

Histochemical study of detailed laticifer structure and rubber biosynthesis-related protein localization in *Hevea brasiliensis* using spectral confocal laser scanning microscopy

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Abstract In *Hevea brasiliensis*, laticifers produce and accumulate rubber particles. Despite observation using histochemical methods, development stage structure and structures with ceasing functions have rarely been described. Spectral confocal laser scanning microscopy with Nile red staining simplifies laticifer structure observation in tangential sections while enhancing the resolution. Laticifer and ray images were extracted from unmixed images and used to monitor changes during growth. A laticifer network structure developed from increased anastomoses between adjoining laticifers outside of the conducting phloem, but because of increased radial division and growth of rays, the network structure ruptured and disintegrated. We also investigated immunohistochemical localization of two rubber particle-associated proteins in the laticifers: small rubber particle protein (SRPP) and rubber elongation factor (REF). Mature bark test results show that SRPP is localized only in the laticifer layers in the conducting phloem; REF is localized in all laticifer layers. Because SRPP plays a positive role in rubber biosynthesis, results show that the rubber biosynthesis capability of laticifers is concentrated where rays and the sieve tube actively transport metabolites.

Keywords *Hevea brasiliensis* · Laticifer structure · Rubber biosynthesis · Nile red · Spectral confocal laser scanning microscopy (SCLSM) · Rubber particle

Abbreviations

CLSM Confocal laser scanning microscopy
SCLSM Spectral confocal laser scanning microscopy
LRP Large rubber particle
SRP Small rubber particle
REF Rubber elongation factor
SRPP Small rubber particle protein

Introduction

Hevea brasiliensis Müll. Arg. is a tropical tree that produces natural rubber (*cis*-1,4-polyisoprene). Among over 2,000 rubber-producing plant species, only *H. brasiliensis* is used in many areas because of its high rubber productivity and its rubber quality, as indicated by indices such as molecular weight. Natural rubber from *H. brasiliensis* has attracted attention as a highly useful industrial polymer because no synthetic substitute has comparable physical properties (Davis 1997). Consequently, because of the continued growth in the global automotive industry, global rubber demand is increasing (International Rubber Study Group: <http://www.rubberstudy.com/>). Consequently, further improvement of natural rubber production is necessary. In addition to simply planting more acreage, technical production improvements and genetic modification of *H. brasiliensis* and other rubber-producing plants may be possible. Fundamental knowledge of rubber biosynthetic processes at the tissular, cellular, and molecular levels is necessary because such processes directly affect production and accumulation of rubber.

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The selection of staining and observation techniques is crucial for detailed structural analysis of laticifers. An observation technique using confocal laser scanning microscopy (CLSM) with appropriate fluorescent probes has been developed for simple visible detection of cellular and tissular functions and for improving image resolution. Spectral imaging can be performed with successive volumes by acquiring a series of sections along the optical axis (Z-axis), thereby providing informative details of the spatial relations among structures within target cells or tissues (Dickinson et al. 2001). This technique is used extensively in both medical and biological applications because it enables the use of image processing techniques such as contrast enhancement, superposition of successive optical sections, stereoscopy, and quantification (Postberg et al. 2005; Tan et al. 2005; Wan et al. 2005; Wu et al. 2005). However, conventional CLSM had limited ability to distinguish overlapping spectra or slight spectral differences in complex systems. Spectral CLSM (SCLSM) is a recently developed high-performance technique that compensates for deficiencies of CLSM (Larson 2006). For this study, we applied SCLSM to enhance resolution and to examine the detailed structure of laticifers.

Many attempts have been made to examine the anatomical structure of laticifers and to characterize rubber particles, the most numerous organelle in the laticifer, where rubber biosynthesis occurs at the surface. Regarding the laticifer structure, they have been stained conventionally with Sudan III, Sudan IV, or Oil red O, and have been observed using light microscopy (Wimalaratna 1973; Richardson 1983; Hamzah et al. 1988; Omman and Reghu 2003). Diagrams of the three-dimensional structures in *H. brasiliensis* bark have been obtained based on observations using light microscopy (Riches and Gooding 1952; Fahn 1979; Nicole et al. 1986). These show that laticifers are arranged in concentric layers in the bark. However, detailed structures of the laticifer in each layer, such as those in tangential sections, remained unclear because of the limitation of stain specificity and observation techniques.

It has been suggested that rubber particles are necessary components in rubber synthesis (Lynen 1969). In *H. brasiliensis*, they have bimodal size distribution in fresh latex. Large rubber particles (LRPs) and small rubber particles (SRPs) show differences not only in molecular weight distribution and molecular structure but also in their surface proteins (Bahri and Hamzah 1996; Sakdapipanich et al. 1999; Ohya et al. 2000; Singh et al. 2003; Tarachiwin et al. 2005). Immunogold electron microscopy has shown that 14- and 24-kDa proteins are localized abundantly on LRPs and SRPs, respectively (Bahri and Hamzah 1996; Singh et al. 2003). Coding genes of 14 kDa rubber elongation factor (REF) and 24 kDa SRP protein (SRPP) have been cloned (Attanyaka et al. 1991; Goyvaerts et al. 1991; Oh

et al. 1999). In vitro rubber biosynthesis assays using rubber particles have shown that SRPs have much higher enzymatic activity than LRPs do (Archer et al. 1963; Ohya et al. 2000). However, rubber particles used in previous studies were prepared from fresh latex obtained from several laticifer layers by tapping the bark. Consequently, the relation between the laticifer structure and rubber particle distribution in laticifer layers has remained unclear.

