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Pathways of extra- and intercellular diffusion of colored substances in the blackened xylem of *Diospyros kaki*

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Abstract

Some species of *Diospyros* form the black wood that is known as ebony. Infrequently, *D. kaki* forms wood with black patterning that is called "kurogaki" in Japan and is highly valued. To understand the mechanism of formation of the blackened xylem, we attempted to clarify the pathways of diffusion of colored substances from the site of their biosynthesis to their deposition in secondary xylem. We observed colored substances in pits and intercellular spaces and we recorded the deposition of colored substances in the cell walls of wood fibers. In gray and black regions, we found colored substances in the pits between xylem cells, in blind pits and in intercellular spaces. At the boundary between whitish and black regions, areas of coloration in the cell walls of wood fibers gradually increased in size from the whitish to the black regions. Heavy deposition of colored substances was observed in the inner region of the secondary walls of wood fibers. Furthermore, coloration of the outer region of the secondary walls and compound middle lamella (CML) of wood fibers gradually became stronger toward the black regions. Our observations suggest that pit-pairs and intercellular spaces might provide the pathways for inter- and extracellular diffusion of colored substances, respectively. In addition, colored substances might penetrate the cell walls of wood fibers, moving from the lumen into the cell wall.

Keywords: Colored substance, *Diospyros kaki*, Intercellular space, Kurogaki, Pit

Introduction

The genus *Diospyros* includes about 500 species of evergreen and deciduous trees and shrubs. It is widely distributed from tropical to temperate zones [1]. Some species of *Diospyros* form blackened xylem that is distributed evenly and/or in streaks in the tree trunks [2]. Such blackened wood is known as ebony, a very valuable wood that is used for furniture, carvings and musical instruments. Hillis and Soenardi [2] proposed that ebony is formed differently from normal heartwood as a response to invasion by fungi. However, limited information is available about the details of the mechanism of formation of ebony.

The wood of *Diospyros kaki* (Japanese persimmon) is usually light orange-brown in color and the colors of the sapwood and heartwood are similar. Occasionally, however, *D. kaki* forms black-patterned wood that is called "kurogaki" in Japan. For centuries, kurogaki has been highly valued and used for the alcove posts of Japanese tea rooms and for tea utensils. Moreover, objects made of kurogaki have been stored as treasure in the Shosoin Repository at the Todaiji Temple, which is a World Heritage site and one of the historic monuments of ancient Nara (eighth century).

The patterns of black pigmentation in kurogaki are complex, with even distribution and/or streaks, as is the case in other blackened species of *Diospyros*.

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Both the localization of black pigmentation and the beauty of patterns of pigmentation strongly affect the economic value of the wood. Clarification of the mechanism of formation of kurogaki might provide useful information for manipulation of patterns of pigmentation.

The mechanism of formation of kurogaki has been examined in several studies. Matsushita et al. [3] reported that 4,8-dihydroxy-5-methoxy-2-naphthaldehyde was the most abundant specific extractive in the black parts of kurogaki. This extractive was localized in xylem parenchyma cells and adjacent axial elements in black wood, as demonstrated by time-of-flight secondary ion mass spectrometry (ToF-SIMS) [4]. These observations indicated that 4,8-dihydroxy-5-methoxy-2-naphthaldehyde might be involved in the blackening of xylem. In other studies, high concentrations of boron were detected in black parts of kurogaki by inductively coupled plasma-mass spectrometry (ICP-MS), ICP emission spectrometry and a modified curcumin–acetic acid method [5–7]. Furthermore, blackening was found to be associated with antifungal properties [5], suggesting a role of boron in the synthesis of antifungal compounds and blackening of xylem. Although there is considerable information about extractives and trace elements that might be related to blackening, the mechanism of formation of pigmented patterns remains to be clarified.

To investigate the mechanism of formation of pigmented patterns in kurogaki of *D. kaki*, it is necessary to examine the diffusion of colored substances from the sites of their biosynthesis to their deposition in secondary xylem. Hillis and Soenardi [2] reported the distribution of colored substances that were deposited on various secondary xylem cells in ebony. Their report was mainly based on macroscopic observations, and their optical microscopic observations did not include information about fine structures, such as pits and intercellular spaces. It has been reported that pits and intercellular spaces might function as pathways for diffusion of heartwood substances in *Acacia mangium* and *Albizia julibrissin* [8, 9]. Therefore, it is possible that pits and intercellular spaces might provide the pathways for diffusion of colored substances during the formation of the black patterns in *D. kaki*.

In the present study, we examined the distribution of colored substances at the cellular level in kurogaki by light microscopy. In particular, we focused on pits and intercellular spaces in an effort to evaluate their possible roles in the diffusion of colored substances. In addition, to investigate the penetration of colored substances into cell walls, we monitored their deposition in the cell walls of wood fibers in blackened xylem. Finally, we considered the significance of various pathways in the diffusion of colored substances during the formation of blackened xylem in *D. kaki*.

