Journal of Plant Physiology 220 (2018) 136–144

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Research Paper

In vivo cytological and chemical analysis of Casparian strips using stimulated Raman scattering microscopy

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ARTICLE INFO

Keywords:
Casparian strip
Lignin
Suberin
Label-free analysis
Stimulated Raman scattering microscopy
Maize

ABSTRACT

The Casparian strip, a barrier to the apoplastic movement of solutes from the cortex to the stele, is essential for the exclusion of salts, selective nutrient uptake, and many other processes. To date, extensive studies have focused on the physiological functions of endodermal Casparian strips. However, the chemical deposition nature of Casparian strips, as well as its relevance with respect to diffusion barrier functions, remains to be further elucidated. Here, we revealed three developmental stages of Casparian strips in maize primary roots using a traditional fluorescent staining method. Apoplastic permeability tests demonstrated that the barrier function of Casparian strips is largely related to their developmental stage and the pattern of lignin and suberin deposits. Fourier transform infrared (FTIR) analysis showed that the Casparian strips from the roots exhibited significant absorption bands characteristic of lignin and suberin, implying that the Casparian strips in maize primary roots consist largely of lignin and suberin. Furthermore, we developed a new method for label-free, *in vivo* structural, and biochemical analysis of Casparian strips based on stimulated Raman scattering (SRS) microscopy. Using SRS microscopy, we found that lignin and suberin accumulate simultaneously during the Casparian strip formation process. Based on these results, we propose a potential application of SRS for the chemical composition analysis of plant Casparian strips *in situ*.

1. Introduction

Plants develop various mechanisms and strategies to survive different environmental stresses. The roots of virtually all vascular plants have an endodermis, which helps mediate the movement of water, ions, and hormones into and out of the vascular system, thus providing a barrier to the apoplastic movement of ions and other solutes (Bonnett, 1968; Naseer et al., 2012; Peterson et al., 1993; Shen et al., 2015). Casparian strips, which develop from the endodermis of primary roots, act as physical apoplastic transport barriers and are involved in blocking the nonselective apoplastic bypass flow of water and ions into the stele (Ma and Peterson, 2003). Previous reports indicated that the properties of apoplastic barriers in the root are tissue-specific and respond to different environmental stress conditions, such as salt (Karahara et al., 2004; Krishnamurthy et al., 2009; Shen et al., 2015), alkaline (Degenhardt and Gimmler, 2000), osmotic (Shen et al., 2015), and cold stresses (Yang et al., 2015). Therefore, the physiological functions of endodermal Casparian strips in roots have received considerable attention.

For a further mechanistic dissection of Casparian strip formation, a better understanding of the chemical compositions of early Casparian strips is essential. Nevertheless, the chemical nature of the Casparian strip polymer has remained a contentious issue for more than a century. It is pointed out that its resistance to chemical treatments does not allow determination of whether it is composed of lignin or suberin (Caspary, 1865). Consequently, it was concluded that Casparian strips are composed of suberin, an aliphatic polyester that is the main component of cork, whereas evidence that Casparian strips largely consist of a lignin-like polymer was also found (Espelie and Kolattukudy, 1979; Van Fleet, 1961). Furthermore, it is worth noting that the total amounts of suberin and lignin in isolated endodermal cell walls vary between dicotyledonous and monocotyledonous species (Zeier et al., 1999a).

Lignin and suberin may play different roles in apoplastic transport. In *Arabidopsis*, the endodermal differentiation process is characterized by two developmental stages: the primary stage of the establishment of the Casparian strips and the secondary stage of the deposition of suberin lamellae that eventually coat the entire endodermal cell (Barberon and Geldner, 2014). The initial endodermal diffusion carrier is

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https://doi.org/10.1016/j.jplph.2017.11.002
Received 25 July 2017; Received in revised form 7 November 2017; Accepted 9 November 2017
Available online 14 November 2017
0176-1617/

composed of a lignin polymer, whereas suberin is neither present nor required in early Casparian strips (Naseer et al., 2012). In maize roots, the development of endodermal cells was observed in three stages on the basis of the morphology of suberin stained by the hydrophobic dye Sudan III and cell walls stained by toluidine blue: the primary stage with the Casparian strip present, the secondary stage showing the deposition of suberin lamellae on the internal wall surface, and the tertiary stage displaying strong U-shaped cell walls (Schreiber et al., 1999; Shen et al., 2015; Zeier et al., 1999b). However, the traditional histochemistry of Casparian strips was limited and could not accurately differentiate the endodermal cells and the deposition of lignin and suberin separately.

Over the past decade, coherent Raman microscopy (CRM) techniques based on coherent anti-Stokes Raman scattering (CARS) or stimulated Raman scattering (SRS) have emerged as powerful biomedical imaging modalities that allow label-free imaging based on the intrinsic vibrational signatures of molecules in the sample (Min et al., 2011). SRS microscopy, which has almost identical spectra to spontaneous Raman scattering and the absence of the nonresonant background, is much more convenient for quantitative chemical imaging (Freudiger et al., 2008; Ploetz et al., 2007; Wang et al., 2016; Yu et al., 2012). Here, we present a new method for label-free, *in vivo* structural, and biochemical analysis of Casparian strips based on SRS microscopy. The purpose of our study is to examine the developmental stages and barrier function of Casparian strips in maize primary roots, with a particular focus on the deposition of lignin and suberin. In addition, the role of SRS microscopy in quantitative label-free chemical imaging for Casparian strips *in vivo* is discussed in an attempt to provide a foundation for further investigations into the chemical nature of plant cell walls *in situ*.