This study was designed to examine the laticifers' detailed structure using precise microscopic techniques, and to investigate the in situ rubber biosynthesis capability of *H. brasiliensis*' laticifers. The combination of SCLSM and Nile red staining was evaluated and then applied to image analysis of laticifers. Using this technique, we assessed the structure of laticifers during growth. Furthermore, immunohistochemical localization of SRPP was used to evaluate the in situ rubber biosynthesis capability.

Materials and methods

Plant materials

Branches of about 60-mm diameter and latex were collected from 10-year-old *H. brasiliensis* trees (clone RRIM600) growing on a commercial plantation in PTPN VIII, Cikumpay, Purwakarta, West Java, Indonesia. Trunks of about 145-mm diameter were collected from 8-year-old *H. brasiliensis* trees (clone PB260) growing on a commercial plantation in Medan, North Sumatra, Indonesia.

Tissue preparation

For structural observation, branches were used as a model to observe differences in laticifer structure by layer. They were cut into about 5-mm² blocks containing epidermis, phloem, cambium, and a slight amount of xylem. The blocks were quickly embedded in compound (TISSUE-TEK OCT; Sakura Finetechnical Co. Ltd., Tokyo, Japan) and snap-frozen in liquid nitrogen. Then 20–60-μm-thick cryosections were prepared using a cryostat (CM-1850; Leica Microsystems, Nussloch, Germany) at –20°C: 30-μm-thick cross-sections, 50-μm-thick longitudinal and 60-μm-thick tangential sections for fluorescent staining, and 20-μm-thick cross-sections for Oil red O staining. To observe the laticifers' detailed structure, 60-μm-thick tangential sections containing laticifers were obtained in order from layers ranging from the cortex to cambium.

Histochemical staining

Cross-sections of bark from branch were prepared to compare the staining ability of Nile red and the conventional

dye, Oil red O. The sections were mounted on a glass slide, and OCT compounds were removed with distilled water. For Oil red O staining, the sections were stained with 0.5 µg/ml Oil red O (Sigma-Aldrich Corp., MO, USA) in 2-propanol for 10 s and washed with 45% acetic acid. The stained sections were mounted in 60% glycerol and sealed with nail varnish. The fluorescent reagent Nile red acts as a hydrophobic probe; its maximum fluorescence changes because of the relative hydrophobicity of the surrounding environment (Fowler and Greenspan 1985). Nile red was applied to examine its capability for rubber staining. For Nile red staining, the sections were stained with 50 µg/ml Nile red (Molecular Probes Inc., Eugene, OR, USA) in 50% ethanol for about 10 s and washed with distilled water. Cell walls were counterstained with 1 mg/ml Fluorescent Brightener 28 (Sigma-Aldrich Corp., MO, USA) in distilled water for 10 s. The sections were mounted in Vectashield mounting medium (Vector Laboratories Inc., CA, USA) and sealed with nail varnish. Latex was air-dried on a glass slide and then stained with Nile red.

Tissue observation using SCLSM

Observation conditions

Stained sections were analyzed using a true spectral imaging confocal laser scanning microscope system (Digital Eclipse C1si; Nikon Corp., Tokyo, Japan) equipped with CFI Plan Apo10×, 20×, 40×, and VC 100× H, and software (EZ-C1 3.40; Nikon Corp.). Fluorescence was excited using the 405 nm line of a blue diode laser (17 mW) and the 488 nm line of a solid laser (20 mW) with laser power set as 1.0–5.0%.

Image acquisition

Emission spectra between 423 and 743 nm were recorded using all 32 channels, each with a 10-nm fluorescence detection bandwidth. Images of 1,024 × 1,024 pixels were acquired and averaged from three successive scans to obtain an improved signal-to-noise ratio. Images of 512 × 512 pixels were acquired and averaged in two successive scans to obtain 3D images for data analyses.

Acquisition of reference spectra

Regions of interest (ROI) representing cellular constituents or subcellular components were used to calculate fluorescence spectra. To acquire reference spectra, fluorescence spectra from 30 locations in a single stained section or latex with higher resolution (1,024 × 1,024 pixels, ten scans, 5-nm fluorescence detection bandwidth) were averaged.

Unmixing for hyperspectral imaging

Spectral unmixing was performed on acquired images using software (EZ-C1 3.40; Nikon Instruments Co.). Reference spectra used for unmixing were two Nile red spectra (maximum fluorescence: 547, 575 nm) and a Fluorescent Brightener 28 spectrum (maximum fluorescence: 444 nm).

Volume rendering (3D rendering)

For rendering, 40–150 images were acquired with a scan range of the Z-axis of 20–50 µm and step size of 0.35–1.00 µm. All images were unmixed and used for volume rendering (spin 0°, tumble 0°, method maximum).

Digital image analysis

NIS-Elements AR software (Nikon Corp.) was used for digital image analysis. All measurements were performed using eight volume-rendered images that had been selected randomly from each layer of the same section. To calculate the number of anastomoses in each laticifer layer, the length of all laticifers and number of anastomoses in images of 512 × 512 pixels were measured using a measurement function. Images of laticifers were extracted from volume-rendered images and converted to a grayscale image with contrast adjustment. The area of laticifers and the other cells in luminance values of 50–255 were measured individually using the Object Count function. The area of rays surrounded by laticifers was selected and measured.