Materials and methods

Three wood samples of *D. kaki* were obtained from commercially available wood. These samples contained some black regions as streaks (Fig. 1a). The samples were divided into three parts by color, namely, whitish (Fig. 1b), gray (Fig. 1c), and black (Fig. 1d).

Transverse sections of approximately 20-μm thickness and tangential and radial sections of approximately 40-μm thickness were cut on the freezing stage of a sliding microtome (REM-710; Yamatokohki, Saitama, Japan) and washed with distilled water. To determine the location of colored substances at the microscopic level, we mounted the sections, without staining, on glass slides with 50% aqueous glycerol and examined them under a light microscope (Axio Scope. A1; Carl Zeiss, Oberkochen, Germany) [10]. The color of the blackened wood did not appear to change with time in aqueous glycerol solutions.

For the preparation of approximately 1-μm-thick sections, each sample was trimmed into small blocks (longitudinal × tangential × radial direction, $2 \times 1 \times 3$ mm^3). These blocks were dehydrated through a graded ethanol series and embedded in epoxy resin (EPON812; TAAB, Berkshire, UK). The color of the blocks was not visibly changed by the dehydration process. Transverse and tangential sections of approximately 1-μm thickness were cut with a glass knife on an ultramicrotome (Ultracut N; Reichert, Vienna, Austria). For observations of pits and intercellular spaces, the sections were examined under the light microscope, which was equipped with Zeiss Plan-Neofluor 40/0.75 and 100×/1.3 objective lenses (Carl Zeiss). Microscopic color images (1280×960 pixels) were recorded with a Digital Sight DS-5M camera (Nikon, Tokyo, Japan).

For quantification of the deposition of colored substances in wood fibers, gray-level profiles of images were analyzed with the image-analysis software ImageJ [11]. Color images of approximately 1-μm-thick sections were converted into 8-bit images with 256 gray scales. Then, the gray levels of these images were inverted with ImageJ. Finally, linear profiles of the gray levels of cell walls of wood fibers were generated.

Results

Whitish regions

Figure 2 shows light micrographs of whitish regions of wood samples of *D. kaki*. The cell walls of vessel elements, wood fibers and xylem parenchyma cells were white or pale yellow in color (Fig. 2a–c). Colored substances

