endodermis and exodermis cells are comprised of lignin and suberin in most species; however, the development of Casparian strips can be strikingly different between species (Barnabas and Peterson 1992; Enstone and Peterson 1997; Zeier et al. 1999a). In onion (*Allium cepa* L.) roots, Casparian strip formation begins in the center of the radial endodermal walls then expands symmetrically outwards (Barnabas and Peterson 1992), but in *Arabidopsis*, suberin biosynthesis and accumulation occurs much later than the formation of an endodermal barrier (Naseer et al. 2012). In this study, we used berberine-aniline blue staining and fluorescence microscopy to dissect the development of the endodermal Casparian strips in the Chinese fir primary root, revealing that they initially formed in the cell walls 0.5 cm from the root tip but became thicker and larger in the cells approximately 1–4 cm from the root tip. Endodermal cells in the region approximately 4–8 cm from the root tip displayed even more pronounced Casparian strips. These results suggest that the development of Casparian strips in the endodermis can be divided into three consecutive stages in the primary roots of Chinese fir.

Casparian strips form a network in the primary walls of the endodermal cells, which acts as a physical apoplastic transport barrier with important roles in the exclusion of pathogens, selective nutrient uptake, and water absorption (Julkowska and Testerink 2015; Karahara et al. 2004; Ranathunge et al. 2011). Casparian strips also prevent the radial

loss of oxygen from the roots to the substrate under anoxia/hypoxia (Kotula et al. 2009, 2014, 2017). Previously, the fluorescent dye calcofluor was used as a convenient apoplastic tracer to check the permeability of the endodermal diffusion barrier (Perumalla et al. 1990; Peterson and Perumalla 1990; Wu et al. 2005). In the present study, we demonstrated that calcofluor diffused through the endodermis and then entered the stele tissue 0.5 cm from the root tip, but it was hardly detectable in the root vascular tissue 1 cm from the tip. These results indicate that the barrier functions of the endodermis were established in the secondary development stage of the Casparian strips, when the thicker bands were observed.

The Casparian strips in the endodermal cells are impregnated with lignin and suberin, and can, therefore, be isolated by enzymatic digestion and used for microscopic and analytical investigations (Robards et al. 1976; Karahara and Shibaoka 1992). In this study, we revealed that the Casparian strips isolated from different zones of the primary roots of Chinese fir showed similar mesh morphologies. No pit fields were observed on the surface of the cell walls at the Casparian strips in the primary roots, which was also reported for *Pinus bungeana* (Wu et al. 2005). These results demonstrate that the continuous Casparian strip nets, form from the early stages of root development are important to provide protection from the external environment.

FTIR has been widely used to determine the chemical composition of Casparian strips, revealing that lignin and suberin are the major constituent biopolymers in many plant species (Hartmann et al. 2002; Zeier and Schreiber 1999). The conclusions made using FTIR spectroscopy have been demonstrated to be consistent with those of previous studies with direct chemical analyses performed using gas chromatography and mass spectrometry (Schreiber 1996; Schreiber et al. 1999). FTIR spectroscopy analysis of *P. bungeana* indicated that very little suberin was present in Casparian strips isolated from the needles, but equal amounts of lignin and suberin were detected in the Casparian strips of the root endodermis (Wu et al. 2005). Casparian strips isolated from the roots of three rice (*Oryza sativa*) cultivars were mainly composed of aliphatic suberin, lignin, and cell wall proteins and carbohydrates (Cai et al. 2011). In this study, we used FTIR spectroscopy to evaluate the chemical compositions of roots at different developmental stages, revealing that the Casparian strips in all stages had similar absorption bands at 2918, 2856, 1732, and 1636 cm^{-1} (Fig. 4). This indicated that the Casparian strips of the Chinese fir root endodermis at a variety of developmental stages contained aliphatic lignin and suberin, although the FTIR absorbances from Zone III were higher than Zone II at these bands (Fig. 4). This showed that the contents of lignin and suberin were higher in the Casparian strips at the tertiary stage than the secondary stage.