Heterologous expression of SRPP and REF in *E. coli*

The SRPP gene (GenBank accession no. AJ223388) and the REF gene (GenBank accession no. X56535) were cloned, respectively, in the *XhoI*–*Bam*HI site of pET15b (Novagen Inc., WI, USA) to construct pET15b-SRPP and pET15b-REF. The *E. coli* BL21 (DE3) transformed with either pET15b-SRPP or pET15b-REF was grown to mid-stationary phase in Luria broth containing 50 mg/l ampicillin at 37°C with vigorous aeration. The cultures were induced by adding IPTG to concentration of 0.1 mM, then incubated for another 5 h at 30°C. The cells were harvested, washed with 0.1 M potassium phosphate (pH 7.4) by centrifugation at 5,000×g for 10 min; then disrupted (BugBuster HT; Novagen Inc.). The homogenate was centrifuged at 10,000×g for 10 min. Overexpression of SRPP and REF caused inclusion body formation. Therefore, each precipitant was dissolved in 8 M urea for denaturation. Purification of the proteins containing N-terminal His-tag was achieved using nickel affinity chromatography (HisTrap HP; GE Healthcare, Tokyo, Japan) according to

the manufacturer's instructions. The buffer system chosen was 20 mM phosphate, pH 7.5, containing 500 mM NaCl, 10 mM imidazole, 8 M urea.

Antibody production

For antibody production by Takarabio (Shiga, Japan), 0.1 mg of purified His-tagged protein, either SRPP or REF, was used per mouse. Antibody against either SRPP or REF was produced in five mice. Animals were inoculated four times at 2-week intervals according to the company's standard protocol. Ten days after the final immunization, the animals were desanguinated. Specificity of each antibody against SRPP and REF was confirmed by western blot.

Tissue fixation and embedding for immunohistochemical localization

The tissue blocks were collected from a mature trunk using cork borer, and were immersed immediately in fixation buffer (4% (v/v) paraformaldehyde in PBS) to avoid latex outflow. After fixing overnight, the tissue blocks were rinsed with PBS. Then the blocks were embedded quickly in compound (Tissue-Tek OCT; Sakura Finetechnical Co. Ltd., Tokyo, Japan), and snap-frozen in liquid nitrogen. Then 40- μ m-thick sections were cut and kept at -20°C in a cryostat (CM-1850; Leica Microsystems).

Immunohistochemical localization

All subsequent steps were conducted at room temperature. After removal of OCT compound with PBS, sections were blocked in PBS containing 10% horse serum (Gibco, Auckland, New Zealand) for 20 min. Then 50 μ l of each mouse primary antibody diluted to concentration 10 μ g/ml in fresh PBS, containing 10% horse serum and 0.1% Triton X-100, was added to the sections with subsequent incubation for 6 h. The sections were then rinsed three times for 10 min each with PBS. Then 50 μ l of secondary antibody (Alexa Fluor 488 Donkey anti mouse IgG; Molecular Probes Inc.) diluted to concentration 10 μ g/ml in fresh PBS, containing 10% horse serum and 0.1% Triton X-100, was added to the sections and incubated for another 4 h. The sections were again rinsed three times for 10 min each with PBS, and mounted in mounting medium (Vectashield; Vector Laboratories Inc.). Then they were sealed with nail varnish. The reference spectrum of Alexa Fluor 488 was acquired from a commercial spectra information website (Molecular Probes Inc.). Observations were performed using SCLSM C1si as described above, with the exception that emission spectra between 320 and 750 nm were recorded.

Results and discussion

Histochemical staining

During light microscope observation of Oil red O-stained sections, the cytoplasm of laticifers and lipid bodies in parenchyma cells surrounding laticifers were stained red (Fig. 1a, c). During SCLSM observation of Nile red-stained sections, the cytoplasm of laticifers fluoresced in yellow fluorescence and lipid bodies in parenchyma cells surrounding laticifers fluoresced in orange (Fig. 1b, d). Based on our observation results, both Oil red O and Nile red non-specifically stained the cytoplasm of laticifers and lipid bodies, respectively. Nevertheless, Nile red produced differences in fluorescence, suggesting that the cytoplasm of laticifers and lipid bodies are separable.

Spectral unmixing of Nile red using SCLSM

Latex and lipid-soluble substances in cells and cell walls were selected as ROIs, and reference spectra were acquired using SCLSM (Fig. 2a). Maximum fluorescence wavelengths were 547 nm for latex and 567 nm for lipid-soluble substances when stained with Nile red and 442 nm for cell walls when stained with Fluorescent Brightener 28. For the fluorescence spectra of Nile red-stained latex and lipid-soluble substances, a difference of 20 nm in the maximum fluorescence wavelength was noted along with differently shaped histograms. Results indicate that Nile red fluorescence specific to latex is separable using SCLSM.