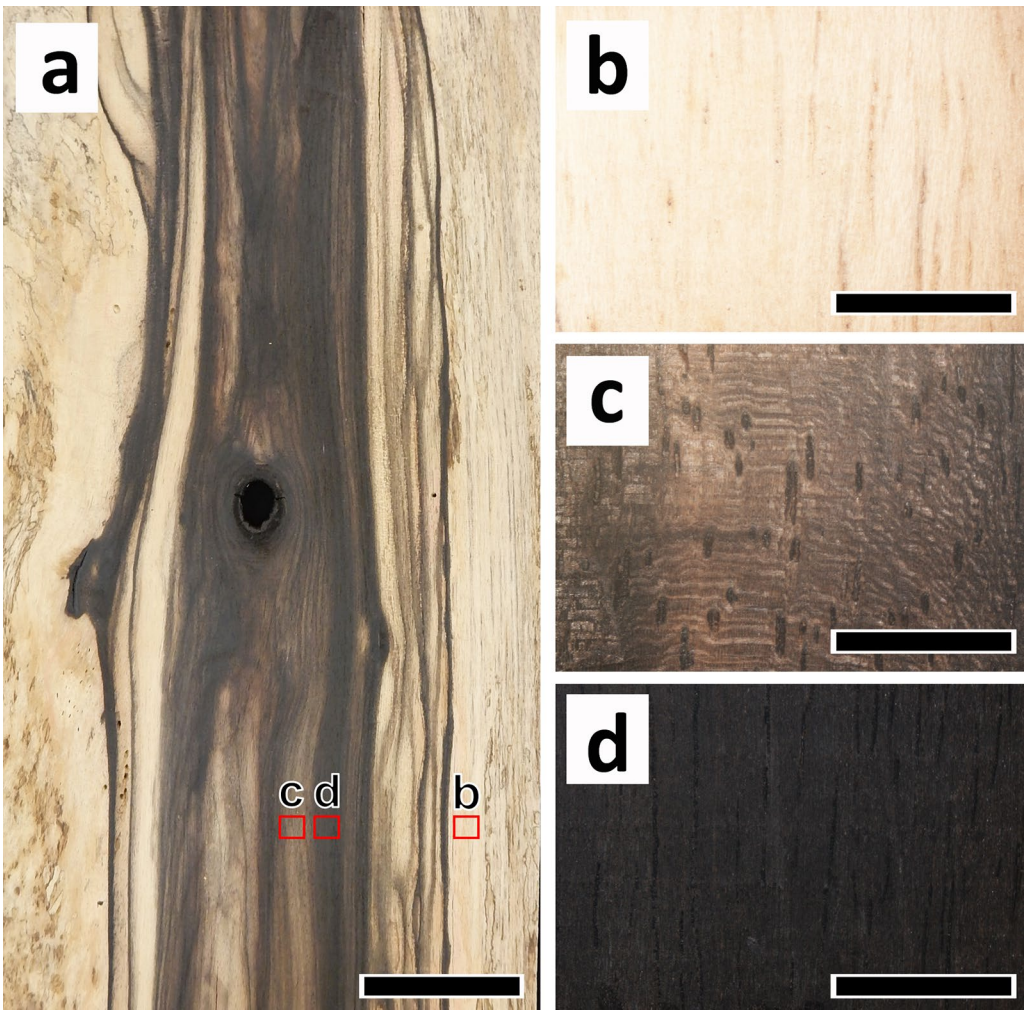

Fig. 1 A photograph (**a**) and micrographs obtained by stereo microscopy (**b**–**d**) of typical wood samples used in this study. Wood of *D. kaki* that contained some black regions was examined (**a**). Wood samples were divided into three parts by color, namely, whitish (**b**), gray (**c**) and black (**d**). Scale bars = 10 cm in **a**; 5 mm in **b**–**d**

were occasionally observed in the lumen of vessel elements, axial parenchyma cells and ray parenchyma cells (black arrowheads in Fig. 2a, b). No deposition of colored substances was evident in the lumen of wood fibers and intercellular spaces (Fig. 2d, e).

Gray regions

Figure 3 shows light micrographs of gray regions from the wood samples of *D. kaki*. Colored substances were often observed in the lumen of vessel elements, wood fibers, axial parenchyma cells and ray parenchyma cells (Fig. 3a–c). In some parts of the gray regions, hardly any colored deposits were found in the lumen of axial parenchyma cells and ray parenchyma cells (Fig. 3d, e, g). Colored deposits were observed in the pits of the wood fibers in such regions (white arrowheads in Fig. 3e–h), even though no colored substances were found in the surrounding xylem parenchyma cells. Colored substances were, however, found on the interior surface of intercellular spaces in the rays (white arrows in Fig. 3i).

Black regions

Figure 4 shows light micrographs of black regions of samples of *D. kaki* wood. The cell walls of wood fibers were predominantly black, whereas walls of vessel elements, axial parenchyma cells and ray parenchyma cells were generally brown (Fig. 4a). Large amounts of colored substances were observed in the lumen of almost all vessel elements (Fig. 4a, b). By contrast, ray parenchyma cells and axial parenchyma cells contained only small amounts

