Casparian Strips in Needles are More Solute Permeable than Endodermal Transport Barriers in Roots of *Pinus bungeana*

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The structure and development of endodermal Casparian strips in *Pinus bungeana* needles and roots were studied by scanning electron microscopy and fluorescence microscopy. Primary pit fields (PFs) frequently occurred in radial walls of Casparian strips isolated from needles, whereas PFs were never detected in Casparian strips from roots. Formation of Casparian strips in needles as well as roots started at the outer parts of the radial walls and they finally occupied the entire radial walls of the endodermis. Fourier transform infrared (FTIR) spectroscopy of Casparian strips isolated from roots revealed significant absorption bands characteristic for suberin. However, in Casparian strips of needles, evidence for suberin was rarely detected by FTIR spectroscopy. The apoplastic permeability of Casparian strips in needles and roots was probed by the apoplastic tracers calcofluor and berberine. Casparian strips in roots efficiently blocked the apoplastic transport (AT) of calcofluor and berberine. Casparian strips in needles blocked the AT of calcofluor, but diffusion of berberine was not inhibited and berberine thiocyanate crystals were detectable in the vascular tissue of the needles. From the data presented, it must be concluded that Casparian strips in needles, which are characterized by the absence of suberin, are more solute permeable compared with Casparian strips in roots.

Keywords: Apoplast — Casparian strips — Endodermis — FTIR spectroscopy — Permeability — *Pinus bungeana*.

Abbreviations: AT, apoplastic transport; FTIR spectroscopy, Fourier transform infrared spectroscopy; PF, pit field.

Introduction

Leaves are involved in many essential physiological processes, such as photosynthesis, transpiration and foliar application. Assimilates produced by leaves during photosynthesis are required for growth and energy supply, and they have to be transported from their source to the vascular bundles. Although symplastic transport can be of importance, transport in the apoplast of the mesophyll tissue seems to dominate (Geiger 1975). Similarly, in transpiration, water travels across the bundle endodermis or sheath to the mesophyll via the apoplast. In pine needles, the vascular tissue and the associated transfusion tissue are surrounded by an endodermis with Casparian strips (Esau 1977). Hardly anything is known about the physiological function of this needle endodermis.

Casparian strips are well known as characteristic modifications of the radial walls of the root endodermis (Esau 1977). The structure (Kroemer 1903, Bonnett 1968, Ma and Peterson 2003), chemical nature (Schreiber et al. 1994, Zeier and Schreiber 1999, Hartmann et al. 2002) and physiological function (Nagahashi et al. 1974, Steudle and Peterson 1998) of endodermal Casparian strips in roots have been the subject of many investigations. It has been shown that environmental stimuli such as light influenced the formation of Casparian strips (Karahara and Shibaoka 1994, Karahara and Shibaoka 1998). Furthermore, various environmental stress factors, such as drought and salinity, can also influence the development and formation of Casparian strips (Stasovski and Peterson 1993, Yokoyama and Karahara 2001, Karahara and Shibaoka 2004). Fairly good concepts about the structure and function of Casparian strips in roots acting as apoplastic transport (AT) barriers have been established (Hose et al. 2001, Enstone et al. 2003).

Still today the function of Casparian strips occurring in aerial organs of plants is less clear (Lersten 1997). Even the question whether the endodermis of conifer needles possesses Casparian strips still remains controversial (Buchholz 1951, Gambles and Dengler 1973, Pant and Basu 1977, Carde 1978, Hu and Wang 1984, Lin and Hu 2000, Boddi et al. 2002). With a recent approach, we successfully isolated Casparian strips from the needles of *Pinus bungeana* for the first time (Wu et al. 2003). The function of Casparian strips in pine needles, however, still remains unclear.

Therefore, we intended to study the fine structure, the development, the chemical composition and the potential function of endodermal Casparian strips in *P. bungeana* needles as an apoplastic barrier in more detail. Furthermore, the structure and function of Casparian strips in needles and roots of *P. bungeana* were compared. This should finally help to understand better the role of Casparian strips in the needles of *P. bungeana*.