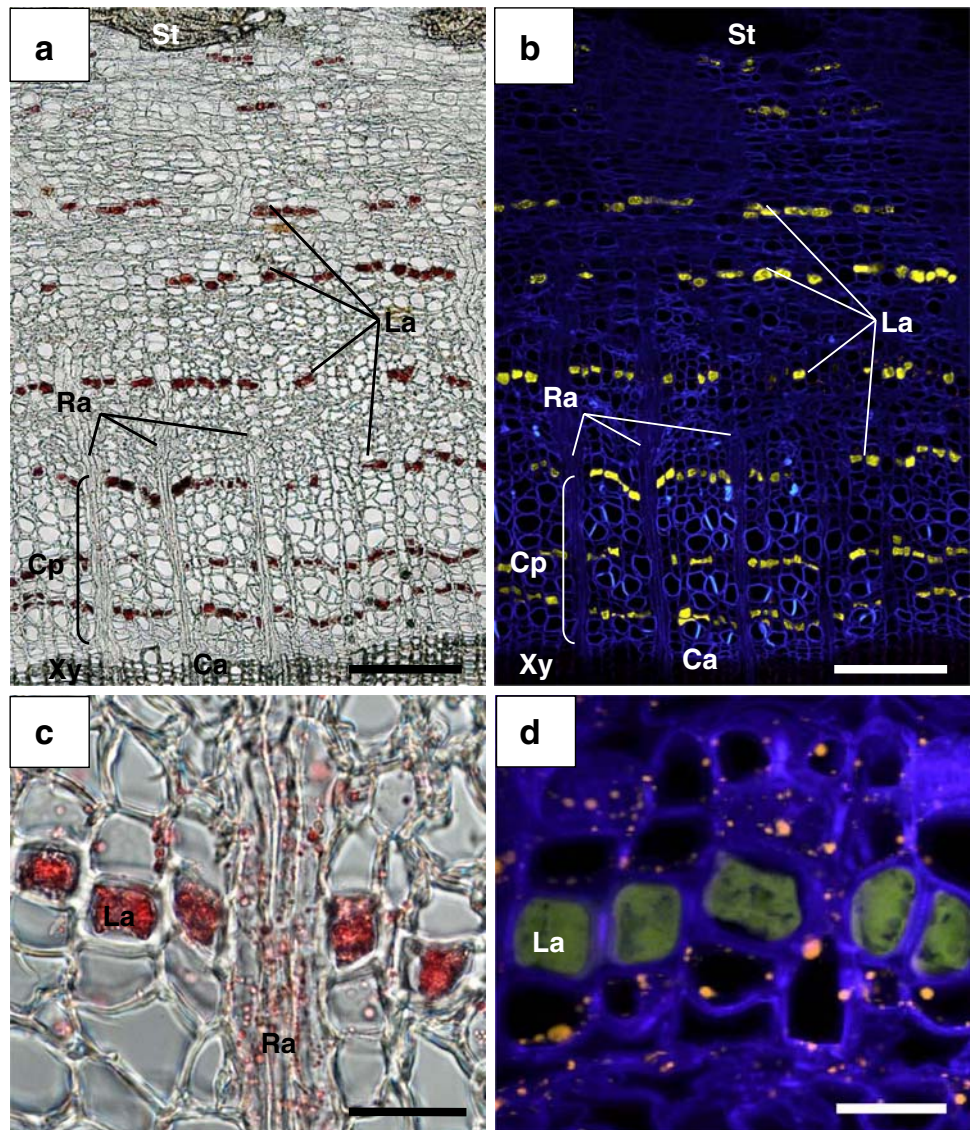
As a result of spectral unmixing using the acquired reference spectra, a peak at 442 nm was identified in green, one at 547 nm in magenta, and one at 567 nm in blue; unmixed images were obtained (Fig. 2b). Extraction of respective fluorescence spectra allowed separation of laticifers in magenta, lipid-soluble substances in blue, and cell walls in green (Fig. 2c–e). Spectral unmixing using SCLSM enabled extraction of images of laticifers in Nile red-stained tissue; they are useful for detailed image analyses.

Laticifer morphology

Laticifers are categorized into two types, non-articulated and articulated, based on their morphology (De Bary 1877; Fahn 1979). The presence of both non-articulated and articulated laticifers in *H. brasiliensis* has been reported (Zhao 1987). The former are present in the cotyledons, leaves, flowers, fruit, roots, and stems, whereas the latter are present in secondary phloem in the bark.

The present study confirmed the presence of concentric-layered articulated laticifers in secondary phloem in the bark. Furthermore, non-articulated laticifers were observed at the outermost laticifer layer in the bark.

Fig. 1 Comparison of staining ability in cross-sections of *H. brasiliensis* bark. **a, c** Bright-field images of Oil red O staining. **b, d** Fluorescence images of Nile red staining with counter-staining with Fluorescent Brightener 28. Scale bar 200 μm (**a, b**), 50 μm (**c, d**). *Ca* cambium, *Cp* conducting phloem, *La* laticifers, *LS* lipid-soluble substances, *Ra* rays, *St* stone cells, *Xy* xylem. With Nile red, the cytoplasm of laticifers and lipid-soluble substances exhibited differing fluorescence, yellow and orange, respectively