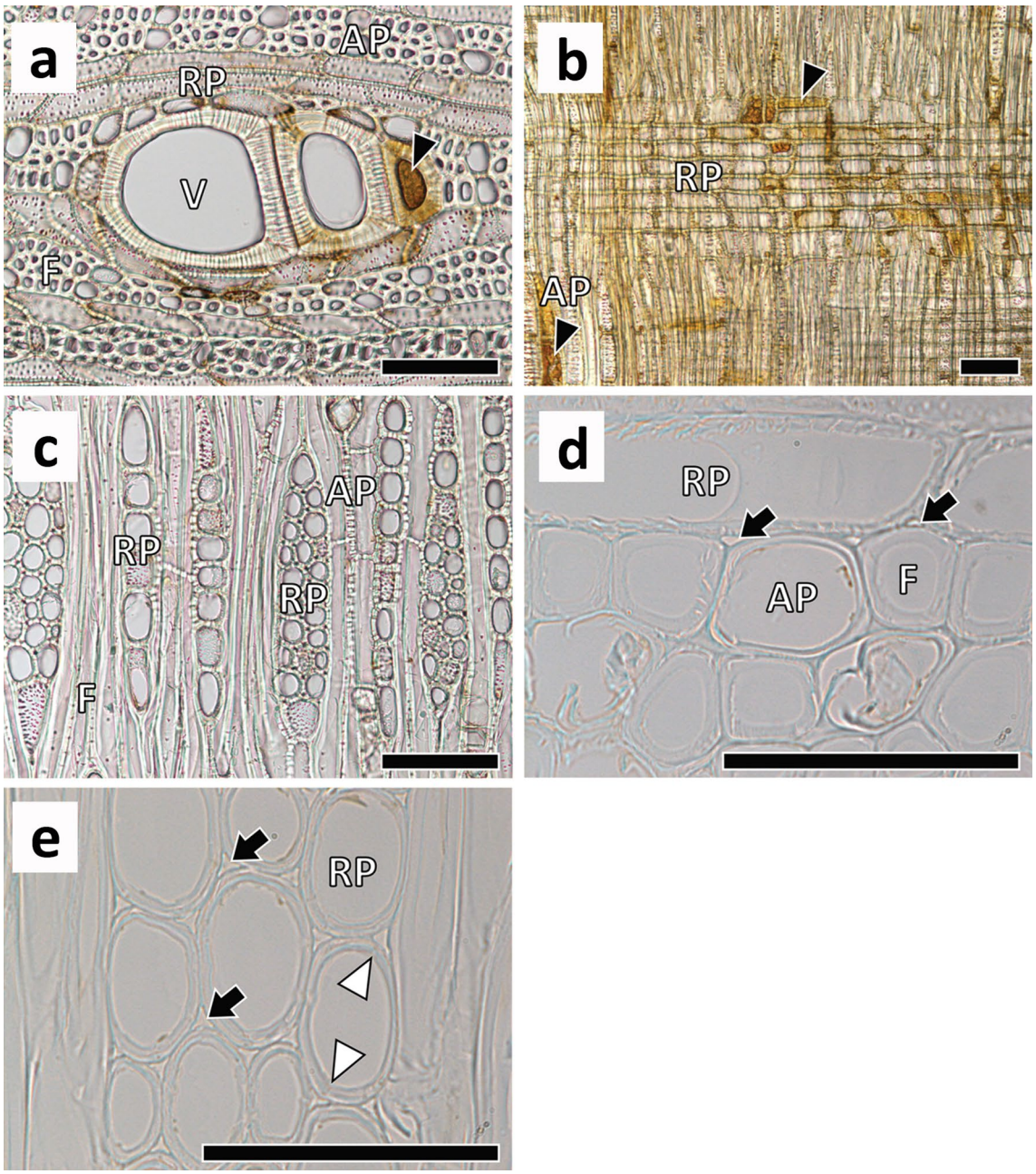

Fig. 2 Light micrographs of transverse (**a**, **d**), radial (**b**) and tangential (**c**, **e**) sections of whitish regions of samples of *D. kaki* wood. Black arrowheads indicate colored substances. Black arrows indicate intercellular spaces in the rays where no colored substances were found. White arrowheads indicate blind pits in ray parenchyma cells. AP: axial parenchyma cell; F: wood fiber; RP: ray parenchyma cell; V: vessel element. Scale bars = 100 µm in **a**–**c**; 50 µm in **d** and **e**

of colored substances (Fig. 4a–c). No differences in terms of the distribution of colored substances were found between ray structures, namely, uniseriate or multiseriate rays, and the various types of ray parenchyma cell, namely, procumbent and upright cells. Deposits of colored substances were observed in pits of vessel elements, wood fibers and axial parenchyma cells (white arrows in Fig. 4a, d) and in intercellular spaces (black arrows in Fig. 4d). In addition, colored substances were observed in the pits between ray parenchyma cells (red arrowheads in Fig. 4e) and the blind pits of ray parenchyma cells that faced the intercellular spaces (white