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Results

Scanning electron microscopy

Scanning electron microscopy of Casparian strips isolated enzymatically from 2-year-old needles and primary roots of saplings demonstrated that the anticlinal walls of the endodermis had resisted the enzymatic degradation (Fig. 1). During isolation of Casparian strips, the outer tangential walls of the endodermal cells were more resistant to enzymatic degradation (Fig. 1B) than the inner tangential walls (Fig. 1A). The inner anticlinal walls oriented parallel to the longitudinal axis of the needle were characterized by a pronounced undulation (Fig. 1C) or by the occurrence of primary pit fields (PFs) (Fig. 1D). The undulation and primary PFs did not occur simultaneously in anticlinal walls. However, there was no PF and no undulation in the transverse walls of the Casparian strips isolated from 2-year-old needles (Fig. 1E). The undulation was also visible in the inner anticlinal walls of the Casparian strips isolated from primary roots (Fig. 1F and G). Primary PFs were never detected in anticlinal walls of Casparian strips isolated from roots.

Fluorescence microscopy

Cross-sections of the cotyledons showed no fluorescence in the radial walls of the endodermis, whereas the xylem exhibited a strong fluorescence after staining with berberine hemisulfate (Fig. 2A). In primary (Fig. 2B) and 2-year-old needles (Fig. 2C), Casparian strips were visible in the radial endodermal walls. In 2-year-old needles (Fig. 2C) and in roots (Fig. 2D), Casparian strips occupied nearly the entire radial walls of the endodermis. Stronger fluorescence was emitted from Casparian strips in primary needles (Fig. 2B) compared with those in 2-year-old needles (Fig. 2C) and in roots (Fig. 2D). In primary needles, Casparian strips were not formed in all endodermal cells, whereas they were present in all endodermal cells.
Casparian strips in needles of *Pinus bungeana*

**Fig. 2** Fluorescence micrographs of free-hand cross-sections of needles (middle portion) and primary roots of *P. bungeana* sequentially stained with berberine hemisulfate and toluidine blue O. (A) Cotyledon. (B) Primary needle. (C) Two-year-old needle. (D) Primary root. En, endodermis; Cs, Casparian strip; Rh, root hair; Xy, xylem. Scale bars = 100 µm.

**Fig. 3** Fluorescence micrographs of isolated Casparian strips sequentially stained with berberine hemisulfate and toluidine blue O. (A) Casparian strips isolated from primary needles of *P. bungeana* saplings. (B) Casparian strips isolated from 2-year-old needles of *P. bungeana* saplings. (C) Casparian strips isolated from 2-year-old needles of a 10-year-old *P. bungeana* tree. (D) Casparian strips isolated from primary roots of 2-year-old *P. bungeana* saplings. Tw, transverse walls of endodermal cells; Rw, radial walls of endodermal cells; Pf, pit fields. Scale bars = 100 µm.
Casparian strips in needles of *Pinus bungeana*

of 2-year-old needles and in roots. Casparian strips were not detectable in radial hypodermal walls of needles or roots.

Casparian strips isolated from primary needles (Fig. 3A) and 2-year-old needles of saplings (Fig. 3B), from 2-year-old needles of 10-year-old trees (Fig. 3C) and from primary roots of saplings (Fig. 3D) showed a bright yellow-green fluorescence and they formed continuous nets (Fig. 3). The mesh of isolated Casparian strips of primary needles and 2-year-old needles obtained from saplings was rectangular (Fig. 3A, B). Furthermore, PFs were present in most radial walls, but they were absent in the transverse walls of Casparian strips, consistent with the results obtained by scanning electron microscopy. The mesh of isolated Casparian strips obtained from 2-year-old needles of 10-year-old trees was more square like. PFs again were abundant in radial walls of Casparian strips. As calculated, the distribution of PFs in Casparian strips isolated from 2-year-old needles of 10-year-old trees (11 ± 2/100 µm) was denser and more abundant as compared with Casparian strips of primary (4 ± 1/100 µm) and 2-year-old needles (7 ± 1/100 µm) from saplings. PFs were not found in radial or transverse walls of Casparian strips isolated from primary roots.

**Development of Casparian strips in needle and root endodermis**

The cross-sections of needles and primary root at different developmental stages were observed. Casparian strips could not be detected in cross-sections of the youngest immature needles directly obtained on the first sample date (May 15, 2003) (Fig. 4A). The width of Casparian strips in the radial walls of the endodermis in developing needles increased more strongly within the first month (Fig. 4B). They occupied about 10 µm of the radial endodermal cell walls. In fully developed needles harvested after 60 d, Casparian strips were 11.5 µm in width and nearly completely occupied radial endodermal cell walls (Fig. 4C). With increasing needle age, Casparian strips extended from the outer parts of the radial walls towards the inner parts with an asymmetrical pattern (Fig. 4B, C).