Morphological changes in laticifers by layer

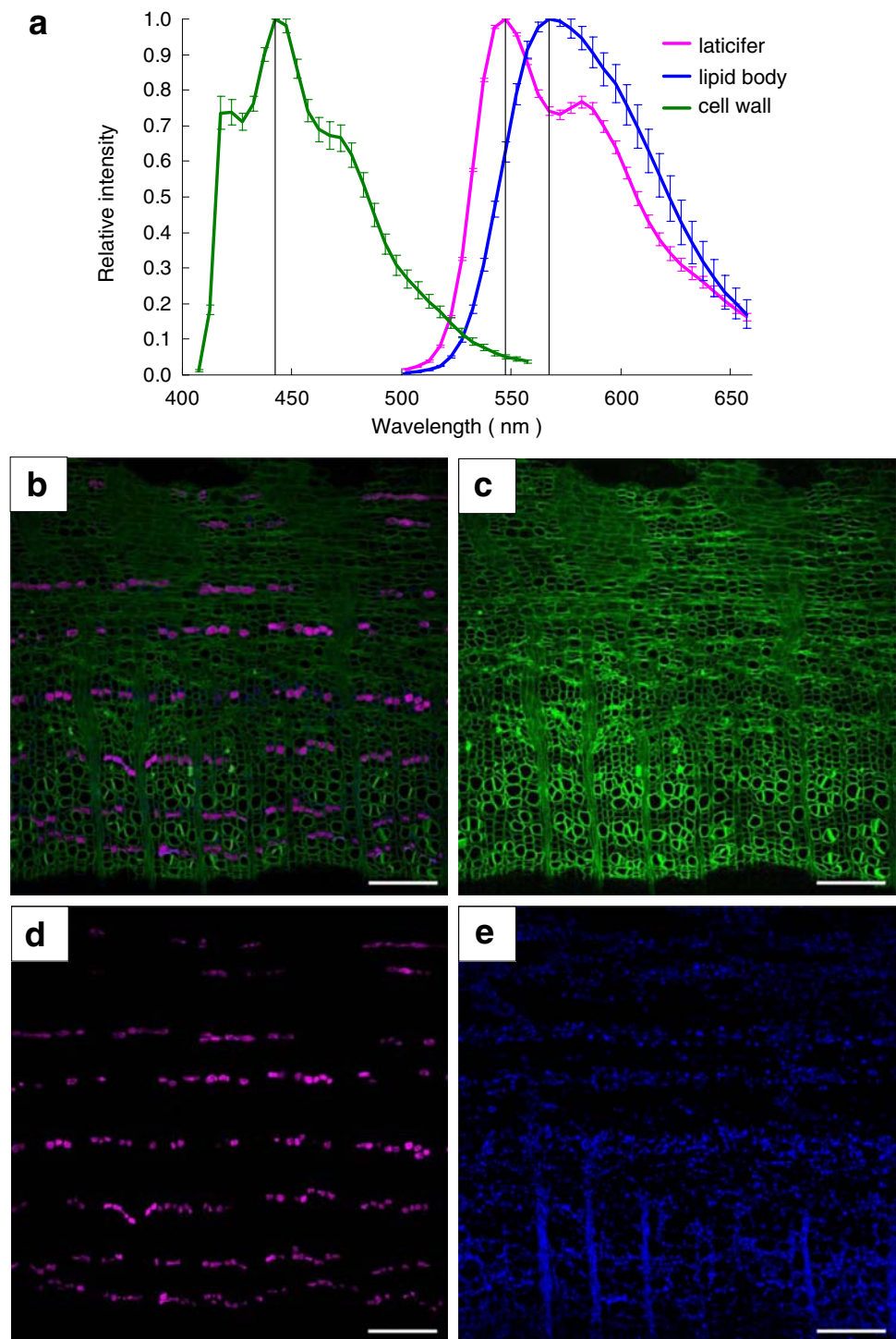
Previously, the presence of anastomoses between adjoining laticifers had been observed using an electron microscope (Hebant et al. 1981). Laticifers in low latex production trees are not continuous; many are actually *cul-de-sacs*, but laticifers in high latex production tree are often continuous (Frey-Wyssling 1930). Therefore, anastomoses between laticifers are believed to enable latex outflow from extensive laticifers in bark during tapping (Archer 1980). Nevertheless, only a few studies have specifically examined the laticifer structure by layer in *H. brasiliensis* (d'Auzac et al. 1989).

Tangential sections of laticifers were prepared to clarify morphological changes in laticifers by layer. Unmixed images of laticifers were acquired using SCLSM, and 3D images were rendered using image processing software (EZ-C1 3.40; Nikon Corp.; Fig. 3a–f).

In conducting phloem where the sieve tubes are functioning, laticifers were narrower, a larger gap separates adjoining laticifers than that in other laticifer layers, and numerous conical ends of laticifers are present (Fig. 3a). In addition, the ends of these laticifers anastomosed to adjoining laticifers in the subsequent layer via multiple anastomoses (Fig. 3b–d). They became part of network structures. The number of anastomoses between laticifers appeared to increase with distance from the cambium (Fig. 3a–d). Analyses of acquired image data, the number of anastomoses formed between adjoining laticifers were determined by layer (Fig. 4). An increase in the number of anastomoses between laticifers with increasing distance from the cambium has been confirmed.

Results of this study revealed that laticifers in conducting phloem are structurally immature and that the network structure of laticifers develops as a result of the

Fig. 2 Spectral unmixing in cross-sections of *H. brasiliensis* bark. **a** Reference spectra used for unmixing. Nile red-stained laticifers and lipid-soluble substances and Fluorescent Brightener 28-stained cell walls were selected as ROIs, and fluorescence spectra from 30 locations in each were averaged. **b** Unmixed image with laticifer display in *magenta*, lipid-soluble substances in *blue*, and cell wall in *green*. **c** Images in which only cell walls were extracted. Sieve plates can be clearly observed in conducting phloem. **d** Images in which only laticifers were extracted. **e** Images in which only lipid-soluble substances were extracted. *Arrows* indicate dense distribution of lipid-soluble substances near laticifers. *Cp* conducting phloem, *Ra* rays. *Scale bar* 200 μ m