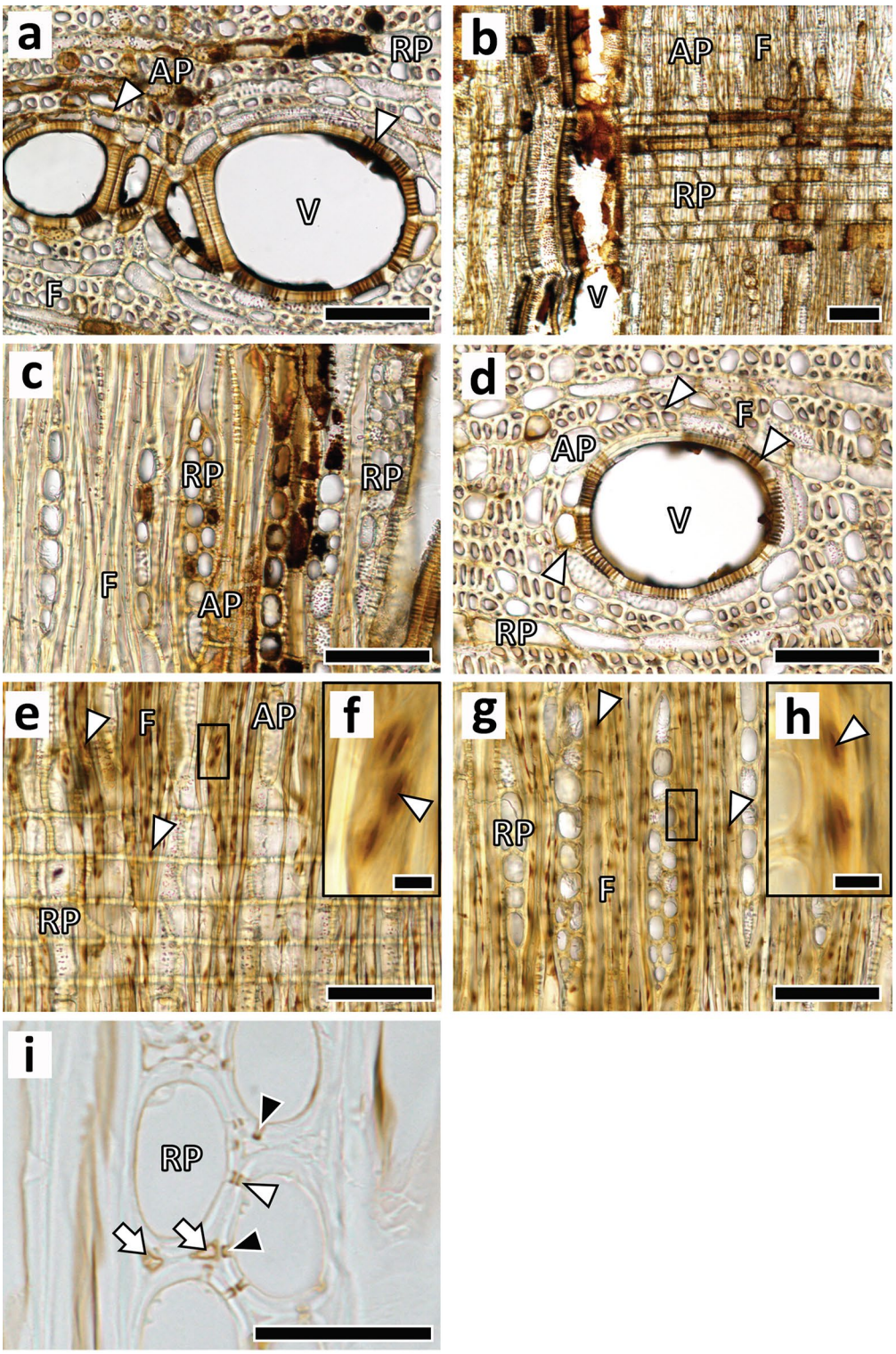

Fig. 3 Light micrographs of transverse (**a**, **d**), radial (**b**, **e**, **f**) and tangential (**c**, **g**, **h**, **i**) sections of gray regions of samples of *D. kaki* wood. **f**, **h** Higher-magnification views of the enclosed areas in **e**, **g**, respectively. White arrowheads indicate colored substances in pits. White arrows indicate colored substances on interior surfaces of intercellular spaces within rays. AP: axial parenchyma cell; F: wood fiber; RP: ray parenchyma cell; V: vessel element. Scale bars = 100 μm in **a**–**d**, **e**, **g**; 5 μm in **f**, **h**; 25 μm in **i**