Casparian strips were found to be faintly visible in the radial wall of all endodermal cells opposite to the protoxylem and the protophloem in the youngest parts of *P. bungeana* roots.
Casparian strips in needles of *Pinus bungeana*

The Casparian strips in needles of *Pinus bungeana* were described starting from 3 mm behind the root tip (Fig. 4D). In the parts of the root 5 mm behind the root tip, most of the radial wall was already occupied by the Casparian strip at the early stage of xylem and phloem development (Fig. 4E, F). With increasing root development (20 mm behind the root tip), Casparian strips completely occupied the whole radial endodermal cell walls (Fig. 4F, G). Similarly to needles, Casparian strips in roots started to form in the outer regions of the radial endodermal cells and they continued to enlarge asymmetrically towards the inner parts of the radial walls (Fig. 4D–H).

**FTIR spectroscopy of Casparian strips**

The spectra of Casparian strips isolated from primary needles, 2-year-old needles and primary roots had a broad absorption band at around 3,330 cm\(^{-1}\), which indicates the occurrence of stretching vibrations of hydrogen bonds in OH or NH groups (Fig. 5A). Similar absorption bands appeared in the spectra of cellulose and lignin, and of lysozyme (Fig. 5B). The two intense bands at 2,920 and 2,852 cm\(^{-1}\) in the spectrum of Casparian strips isolated from roots can be assigned to the asymmetrical and symmetrical stretching vibration of methylene groups, as they occur in suberin. In the spectra of Casparian strips isolated from needles, however, these sharp bands were rarely detectable (Fig. 5A). Similar absorption bands of methylene groups at 2,918 and 2,850 cm\(^{-1}\) were observed with ethyl stearate, the ester of the linear long chain stearic acid with ethanol (Fig. 5B).

The strong absorption band attributed to C–O stretching vibrations in carboxylic esters, which appeared in the spectrum of ethyl stearate at 1,740 cm\(^{-1}\) (Fig. 5A), was also detectable at 1,736 cm\(^{-1}\) in the spectrum of Casparian strips from roots (Fig. 5A, B). The corresponding absorption bands were very weak at 1,737 cm\(^{-1}\) in the spectrum of primary needles and at 1,730 cm\(^{-1}\) in 2-year-old needles. Absorption bands between 1,650 and 1,500 cm\(^{-1}\) can be of very different chemical origins. Bands at 1,600 and 1,500 cm\(^{-1}\) are typical of the vibrations of C atoms in the aromatic ring, as they were in fact present at 1,611 and 1,514 cm\(^{-1}\) in 2-year-old needles, at 1,631 and 1,535 cm\(^{-1}\) in primary needles, and at 1,610 and 1,513 cm\(^{-1}\) in roots (Fig. 5A). In the spectrum of cellulose and lignin, comparable absorption peaks were found at 1,637 and 1,511 cm\(^{-1}\) (Fig. 5B). However, bands at 1,660 and 1,520 cm\(^{-1}\) characteristic for the amide I and amide II bands as they appear in proteins (e.g. lysozyme in Fig. 5B) significantly overlapped with other signals in Fig. 6A. Bands at around 1,060 cm\(^{-1}\), as they occurred in all three samples of isolated Casparian strips (Fig. 5A), are typical for carbohydrates as they can be seen in cellulose (Fig. 5B).

**Permeability tests**

The apoplastic permeability of the endodermis in needles and roots of *P. bungeana* was probed by the two different apoplastic tracers calcofluor and berberine. In needles, calcofluor penetrated and stained the mesophyll below the wound (Fig. 6A). Diffusion of calcofluor into the vascular tissue in the middle of the needle was inhibited efficiently by the Casparian strips of the roots (Fig. 6C, D). Root sections treated with calcofluor showed a bright fluorescence in the epidermis and a light blue fluorescence in the cortex (Fig. 6B). In the control sections, hardly any autofluorescence was detectable in the epidermis and the cortex (Fig. 6D). Diffusion of calcofluor into the central cylinder was prevented efficiently by the Casparian strips of the roots (Fig. 6C, D).

