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NOTE

Temporally and spatially controlled death of parenchyma cells is involved in heartwood formation in pith regions of branches of *Robinia pseudoacacia* var. *inermis*

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Abstract Focussing on four types of parenchyma cell around pith regions of branches of *Robinia pseudoacacia* L. var. *inermis*, we examined the timing and role of cell death during heartwood formation. Large parenchyma cells that were located in the inner part of the pith died within a year. By contrast, other parenchyma cells died within 4 years, with the timing of cell death depending on the type of cell. Axial parenchyma cells of the xylem close to the pith died first. Then, small parenchyma cells died in the perimedullary zone in the outer part of the pith. Finally, ray parenchyma cells in the xylem close to the pith died. Variations in the autofluorescence of cell walls, which might have been due to deposition of heartwood substances, were observed first in xylem ray parenchyma cells and small parenchyma cells in the perimedullary zone. Our results indicate that the initiation of heartwood formation occurs within 4 years in pith regions of branches in *Robinia pseudoacacia* L. var. *inermis*. Moreover, it appears that not only xylem ray parenchyma cells but also small parenchyma cells in the perimedullary zone might be involved in the synthesis of heartwood substances.

Keywords Cell death · Heartwood formation · Pith · *Robinia pseudoacacia* · Xylem parenchyma cell

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Introduction

The formation of heartwood is a unique phenomenon in long-lived woody plants. Heartwood is defined as ''the inner layers of wood, which, in the growing tree, have ceased to contain living cells, and in which the reserve materials (e.g., starch) have been removed or converted into heartwood substances'' [1]. Some species form colored heartwood because xylem parenchyma cells synthesize heartwood substances such as polyphenols that contribute to increases in the decay resistance of tree trunk, prior to their death [2–5]. A full understanding of the mechanism of heartwood formation should provide information that is useful in efforts to control the chemical properties of wood.

Heartwood formation in temperate zone trees results from the death of xylem parenchyma cells that is associated with the annual life cycle of the tree. The death of xylem parenchyma cells progresses from the inner toward the outer region of the stem. Therefore, the death of xylem parenchyma cells might be expected to begin in the tissues at the center of branches and stems, where the pith, consisting of parenchyma cells, is located [6]. If this hypothesis is correct, the parenchyma cells in the pith might be involved in the initiation of heartwood formation. However, the site of initiation of heartwood formation remains to be identified.

The main objective of the present study was to determine the site of initiation of heartwood formation within the branches of *Robinia pseudoacacia* L. var. *inermis*. Since *Robinia pseudoacacia* L. has narrow sapwood [7], we were able to follow the process of cell death in parenchyma cells around the pith within a short distance from the apical portions of branches. We examined the timing of the death of parenchyma cells in the pith and in

the xylem close to the pith. In addition, we examined the distribution of storage starch in parenchyma cells around the pith because the amount of starch in xylem parenchyma cells generally decreases during the conversion of sapwood into heartwood [5, 8, 9]. Moreover, we examined variations in the autofluorescence of cell walls in an attempt to identify the roles of parenchyma cells around the pith. Imaging systems combined with chemical analysis, such as ultraviolet (UV) microspectrophotometry and time-of-flight secondary ion mass spectrometry (ToF-SIMS), are effective tools for the investigation of localization of heartwood substances at cellular level [10–14]. Therefore, in this paper, we used fluorescence microscopy and confocal laser-scanning microscopy with spectrum analysis to analyze the changes in autofluorescence of cell walls due to lignin and deposition of heartwood substances.