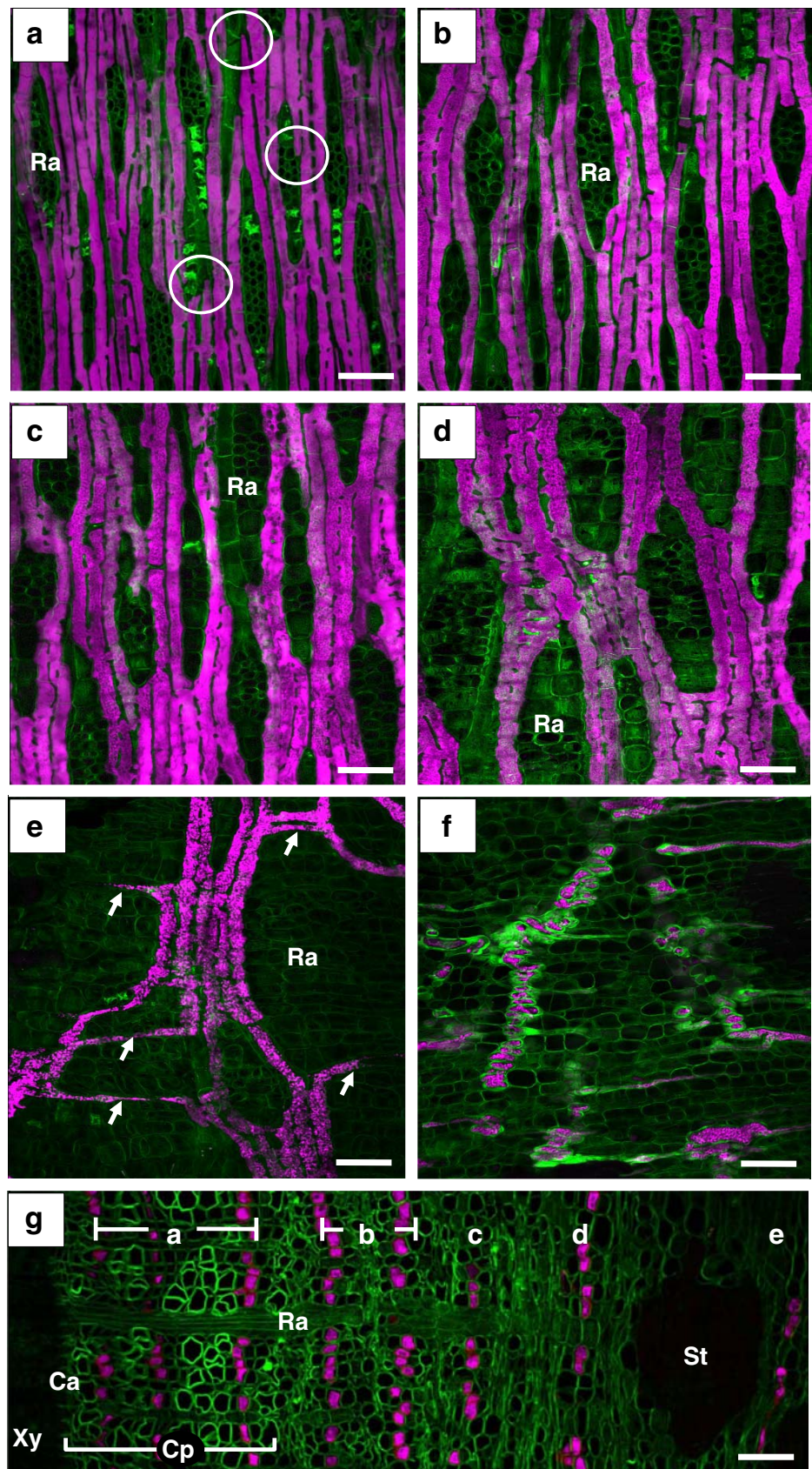
disappearance of the ends of laticifers and an increased number of anastomoses as the distance from the cambium increases.

Laticifers and rays

Many rays between laticifers were observed in all laticifer layers (Fig. 3a–f). Radial division and growth of rays were

also observed, increasing concomitantly with distance from the cambium (Fig. 3a–f). However, laticifers themselves did not divide. Furthermore, laticifers were observed to disintegrate ultimately as a result of radial division and growth and lateral tension as distance from the cambium increased. Unmixed images of laticifers were obtained. The ratio of the area occupied by laticifers and rays was calculated for each layer (Fig. 5). The area of laticifers increased from

Fig. 3 Structure of laticifers by layer in tangential sections of *H. brasiliensis*. **a–f** Unmixed images of tangential sections in which only laticifers and cell walls were extracted. **g** Unmixed images of cross-sections. **a–e** Corresponds to layers **a–e** in **g**, respectively. **f** Non-articulated laticifers which located outermost laticifer layer. **Circles** in **a** indicate ends of laticifers are present. **Arrows** in **e** indicate laticifers network beginning to disintegrate due to the radial development of rays. **Ca** cambium, **Cp** conducting phloem, **La** laticifers, **Ra** rays, **St** stone cells, **Xy** xylem. **Scale bar** 100 μ m (**a–g**)



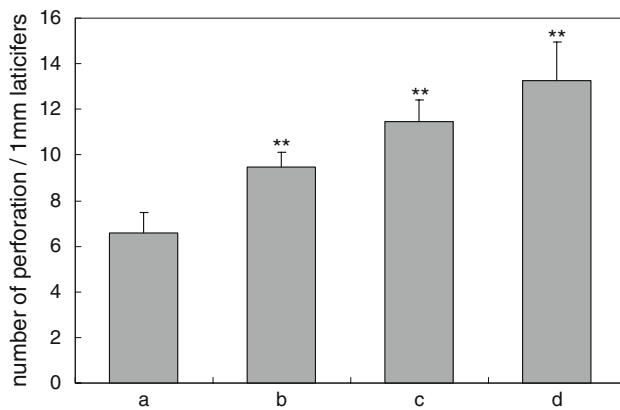


Fig. 4 Number of anastomoses per 1 mm of laticifer in each layer. For each laticifer layer, eight tangential section images were acquired, the total length of laticifers and number of anastomoses in each image were measured, and the number of anastomoses per 1 mm of laticifer was calculated. The mean of eight measurements is shown with the standard deviation. Double asterisk indicates a significant difference at the level of 5% with respect to layer *a*. Note: measurement was not possible for *e* and *f* because of laticifer disintegration