Needles with a damaged cuticle and epidermis allowed berberine to diffuse through the mesophyll across Casparian strips into the vascular tissue in the center of the needle, as can
Fig. 6  Fluorescence micrographs of freehand cross-sections of 2-year-old *Pinus bungeana* needles (A, B, E and F) and primary roots (C, D, G and H). (A) Cross-section of a wounded needle treated with calcofluor. (B) Autofluorescence of a control needle. Exposure times were the same for (A) and (B). (C) Section of a root treated with calcofluor. (D) Autofluorescence of a control root. Exposure times were the same for (C) and (D). (E) Cross-section of a wounded needle treated with berberine hemisulfate and potassium thiocyanate. Fluorescent crystals are visible in the vascular bundle (red arrows). (F) Autofluorescence of a control needle. Exposure times were the same for (E) and (F). (G) Cross-section of a root treated with berberine hemisulfate and potassium thiocyanate. Fluorescent crystals are visible only in the cortex and not in the central cylinder. (H) Autofluorescence of a control root. Exposure times were the same for (G) and (H). Cs, Casparian strips; Co, cortex; En, endodermis; Ep, epidermis; Me, mesophyll; Vb, vascular bundle; W, wound. Scale bars = 200 µm.
be seen from the bright yellow fluorescence of berberine thiocyanate crystals (Fig. 6E). With the exception of the red chlorophyll fluorescence, rarely any autofluorescence was detectable in the control sections (Fig. 6F). In roots, both berberine-treated and control sections exhibited a green-yellowish autofluorescence and the vascular tissue, since berberine thiocyanate crystals were efficiently prevented from crossing the endodermis and entering the vascular tissue, since berberine thiocyanate crystals were found only outside the layer of endodermal cells (Fig. 6G).

**Discussion**

The development of Casparian strips in radial walls of the root endodermis can be strikingly different when comparing different species (Barnabas and Peterson 1992, Enstone and Peterson 1997, Karahara et al. 2004). Formation of Casparian strips in onion root endodermis started in the middle of the radial endodermal walls and they expanded symmetrically to both sides of the radial cell walls (Barnabas and Peterson 1992). In corn roots and other monocotyledonous species, formation of Casparian strips started in the radial walls adjacent to the cortex and they expanded asymmetrically towards the center of the endodermal walls (Van Fleet 1942, Enstone and Peterson 1997). Casparian strip initiation was correlated with the xylem or the oxidation of phloem-associated compounds (Van Fleet 1942, Wilcox 1954). In *P. bungeana*, both foliar and root endodermal Casparian strips started to form in the outermost parts of the radial endodermal walls and they expanded asymmetrically towards the center of the endodermal walls. The early development of foliar and root Casparian strips in the radial wall of endodermis opposite xylem and phloem formation did not seem to support the observation of either Van Fleet (1942) or Wilcox (1954). Additionally, Casparian strips in roots were already nearly completed in very early developmental stages compared with xylem and phloem development. This was different from foliar Casparian strips, where the increase of their radial width was concomitant with xylem and phloem development. From this observation, it can be concluded that Casparian strips in needles are not as essential to plant function as Casparian strips in roots, which were fully developed at a very early stage. This idea is also supported by the lack of Casparian strips in cotyledon endodermis.

Enzymatically isolated endodermal cell walls can be used for microscopic and analytical investigations (Robards et al. 1976, Karahara and Shibaoka 1992, Schreiber et al. 1994). In isolated endodermal cell walls of *Clivia miniata* Reg., numerous PFs were observed on the surfaces of the periclinal walls of the endodermis, but they have never been observed in the radial walls characterized by the deposition of the Casparian strips (Schreiber et al. 1994). In the present investigation, numerous PFs were present in the radial walls of the needle endodermis characterized by Casparian strips, in contrast to the observation on the root Casparian strips, forming a part of symplast interaction in the endodermis which greatly facilitated the intercellular transportation by plasmodesmata. PFs in the radial walls of foliar Casparian strips reduce the barrier properties of the endodermis, again indicating that foliar Casparian strips in needles are less important than in roots.