The development of Casparian strips is usually accompanied with the polymerization and deposition of lignin and suberin (Chen et al. 2011; Krishnamurthy et al. 2009). Casparian strips represent only the primary developmental stage of the endodermis, which is followed by the deposition of suberin lamellae onto the surface of the protoplast next to the endodermal cell walls (Schreiber et al. 1999; Zeier et al. 1999b). Therefore, the chemical analysis of Casparian strips isolated from roots may reflect the presence of both polymers. Additionally, Schreiber et al. (1994) showed that the native structure of Casparian strips is usually destroyed by their enzymatic isolation, making their spatial analysis inaccurate. Confocal Raman microscopy overcomes this problem, as it enables their chemical composition to be assessed nondestructively and in situ, without exogenous labels, making it an appropriate technology for the investigation of Casparian strip composition at different developmental stages. We first detected the confocal Raman signals of lignin and suberin 0.5 cm from the root tip (Fig. 5a, a'), and their signals became stronger in the compound middle lamella of the endodermal cells 1 cm from the root tip, concurrent with the development and thickening of the Casparian strips (Fig. 5b, b'). The confocal Raman signal intensity and range associated with lignin and suberin were highest at 8 cm from the root tip. These results confirmed the findings of our berberine-aniline blue staining assay, and indicated that suberification and lignification began in the outer radial cell walls of the endodermal cells in Chinese fir, concurrent with the development of the Casparian strips. We confirmed that the endodermal cells were more permeable 0.5 cm from the root tip, but had formed effective barriers to water and solutes by the secondary development stage of the Casparian strips. This is consistent with previous studies obtained from Maize (*Zea mays* L.), which reported that the thicker deposition and larger size of Casparian dots at the secondary development stage of the Casparian strips contributed to its barrier functions (Man et al. 2018). The key functions of Casparian strips are to prevent the loss of water from the plant to their surroundings, facilitate the selective absorption of inorganic ions from the external media, and resist pathogen attack (Ma and Peterson 2003; Zeier and Schreiber 1997), making them a vital component of the root system and its ability to respond to environmental change. The early formation of the endodermal Casparian strips demonstrated here suggests that they may provide Chinese fir seedlings with a greater adaptability to the environment, which resulted in the wide distribution of this species across the various biomes in China.

In summary, we described the cytological features of the root endodermis during the development of the Casparian strips. The efficiency of their barrier function was shown to be largely related to their developmental stage and the deposition of lignin and suberin. Furthermore, using confocal

Raman microscopy, we showed that lignification and suberification occur simultaneously during Casparian strip development, which formed an effective endodermal barrier in Chinese fir.

Author contribution statement CS, WS and RL were the equal chief scientist in this study, performed the experiment, wrote the manuscript; LD participants in this study, offered some suggestions about the results, JW participants in this study, guided to complete the confocal Raman experiment; RL and JL designed the experiments, revised manuscripts for submission.

Acknowledgements This work was supported by the State '13.5' Key Research Program of China (no. 2016YFD0600102), the National Natural Science Foundation of China (31670182, 31761133009, 31530084, 31401149, 31501168), and the Fundamental Research Funds for the Central Universities (2017ZY10, BLX201418) and the Program of Introducing Talents of Discipline to Universities (111 project, B13007).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

OpenAccess This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

- Agarwal UP (2006) Raman imaging to investigate ultrastructure and composition of plant cell walls: distribution of lignin and cellulose in black spruce wood (*Picea mariana*). *Planta* 224:1141–1153
- Barnabas AD, Peterson CA (1992) Development of Casparian bands and suberin lamellae in the endodermis of onion roots. *Can J Bot* 70:2233–2237
- Bernards MA (2002) Demystifying suberin. *Can J Bot* 80:227–240
- Brundrett MC, Enstone DE, Peterson CA (1988) A berberine-aniline blue fluorescent staining procedure for suberin, lignin and callose in plant tissues. *Protoplasma* 146:133–142
- Cai X, Chen T, Zhou QY, Xu L, Qu LQ, Hua XJ, Lin JX (2011) Development of Casparian strips in rice cultivars. *Plant Signal Behav* 6:59–65
- Caspary R (1865) Remarks on the protective sheath and the formation of stem and root (translated from German). *Jahrbücher für wissenschaftliche Botanik* 4:101–124
- Chen T, Cai X, Wu X, Karahara I, Schreiber L, Lin JX (2011) Casparian strips development and its potential function in salt tolerance. *Plant Signal Behav* 6:1499–1502
- Das RS, Agrawal YK (2011) Raman spectroscopy: recent advancements, techniques and applications. *Vib Spectrosc* 57:163–176
- Degenhardt B, Gimmler H (2000) Cell wall adaptations to multiple environmental stresses in maize roots. *J Exp Bot* 51:595–603