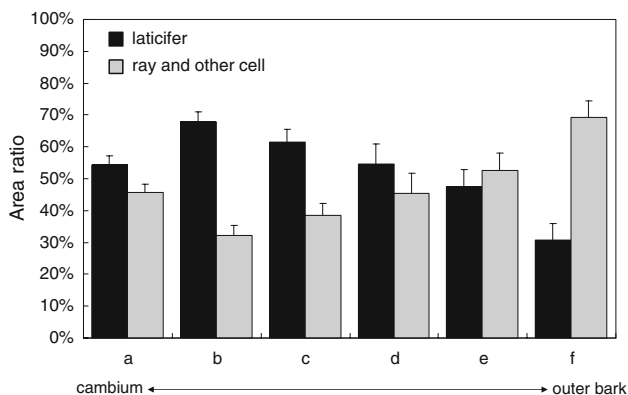


Fig. 5 Area ratio of laticifers and rays in each layer. For each laticifer layer, eight tangential section images were acquired, the area of laticifers and rays in each image was measured, and the area ratio of both was calculated. The mean of eight measurements is shown with the standard deviation

conducting phloem to adjacent layer, then decreased with distance from the cambium. In contrast, the area of rays increased with distance from the cambium. The area distribution of individual rays was determined for each layer (Fig. 6). Results show that the area of all the rays increased concomitantly with distance from the cambium. Moreover, marked division and growth were observed in some rays.

The rays play an important role in supplying necessary metabolites used for rubber biosynthesis in the laticifers (Hebant and Fay 1980). Nevertheless, the present findings suggest that the radial division and the growth of rays cause the laticifers' lateral network structure to disintegrate. Their latex productivity ultimately disappears.

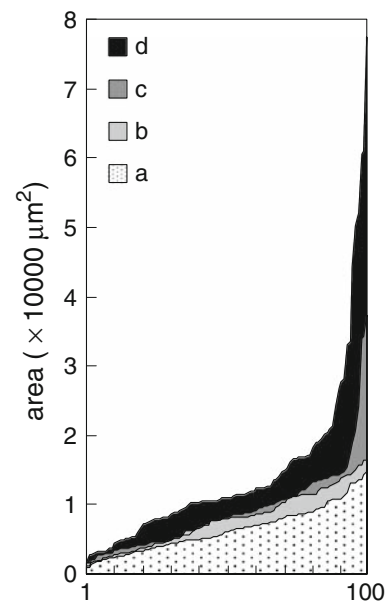


Fig. 6 Area of individual rays appearing in each laticifer layer. The area of individual rays appearing in each laticifer layer was measured for 100 rays and its distribution is shown. Note: measurement was not possible for *e* and *f* because of laticifer disintegration

This study dynamically depicted the process from laticifer origination through growth and disintegration. Analytical results suggest that the structural development of laticifers through increased anastomoses and a balance in division and growth of rays involved in metabolite supply are crucial for efficient latex production.

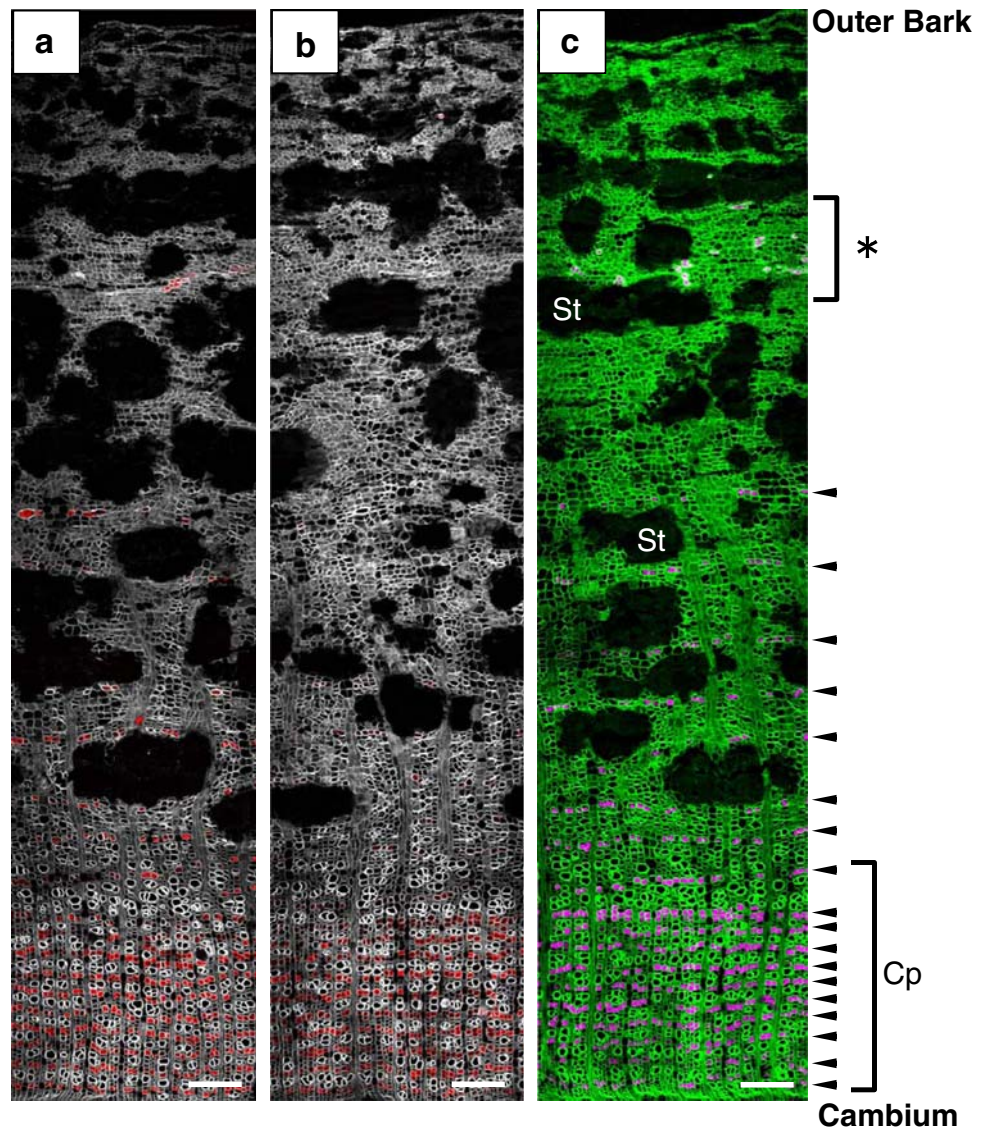
Immunohistochemical localization of rubber particle-associated proteins, REF and SRPP, in bark