Using FTIR spectroscopy, it was demonstrated that lignin was one of the major biopolymers present in Casparian strips of a series of different plant species (Schreiber et al. 1994, Zeier and Schreiber 1999). However, Casparian strips also contained small amounts of suberin, cell wall carbohydrates and proteins (Zeier and Schreiber 1999). These conclusions from FTIR spectroscopy were confirmed by direct chemical analysis of isolated endodermal cell walls using gas chromatography and mass spectrometry (Schreiber 1996, Schreiber et al. 1999). The chemical composition of isolated Casparian strips from *P. bungeana* roots, as can be deduced from FTIR spectroscopy, corresponds very well with previous reports for *Pisum sativum*, which indicated that the Casparian strips of the root endodermis contained an equal amount of suberin and lignin (Zeier et al. 1999). However, FTIR spectroscopy of Casparian strips isolated from needles did not provide evidence that there were significant amounts of suberin. Sharp absorbance bands at 2,920, 1,850 and 1,730 cm\(^{-1}\), which are characteristic of linear, long chain, aliphatic compounds as they occur in suberin (Zeier and Schreiber 1999), were extremely weak and very broad in isolated Casparian strips of *P. bungeana* needles. This result was confirmed by an analytical approach using gas chromatography and mass spectrometry, which did not provide evidence for the occurrence of typical suberin in isolated Casparian strips of needles (data not shown). Thus, from this difference in the content of suberin between Casparian strips in needles and roots, different functional properties as AT barriers can be postulated.

The role of the root endodermal Casparian strips as an AT barrier has been the subject of many investigations. Fluorescent dyes including calcofluor, berberine and Lucifer yellow CH were frequently used for this purpose (Robards 1984, Perumalla et al. 1990, Enstone and Peterson 1992a, Seago et al. 1999). Calcofluor, which binds tightly to cellulose, has been used as an apoplastic tracer to detect the presence of hypodermal Casparian strips in roots of 181 species from 53 families of flowering plants (Perumalla et al. 1990, Peterson and Perumalla 1990). However, the cases of membrane permeability of berberine varied in different organisms. It was found that cultured *Coptis japonica* cells could take up berberine from the medium (Sato et al. 1993, Sakai et al. 2002). The membrane permeability of berberine was also revealed in the intact vacuoles and a tonoplast vesicle system of *C. japonica* (Otani et al. 2005). On the contrary, berberine could not pass the membrane of yeast and some plant species (Sato et al. 1990, Enstone and Peterson 1992a). Enstone and Peterson (Enstone and Peterson 1992a, Enstone and Peterson 1992b) reported that berberine was confined to AT, while the inward diffusion of berberine was arrested at the root endodermis in *Zea mays*, *Allium cepa*.
and *Helianthus annuus*. In order to prevent berberine from relocation after sectioning, a method was developed precipitating berberine by thiocyanate to form immobile crystals (Enstone and Peterson 1992a, Seago et al. 1999). In the present experiment, we demonstrated that berberine hemisulfate did not enter the vascular tissue of root. This result excluded the possibility of berberine hemisulfate in symplastic transport and provided additional evidence that berberine hemisulfate could be used as an apoplastic tracer in the investigation of physiological functions of Casparian strips in this species.

Plants have a variety of structural designs and mechanisms to respond to changes in environmental conditions and to perform physiological functions for better growth (Esau 1977, Peterson and Cholewa 1998, Degenhardt and Gimmler 2000, Enstone et al. 2003). The root is the organ exposed to the soil and is primarily involved in absorption, in which the apoplastic interface between plant and environment is formed by cell wall modifications of the endodermis and hypodermis such as Caspian strips and suberin lamellae (Ma and Peterson 2001, Enstone et al. 2003). The leaf is the key organ exposed to air for photosynthesis and transpiration and has a thick-walled epidermis with a heavy wax cuticle to help keep moisture in and pathogens out (Schönherr 1982). In addition, deeply sunken stomata in the leaf protect the plant through opening and closing in response to environmental stress. In the present study, the physiological function of Casparian strips in needles and roots as apoplastic barriers was compared using two fluorescent apoplastic tracers, calcofluor and berberine. Calcofluor was blocked efficiently at the endodermis by Caspian strips in both needles and roots. Berberine was also blocked by Caspian strips in the endodermis of roots. However, berberine was able to penetrate radial endodermal cell walls with Caspian strips in the needles, since berberine thiocyanate crystals were detectable in the vascular tissue in the center of the needles. Furthermore, crystals were clearly observed in the same regions in continuous sections. These results provide good evidence that Caspian strips in the endodermis of needles obviously have a greater porosity than Caspian strips in roots which contributed to more solute permeability of Caspian strips in needles than endodermal transport barriers in roots of *P. bungeana*. Given the link between permeability of Caspian strips and the role of leaves, this is an important finding, suggesting that the higher permeability of foliar Caspian strips would facilitate the transport of assimilates and water from the mesophyll tissue to the vascular tissue, and vice versa. This difference in function of Caspian strips in needles from roots was indeed supported by the result of FTIR spectroscopy which showed that significantly lower amounts of suberin weaken the barrier properties of foliar Caspian strips.