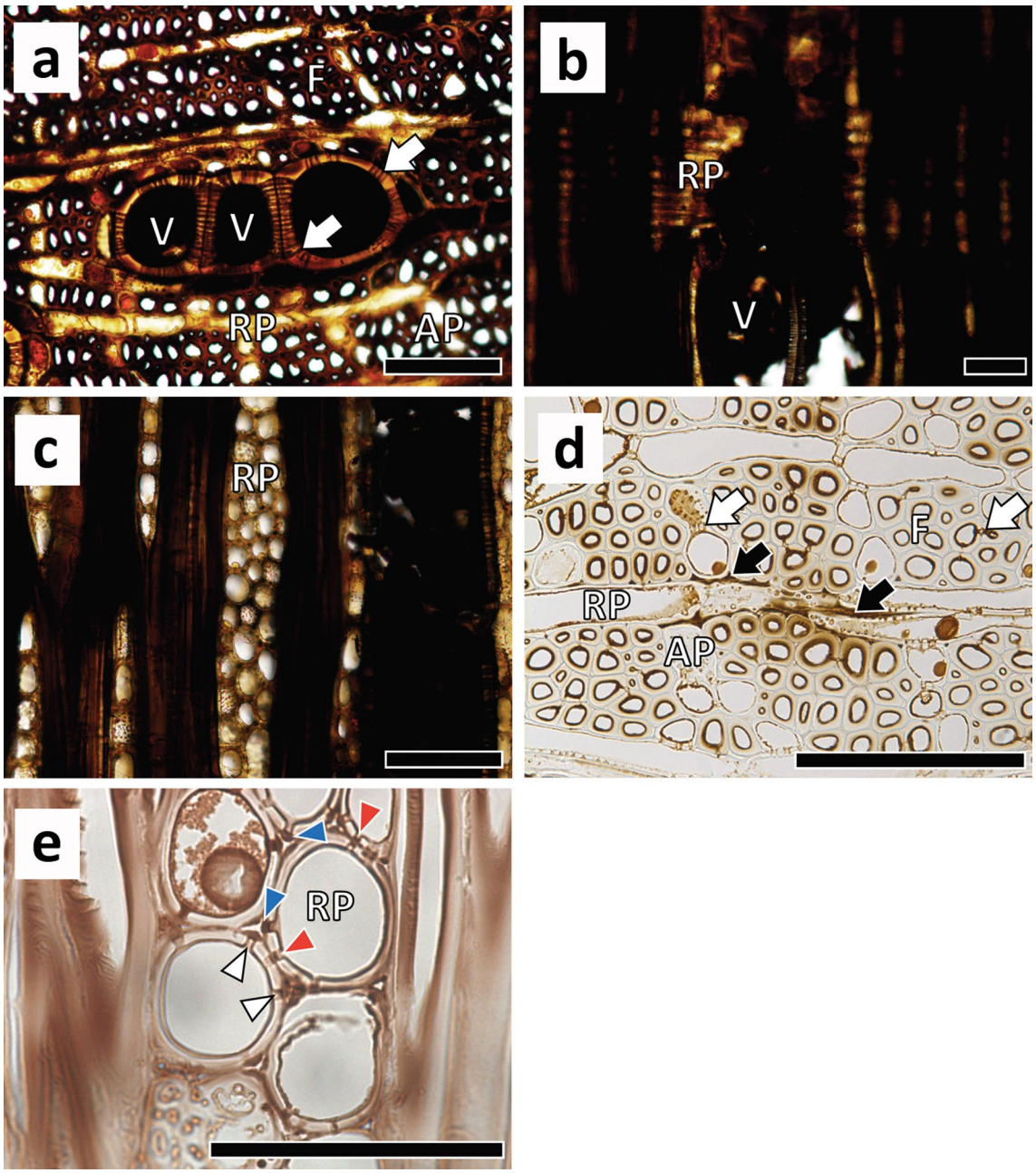

Fig. 4 Light micrographs of transverse (**a**, **d**), radial (**b**) and tangential (**c**, **e**) sections of black regions of samples of *D. kaki* wood. White arrowheads indicate colored substances in blind pits of ray parenchyma cells that faced intercellular spaces. Red arrowheads indicate colored substances in pits between ray parenchyma cells. Blue arrowheads indicate colored substances leaking from blind pits into intercellular spaces. White arrows indicate colored substances in pits. Black arrows indicate colored substances in intercellular spaces. AP: axial parenchyma cell; F: wood fiber; RP: ray parenchyma cell; V: vessel element. Scale bars = 100 µm in **a**–**d**; 50 µm in **e**

arrowheads in Fig. 4e). Furthermore, colored substances appeared to leak out of blind pits into intercellular spaces (blue arrowheads in Fig. 4e).

The boundary between whitish and black regions

Figure 5 shows light micrographs of the boundary between whitish and black regions. Axial parenchyma cells and ray parenchyma cells contained small amounts of colored substances (Fig. 5a). In addition, regions of