- Dieing T, Hollricher O (2008) High-resolution, high-speed confocal Raman imaging. *Vib Spectrosc* 48:22–27
- Enstone DE, Peterson CA (1997) Suberin deposition and band plasmolysis in the corn (*Zea mays* L.) root exodermis. *Can J Bot* 70:1188–1199
- Enstone DE, Peterson CA (1998) Effects of exposure to humid air on epidermal viability and suberin deposition in maize (*Zea mays* L.) roots. *Plant Cell Environ* 21:837–844
- Enstone DE, Peterson CA, Ma F (2003) Root endodermis and exodermis: structure, function and responses to the environment. *J Plant Growth Regul* 21:335–351
- Gierlinger N, Schwanninger M (2006) Chemical imaging of poplar wood cell walls by confocal Raman microscopy. *Plant Physiol* 140:1246–1254
- Hartmann K, Peiter E, Koch K, Schubert S, Schreiber L (2002) Chemical composition and ultrastructure of broad bean (*Vicia faba* L.) nodule endodermis in comparison to the root endodermis. *Planta* 215:14–25
- Hose E, Clarkson DT, Steudle E, Schreiber L, Hartung W (2001) The exodermis: a variable apoplastic barrier. *J Exp Bot* 52:2245–2264
- Ji Z, Ma JF, Zhang ZH, Xu F, Sun RC (2013) Distribution of lignin and cellulose in compression wood tracheids of *Pinus yunnanensis* determined by fluorescence microscopy and confocal Raman microscopy. *Ind Crop Prod* 47:212–217
- Julkowska MM, Testerink C (2015) Tuning plant signaling and growth to survive salt. *Trends Plant Sci* 20:586–594
- Karahara I, Shibaoka H (1992) Isolation of Casparian strips from pea roots. *Plant Cell Physiol* 33:555–561
- Karahara I, Shibaoka H (1994) The Casparian strips in pea epicotyls: effects of light on its development. *Planta* 192:269–275
- Karahara I, Ikeda A, Kondo T, Uetake Y (2004) Development of the Casparian strips in primary roots of maize under salt stress. *Planta* 219:41–47
- Kotula L, Ranathunge K, Schreiber L, Steudle E (2009) Functional and chemical comparison of apoplastic barriers to radial oxygen loss in roots of rice (*Oryza sativa* L.) grown in aerated or deoxygenated solution. *J Exp Bot* 60:2155–2167
- Kotula L, Colmer TD, Nakazono M (2014) Effects of organic acids on the formation of the barrier to radial oxygen loss in roots of *Hordeum marinum*. *Funct Plant Biol* 41:187–202
- Kotula L, Schreiber L, Colmer TD, Nakazono M (2017) Anatomical and biochemical characterisation of a barrier to radial O₂ loss in adventitious roots of two contrasting *Hordeum marinum* accessions. *Funct Plant Biol* 44:845–857
- Krishnamurthy P, Ranathunge K, Franke R, Prakash HS, Schreiber L, Mathew MK (2009) The role of root apoplastic transport barriers in salt tolerance of rice (*Oryza sativa* L.). *Planta* 230:119–134
- Lehmann H, Stelzer R, Holzamer S, Kunz U, Gierth M (2000) Analytical electron microscopical investigations on the apoplastic pathways of lanthanum transport in barley roots. *Planta* 211:816–822
- Ma F, Peterson CA (2003) Current insights into the development, structure and chemistry of the endodermis and exodermis of roots. *Can J Bot* 81:405–421
- Ma J, Hang Z, Yang G, Mao J, Xu F (2011) Ultrastructural topochemistry of cell wall polymers in *Populus nigra* by transmission electron microscopy and Raman imaging. *BioResources* 6:3944–3959
- Man Y, Zhao YY, Ye R, Lin JX, Jing YP (2018) In vivo cytological and chemical analysis of Casparian strips using stimulated Raman scattering microscopy. *J Plant Physiol* 220:136–144
- Nagahashi G, Thomson WW, Leonard RT (1974) The Casparian strips as a barrier to the movement of lanthanum in corn roots. *Science* 183:670–671
- Nakayama T, Shinohara H, Tanaka M, Baba K, Ogawa-Ohnishi M, Matsubayashi Y (2017) A peptide hormone required for Casparian strips diffusion barrier formation in *Arabidopsis* roots. *Science* 355:284–286
- Naseer S, Lee Y, Lapierre C, Franke R, Nawrath C, Geldner N (2012) Casparian strips diffusion barrier in *Arabidopsis* is made of a lignin polymer without suberin. *Proc Natl Acad Sci USA* 109:10101–10106
- Perumalla CJ, Peterson CA, Enstone DE (1990) A survey of angiosperm species to detect hypodermal Casparian bands. I. Roots with a uniseriate hypodermis and epidermis. *Bot J Linn Soc* 103:93–112
- Peterson CA (1988) Exodermal Casparian bands: their significance for ion uptake by roots. *Physiol Plant* 72:204–208
- Peterson CA, Perumalla CJ (1990) A survey of angiosperm species to detect hypodermal Casparian bands. II. Roots with a multiseriate hypodermis or epidermis. *Bot J Linn Soc* 103:113–125
- Peterson CA, Murrmann M, Steudle E (1993) Location of the major barriers to water and ion movement in young roots of *Zea mays* L. *Planta* 190:127–136
- Priestley JH, North EE (1922) Physiological studies in plant anatomy: III. The structure of the endodermis in relation to its function. *New Phytol* 21:113–139
- Ranathunge K, Kotula L, Steudle E, Lafitte R (2004) Water permeability and reflection coefficient of the outer part of young rice roots are differently affected by closure of water channels (aquaporins) or blockage of apoplastic pores. *J Exp Bot* 55:433–447
- Ranathunge K, Lin J, Steudle E, Schreiber L (2011) Stagnant deoxygenated growth enhances root suberization and lignifications, but differentially affects water and NaCl permeabilities in rice (*Oryza sativa* L.) roots. *Plant Cell Environ* 34:1223–1240
- Robards AW, Payne HL, Gunning BES (1976) Isolation of the endodermis using wall-degrading enzymes. *Cytobiology* 13:85–92
- Schreiber L (1996) Chemical composition of Casparian strips isolated from *Clivia miniata* Reg. roots: evidence for lignin. *Planta* 199:596–601
- Schreiber L, Breiner H, Riederer M, Düggelin M, Guggenheim R (1994) The Casparian strips of *Clivia miniata* Reg. roots: isolation, fine structure and chemical nature. *Bot Acta* 107:353–361
- Schreiber L, Hartmann K, Skrabs M, Zeier J (1999) Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls. *J Exp Bot* 50:1267–1280
- Shen J, Xu G, Zheng HQ (2015) Apoplastic barrier development and water transport in *Zea mays* seedling roots under salt and osmotic stresses. *Protoplasma* 252:173–180
- Shi J, Zhen Y, Zheng R (2010) Proteome profiling of early seed development in *Cunninghamia lanceolata* (Lamb.) Hook. *J Exp Bot* 61:2367–2381
- Stasovski E, Peterson CA (1993) Effects of drought and subsequent rehydration on the structure, vitality, and permeability of *Allium cepa* adventitious root. *Can J Bot* 71:700–707
- State Forest Administration P. R. China (2014) Forest resources in China (2009–2013). China Forestry Publishing House, Beijing
- Steudle E (2000) Water uptake by roots: effects of water deficit. *J Exp Bot* 51:1531–1542
- Steudle E, Peterson CA (1998) How does water get through roots? *J Exp Bot* 49:775–788
- Van Fleet DS (1961) Histochemistry and function of the endodermis. *Bot Rev* 27:165–221
- Weis C, Hildebrandt U, Hoffmann T, Hemetsberger C, Pfeilmeier S, König C, Schwab W, Eichmann R, Hükelhoven R (2014) CYP83A1 is required for metabolic compatibility of *Arabidopsis* with the adapted powdery mildew fungus *Erysiphe cruciferarum*. *New Phytol* 202:1310–1319
- Wu XQ, Lin JX, Lin QQ, Wang J, Schreiber L (2005) Casparian strips in needles are more solute permeable than endodermal transport barriers in roots of *Pinus bungeana*. *Plant Cell Physiol* 46:1799–1808