### Materials and Methods

#### Plant materials

Seeds were obtained from *P. bungeana* Zucc. ex Endl. trees growing at the Institute of Botany, Chinese Academy of Sciences, PR China. Needles from cotyledons, primary needles and 2-year-old needles, and primary roots were sampled from 2-year-old saplings grown in the greenhouse at 25°C in 5 liters pots filled with forest soil. In addition, 2-year-old needles and developing needles were obtained from 10-year-old *P. bungeana* plants for developmental studies. Needles were typically harvested at intervals of 10 d from May 15 to July 15, 2003. Sections were taken from the middle segments of the needles.

#### Isolation of Caspian strips from needles and roots

Freshly harvested primary roots and needles were cut into pieces 5–10 mm in length using a razor blade. Enzymatic isolation was performed, following the methods described previously (Karahara and Shibaoa 1992, Schreiber et al. 1994). Two weeks after incubation in the enzymatic solution containing cellulose (Yakult, Japan) and pectinase (Yakult, Japan), isolated Caspian strips could be sampled. They were thoroughly washed, and stored in deionized water at 4°C.

#### Scanning electron microscopy

Caspian strips isolated enzymatically from primary roots and needles were mounted on aluminum stubs, air dried, and sputtered with gold (~20 nm). The samples were observed at 15 kV in a scanning electron microscope (XL30, Philips, Eindhoven, The Netherlands).

#### Microscopy and staining procedures

Freehand cross-sections of 20–25 µm thickness were obtained from saplings using a razor blade. For staining, cross-sections of needles and isolated Caspian strips were mounted on microscope slides, stained with berberine hemisulfate, and counter-stained with toluidine blue O for better contrast, as described previously (Brundrett et al. 1988). Samples were observed with a fluorescence microscope (Q500 IW, Carl Zeiss, Göttingen, Germany) with UV illumination using the Zeiss filter set 04 (exciter filter BP 450–490 nm, chromatic beam filter FT 510 nm, and barrier filter LP 515 nm). The width of the Caspian strips in the endodermal radial walls was measured with AxioVision 4.0 software (Carl Zeiss, Göttingen, Germany).

#### FTIR spectroscopy

FTIR spectra of Caspian strips isolated from *P. bungeana* needles and primary roots were recorded using an FTIR spectrometer (Magna-IR 750, Thermo Nicolet, Kanagawa, Japan) equipped with a mercury–cadmium–telluride detector. The FTIR spectra were recorded in the absorbance mode by accumulating 128 scans with a resolution of 8 cm⁻¹ in the spectral range from 3,500 to 600 cm⁻¹. Solid-state IR spectra of cellulose, lignin, ethyl stearate and lysozyme from the Thermo Nicolet Corporation database served as references for cell wall carbohydrates, lignin, aliphatic suberin and cell wall proteins, respectively.

#### Permeability tests

The apoplastic fluorescent dyes calcofluor and berberine were used for permeability tests. Previously published methods (Perumalla et al. 1990, Enstone and Peterson 1992b) with some modifications were applied. Needles of *P. bungeana* were carefully wounded by scratching the cuticle, since the highly impermeable cuticle would not allow the dyes to enter the needle. Care was taken that only epidermal and subepidermal tissue was affected by wounding without damaging the endodermal cell layer. The wound was covered for 12 h with small
pieces of cotton that had been soaked either in 0.01% berberine (w/v) dissolved in 0.05 M phosphate buffer (pH 6.0) or in 0.01% calcofluor (w/v). Berberine was precipitated by a subsequent treatment with 0.05 M potassium thiocyanate. Roots were carefully excised from the saplings and the cut edges were sealed with paraffin wax. Root samples were immersed for 12 h in the fluorescent dye solutions described above. After incubation, root and needle samples were rinsed in phosphate buffer and sectioned with double-edged razor blades. In order to avoid the possibility of artifacts produced by sectioning, freehand cross-sections were made in the present experiment, changing the blade after each section. Then the sections were mounted in glycerin, and directly observed using fluorescence microscopy. For calcofluor-treated sections, a blue fluorescence filter was chosen including exciter filter BP 365 nm, chromatic beam filter FT 395 nm and barrier filter LP 420 nm. For berberine thiocyanate-treated sections, a green fluorescence filter was chosen including exciter filter BP 450–490 nm, LP 420 nm.

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