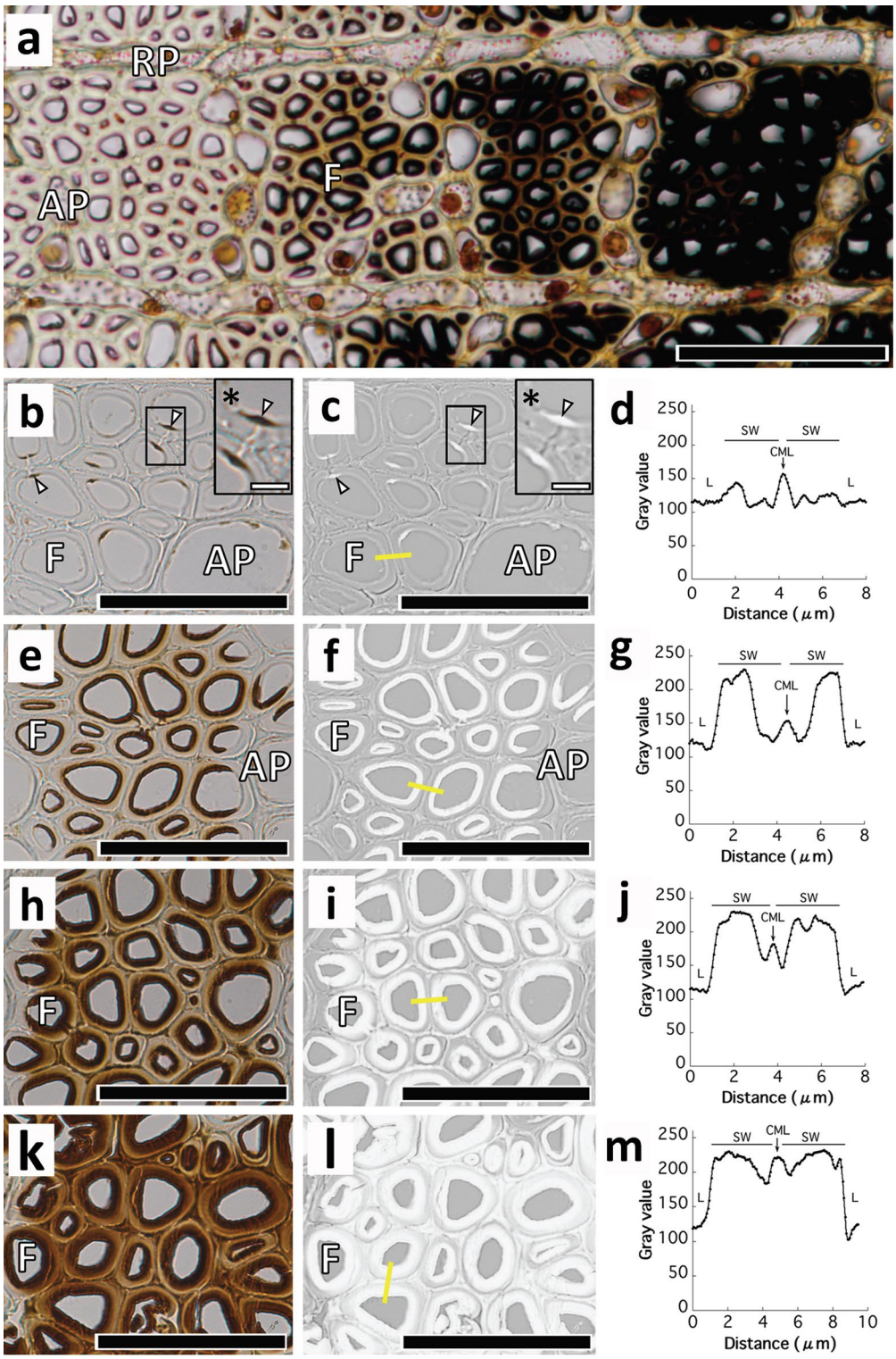

Fig. 5 Light micrographs (**a**, **b**, **e**, **h**, **k**), inverted gray-scale images of panels **b**, **e**, **h**, **k** (**c**, **f**, **i**, **l**) and gray-level profiles of cell walls (**d**, **g**, **j**, **m**) of transverse sections at the boundary between whitish and black regions in samples of *D. kaki* wood. Representative gray-level profiles in **d**, **g**, **j**, **m** were obtained from the yellow-lined sites in the gray-scale images **c**, **f**, **i**, **l**, respectively. Asterisks in **b**, **c** show higher-magnification images of a pit region (the enclosed area). White arrowheads indicate deposits of colored substances on the inner surfaces of wood fibers in pit regions. AP: axial parenchyma cell; CML: compound middle lamella; L: lumen; RP: ray parenchyma cell; SW: secondary wall; F: wood fiber. Scale bars = 100 μm in **a**; 50 μm in **b**, **c**, **e**, **f**, **h**, **i**, **k**, **l**; 5 μm in enclosed area of **b**, **c**

dense coloration in the cell walls of wood fibers gradually increased in size from the whitish to the black regions (Fig. 5a). Near the whitish regions, colored substances were observed on the inner surfaces of wood fibers in pit regions (white arrowheads in Fig. 5b). In the inner regions of secondary walls of wood fibers, there were heavy deposits of colored substances in areas closer to black regions (Fig. 5e, h, k). The width of the pigmented part increased toward the black regions (Fig. 5e, h, k). In the outer region of secondary walls and compound middle lamella (CML), coloration gradually became stronger toward the black regions (Fig. 5e, h, k). The patterns of accumulation of colored substances in cell walls of wood fibers were clearly apparent on inverted gray-scale images and in the gray-level line profiles of cell walls (Fig. 5c, d, f, g, i, j, l, m). Initially, the gray value of the inner region of secondary walls increased (cf. Fig. 5g with Fig. 5d). Then, the width of high gray-value areas increased toward the black regions (Fig. 5j, m). In addition, gray values of the outer region of secondary walls and the CML gradually increased toward the black regions (Fig. 5g, j, m).

Discussion

In the gray regions of the xylem of *D. kaki*, we found that colored substances had accumulated in both axial and ray parenchyma cells (Fig. 3). Matsushita et al. [4] demonstrated that 4,8-dihydroxy-5-methoxy-2-naphthaldehyde was the most abundant specific extractive in the black regions of kurogaki, and it was localized in xylem parenchyma cells, as shown by ToF-SIMS. These results indicated that xylem parenchyma cells synthesize chemical compounds that are related to the blackening of xylem. However, the pathways for diffusion of colored substances from the sites of their biosynthesis to their deposition in secondary xylem of *D. kaki* remained to be clarified. In the present study, we determined the pathways for diffusion of colored substances by monitoring their locations at the microscopic level.