2. Materials and methods

2.1. Plant materials

Maize (*Zea Mays* L. cv. Zhengdan 958) kernels were soaked in distilled water for 24 h at room temperature, sterilized with 0.2% NaClO for 15 min, rinsed three times with sterile water, and germinated on filter paper moistened with distilled water for two days in darkness at 28 °C. For further culture, seedlings with roots approximately 3 cm long were chosen and grown in a growth chamber (16 h light at 500–600 μmol m^{-2} s^{-1} photosynthetically active radiation, 22 °C, 70% relative atmospheric humidity; 18 °C and 90% relative atmospheric humidity during the dark period) for a further four days. The seedlings were watered with sterilized distilled water every day. Under these growth conditions, the maize seedlings developed primary roots 14–17 cm in length.

2.2. Histochemistry of Casparian strips

Freehand cross-sections of 20–25 μm thickness were cut at the following distances from the root tip: 2, 4, 6, 8, 10, and 12 cm, using a razor blade. To check the Casparian bands, sections were stained with 0.1% (w/v) berberine hydrochloride for 1 h and counter-stained with 0.5% (w/v) aniline blue for another hour (Brundrett et al., 1988; Schreiber et al., 1994). Stained sections were viewed with a Leica TCS SP5 confocal laser microscope using an excitation wavelength of 450–490 nm and a detection wavelength of 520 nm. To detect suberin lamellae, sections were stained for 1 h with 0.01% (w/v) Fluorol Yellow 088 (FY 088) as described previously (Brundrett et al., 1991; Shiono et al., 2014) and then examined under a Leica TCS SP5 confocal laser microscope under excitation at 450–490 nm, emission at 520 nm, and dichroic mirror at 510 nm.

2.3. Permeability tests

The permeability of the root apoplastic barriers was tested using the apoplastic fluorescent dyes Calcofluor and berberine as reported previously using described procedures with some modifications (Wu et al., 2005; Pecková et al., 2016; Weis et al., 2014). Fresh primary roots were cut into segments approximately 5 mm long at the following distances from the root tip: 2, 4, 6, 8, 10, and 12 cm, using a razor blade, and the cut edges were sealed with paraffin wax. Root segments of maize seedlings were carefully wounded by scratching the exodermis, since this highly impermeable tissue does not allow dyes to enter the cortex. The segments were soaked either in 0.05% (w/v) berberine hemisulfate dissolved in 0.05 M phosphate buffer (pH 6.0) for 12 h and then in 0.05 M potassium thiocyanate (KSCN) for 2 h to precipitate crystals of berberine thiocyanate or in 0.01% (w/v) Calcofluor for 24 h. After incubation, root sections were rinsed in phosphate buffer and sectioned with double-edged razor blades. Then, the sections were mounted in glycerin and were directly observed using a Leica DM2500 fluorescence microscope. For berberine thiocyanate-treated sections, the specimens were examined with excitation filter BP 450–490 nm, chromatic beam filter FT 510 nm, and barrier filter LP 515 nm. For the Calcofluor-treated sections, the specimens were observed with excitation filter BP 365 nm, chromatic beam filter FT 395 nm, and barrier filter LP 420 nm.

2.4. Isolation of Casparian strips from roots

Primary roots of six-d-old maize plants, which were approximately 14–17 cm long, were divided into three zones: zone I (2–4 cm), zone II (4–8 cm), and zone III (8–12 cm). Root sections were separately isolated from zone II and zone III using a razor blade. Enzymatic isolation and purification procedures of Casparian strips were carried out following a method previously described in detail (Schreiber et al., 1994). Root sections were incubated at 37 °C in an enzymatic solution containing 2.5% (w/v) cellulose (Onozuka R-10) and 1.5% (w/v) Pectinase Y-23 dissolved in 0.01 M citric buffer adjusted to pH 5. After one day, root segments were washed three times in deionized water, followed by separating the isolated rhizodermal and hypodermal cell walls. Then, the endodermal cell walls and xylem vessels were isolated using two forceps under a binocular microscope. The separated networks of the Casparian strips from zone II and zone III were stored in sterilized distilled water at 4 °C.

2.5. FTIR spectroscopy

Casparian strips isolated from primary roots were transported to the Medium Instrument Lab, Chemistry College, Peking University. FTIR spectra of Casparian strips were recorded using an FTIR spectrometer (Spotlight 200, PerkinElmer, America). The FTIR spectra were recorded with a resolution of 2 cm^{-1} in the spectral range from 4000 to 650 cm^{-1}. Samples were scanned three times.

2.6. SRS microscopy

Freehand cross-sections of 20–25 μm thickness were cut at the following distances from the root tip: 2, 4, 6, 8, 10, and 12 cm, using a razor blade. An SRS imaging microscope using a mode-locked Nd:YVO4 laser (High QLaser) was used to generate a 7-ps, 76 MHz pulse train of both 1064 nm (1 W average power) and 532 nm (5 W average power) laser beams. The 1064 nm output was used as the Stokes light. The 532 nm beam was 50/50 split to pump two optical parametric oscillators (Levante Emerald, A·P·E Angewandte Physik und Elektronik GmbH). The output wavelengths of the optical parametric oscillators were selected at 816.4 and 909.6 nm to use as pump beams to induce the stimulated Raman signal for the 2850 cm^{-1} suberin CH_2 stretching vibration and the 1600 cm^{-1} lignin aromatic ring vibration, respectively. All pump and Stokes beams were directed into an Olympus laser scanning microscope scanning unit (BX62WI/FV300; Olympus) and focused by a high numerical aperture water-immersion objective (UPLSApo 60 × 1.20 NA W; Olympus). The light transmitted through