Reportedly, SRPs have much higher rubber biosynthesis activity than LRPs (Archer et al. 1963; Ohya et al. 2000). Nevertheless, rubber particle distribution in tissues has remained unclear. Therefore, immunohistochemical localization of their main surface proteins, SRPP and REF, was investigated in this study.

Both SRPP and REF were detected specifically in laticifers. In branches, both SRPP and REF were detected abundantly in all laticifer layers (data not shown). Furthermore, in mature bark, REF was detected abundantly in all laticifer layers (Fig. 7a). In contrast, SRPP was detected abundantly in laticifer layers in the conducting phloem, but very slightly in the outer conducting phloem (Fig. 7b). Based on these results, a distribution model of rubber particles in laticifers layers is presented in Fig. 8.

It has been suggested that recombinant SRPP has positive role in rubber biosynthesis (Oh et al. 1999), this

Fig. 7 Immunohistochemical localization of REF (a) and SRPP (b) in cross-section of mature bark of *H. brasiliensis*. c Unmixed image of the same field is shown in (a) and (b). Laticifers are shown in magenta and cell wall in green. Arrows indicate each laticifer layer. Asterisk indicates non-articulated laticifer. Cp conducting phloem, St stone cells. Scale bar 200 μ m (a–c)



study used immunohistochemical localization of SRPP to evaluate the in situ rubber biosynthesis capability. SRP localized in all laticifer layers in branches and in the laticifer layers in conducting phloem in mature bark. Thus, the laticifers' rubber biosynthesis capability is limited where the rays and sieve tube are functionally active to transport metabolites.

Furthermore, it has been suggested that LRP was derived from the aggregation and/or association of functional groups at both terminal end groups of rubber molecules (Tarachiwin et al. 2005). Together, our results that LRP localized in all laticifer layers including those where laticifer structures were disintegrated and rays were not functioning suggest that the role of REF might relate to termination of rubber elongation.

Conclusion

This study developed a precise microscopic observation technique combining Nile red staining and SCLSM to observe the laticifer structure. Spectral unmixing of Nile red-stained tissues using SCLSM showed that Nile red is effective as a latex-specific probe. Image analysis of unmixed images using SCLSM enabled in situ three-dimensional identification of laticifer structures. This technique is suited to observation in tangential sections, which had proven difficult in previous observations. Consequently, detailed image analyses of laticifers and rays were possible, as was quantitative determination of results of histological observations.

This study dynamically depicted the process from origination to growth and disintegration of laticifers. Analytical

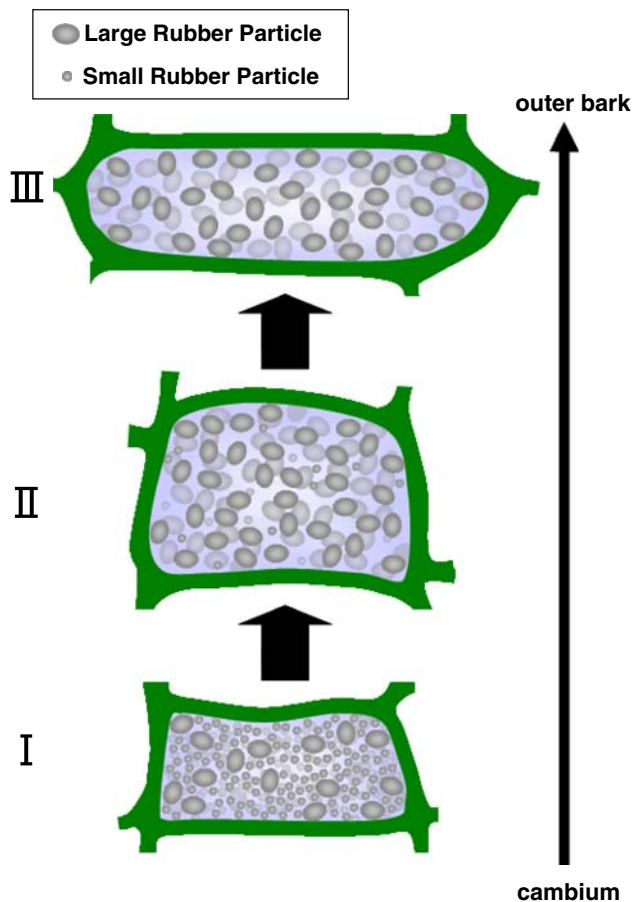


Fig. 8 Model showing the presence of rubber particles, LRPs and SRPs, in the laticifers

results suggest that the structural development of laticifers as a result of increased anastomoses and a balance in division and growth of rays involved in the metabolite supply is crucial for efficient latex production. Furthermore, immunohistochemical analyses suggest that the laticifers' rubber biosynthesis capability is limited between where rays and sieve tubes are functionally active to transport metabolites.

Future studies are necessary to clarify rubber particle formation and the subcellular origin and mutual relation of SRPs and LRPs to elucidate the rubber biosynthesis mechanism.

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