In gray regions, some ray parenchyma cells contained colored substances and neighboring wood fibers and intercellular spaces also contained colored substances (Fig. 3). ToF-SIMS observations revealed that 4,8-dihydroxy-5-methoxy-2-naphthaldehyde was distributed in ray parenchyma cells and in some adjacent axial elements [4]. During heartwood formation, heartwood substances, which are heartwood-specific phenolic compounds that affect coloration of heartwood, migrate from xylem parenchyma cells to neighboring cells [12–14]. Therefore, it seems plausible that colored substances might migrate from xylem parenchyma cells to adjacent cells via a similar pathway in *D. kaki*. In addition, although some xylem parenchyma cells contained large amounts of colored substances in the gray regions (Fig. 3a–c), small amounts of colored substances were detected in xylem parenchyma cells in the black regions (Fig. 4a–c). This observation suggests that most of the colored substances synthesized in xylem parenchyma cells might migrate from cells during the formation of the blackened xylem.

In gray regions and black regions, colored substances were detected in pits of vessel elements, wood fibers and xylem parenchyma cells (Figs. 3, 4). It has been reported, similarly, that heartwood substances were localized in pits, an observation that suggests that pit-pairs might function as intercellular diffusion pathways for heartwood substances [8, 9, 12, 13, 15]. Accordingly, present observation also indicates pit-pairs might function similarly as pathways for intercellular migration of colored substances in *D. kaki*.

We did not detect any deposits of colored substances in the intercellular spaces and blind pits of whitish regions (Fig. 2d, e). By contrast, colored substances were observed in intercellular spaces in gray and black regions (Figs. 3i and 4d, e). In addition, we noted that black substances appeared to leak from blind pits into intercellular spaces in the black regions (Fig. 4e). These observations suggest that colored substances might migrate from ray parenchyma cells to intercellular spaces via blind pits and then diffuse through the network of intercellular spaces. It has been suggested that intercellular spaces provide routes for gas exchange [16–19] and serve as a system for the transport and storage of water and nutrients [20–24]. It has also been suggested that intercellular spaces function as the extracellular pathway for diffusion of heartwood substances [8, 9, 12]. Thus, it seems reasonable that intercellular spaces might also function as the extracellular pathway for diffusion of colored substances during the blackening of xylem in *D. kaki*. A further study on the three-dimensional structure of intercellular spaces is needed to understand how colored substances diffuse via intercellular spaces during blackening of xylem in *D. kaki*.

At the boundary between whitish and black regions, the extent of coloration of cell walls of wood fibers gradually increased from the whitish to the black region (Fig. 5a). Heavy accumulation of colored substances was evident within the inner regions of secondary walls (Fig. 5e, h, k). Furthermore, the intensity of pigmentation of the outer parts of secondary walls increased toward the black regions (increase in gray value and width of the high gray value regions of secondary walls; Fig. 5d, g, j, m). Therefore, we can conclude that the deposition of colored substances progresses from the inner to the outer part of the cell wall in wood fibers in *D. kaki*. In addition, gray values of CML gradually increased toward the black region (Fig. 5d, g, j, m). This observation indicates that colored substances

diffuse into the CML and are gradually deposited during the blackening of xylem of *D. kaki*. Deposition of heartwood substances in cell walls and CML has been similarly demonstrated using different microscopy techniques, such as confocal laser-scanning microscopy, transmission electron microscopy and confocal Raman microscopy [14, 15, 25, 26]. Belt et al. [25] noted that, in the heartwood of *Pinus sylvestris*, pinosylvin was detectable both in cell walls and in CML, but resin acid was distributed only in the cell walls, suggesting that the patterns of distribution might differ between extractives. Streit and Fengel [14] suggested two possible pathways for penetration of phenolic compounds in the heartwood of *Schinopsis balansae*, namely, a pathway from the lumen into the cell wall and a pathway from pits and intercellular spaces into CML. Similarly, a pathway from pits and intercellular spaces into CML could exist during the formation of blackened xylem in *D. kaki*.

Conclusion

Our observations suggest the following pathways for diffusion of colored substances during formation of blackened xylem in *D. kaki*: (1) pit-pairs and intercellular spaces might provide the intercellular and extracellular diffusion pathways, respectively; and (2) colored substances might penetrate fiber cell walls via a pathway from the lumen into the cell wall.

Abbreviations

CML: Compound middle lamella; ICP-MS: Inductively coupled plasma-mass spectrometry; ToF-SIMS: Time-of-flight secondary ion mass spectrometry.

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Authors' contributions

KI, SN, TB, YM, PK, IA, RF and SN conceived and designed the experiments. KI, IA, SN performed the experiments and analyzed the data. KI wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

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Competing interests

The authors declare that they have no competing interests.

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