- Yang J, Ding C, Xu B, Chen C, Narsai R, Whelan J, Hu Z, Zhang M (2015) A Casparian strips domain-like gene, *CASPL*, negatively alters growth and cold tolerance. *Sci Rep* 5:14299
- Yokoyama M, Karahara I (2001) Radial widening of the Casparian strips follows induced radial expansion of endodermal cells. *Planta* 213:474–477
- Zeier J, Schreiber L (1997) Chemical composition of hypodermal and endodermal cell walls and xylem vessels isolated from *Clivia miniata*: identification of the biopolymers lignin and suberin. *Plant Physiol* 113:1223–1231
- Zeier J, Schreiber L (1998) Comparative investigation of primary and tertiary endodermal cell walls isolated from the roots of five monocotyledonous species: chemical composition in relation to fine structure. *Planta* 206:349–361
- Zeier J, Schreiber L (1999) Fourier transform infrared-spectroscopic characterization of isolated endodermal cell walls from plant roots: chemical nature in relation to anatomical development. *Planta* 209:537–542
- Zeier J, Goll A, Yokoyama M, Karahara I, Schreiber L (1999a) Structure and chemical composition of endodermal and rhizodermal/hypodermal walls of several species. *Plant Cell Environ* 22:271–279
- Zeier J, Ruel K, Ryser U, Schreiber L (1999b) Chemical analysis and immunolocalisation of lignin and suberin in endodermal and hypodermal/rhizodermal cell walls of developing maize (*Zea mays* L.) primary roots. *Planta* 209:1–12

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.