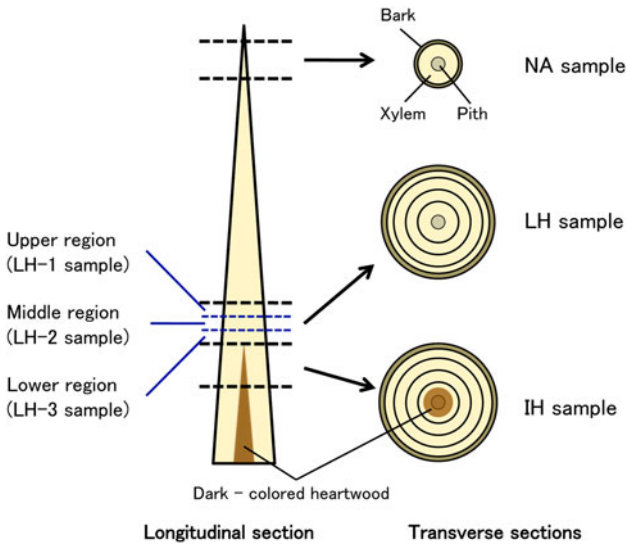


Fig. 1 A schematic diagram showing the positions of samples that were collected from branches of *Robinia pseudoacacia* L. var. *inermis*. Three samples were collected from each branch, namely, tissue near the apical region (NA sample), tissue with four annual rings in xylem that lacked dark-colored heartwood (LH sample), and tissue with four annual rings in xylem that included dark-colored heartwood (IH sample). In addition, LH sample was divided into three parts, namely, *upper* (LH-1 sample), *middle* (LH-2 sample) and *lower region* (LH-3 sample)

Materials and methods

Sample collection

Two *Robinia pseudoacacia* L. var. *inermis* trees, growing in the Field Museum Tama Hills of the Tokyo University of Agriculture and Technology in Hachioji-Tokyo (35°63′N, 139°37′E), Japan, were used in this study. A four-year-old branch with a diameter of approximately 3 cm was collected from each tree in November 2010. Since Nobuchi et al. [7] observed that heartwood formation in *Robinia pseudoacacia* L. started in July and continued until at the end of the following March, we expected to observe the process of heartwood formation in November.

Light and fluorescence microscopy

We collected samples from three different parts of each branch as follows: tissues near the apical regions (NA samples); tissues with four annual rings in xylem that lacked dark-colored heartwood (LH samples); and tissues with four annual rings in xylem that included dark-colored heartwood (IH samples) (Fig. 1). In addition, LH samples were divided into three parts, namely, upper (LH-1 samples), middle (LH-2 samples) and lower regions (LH-3 samples). We identified the presence of heartwood by examining the color of tissues with the naked eye.

All samples (diameter in 1–3 cm and height in 3 cm) were fixed overnight at room temperature in a 4% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and then the samples were washed with the same buffer. Transverse sections of approximately 16-μm thickness and radial sections of approximately 40-μm thickness were cut on a sliding microtome (Yamatokohki, Saitama, Japan).

For light microscopy, sections were stained with a 1% aqueous solution of acetocarmine for observation of nuclei [15–18], with a 1% aqueous solution of iodine–potassium iodide for observation of starch grains [15, 19, 20], and with a 1% aqueous solution of safranin for observation of cell walls [16–18]. Stained sections were observed under a light microscope (Axioscop; Carl Zeiss, Oberkochen, Germany).

For observations of changes in autofluorescence of cell walls during heartwood formation, unstained transverse sections of approximately 16-μm thickness were examined with a fluorescence microscope (Axioscop) under epifluorescence illumination (excitation/emission combination BP353-377/LP397). Lignin and heartwood substances such as polyphenols were expected to emit autofluorescence under UV excitation [21, 22].

Recording of emission spectra of the autofluorescence of cell walls by confocal laser-scanning microscopy

Unstained transverse sections of approximately 16-μm thickness were mounted on glass slides in glycerol. Sections were examined in "lambda scanning" mode with

single-photon excitation under a confocal laser-scanning microscope (LSM 710 NLO; Carl Zeiss) equipped with a Zeiss Plan-Apochromat 40×/0.95 objective lens (Carl Zeiss). Sequential single-plane images (512 × 512 pixels) were obtained at wavelengths from 462 to 723.9 nm in steps of 9.7 nm under fluorescence excitation at 405 nm. The relative intensity of autofluorescence at each wavelength was recorded from six randomly chosen areas (0.415 × 0.415 μm^2) of walls of xylem and pith cells, and then the average value from the six areas was calculated.

Results and discussion

Figure 2 shows the region around the pith in a branch of *Robinia pseudoacacia* L. var. *inermis*. The region included two types of xylem parenchyma cell, namely, axial parenchyma cells and ray parenchyma cells. The two types of parenchyma cell of the pith were of different size. Small parenchyma cells were located in the perimedullary zone that formed the outer part of the pith, while large parenchyma cells were located in the inner part of the pith. The perimedullary zone consisted of four to six layers of small parenchyma cells. We shall refer to such small parenchyma cells in the perimedullary zone as "perimedullary cells".

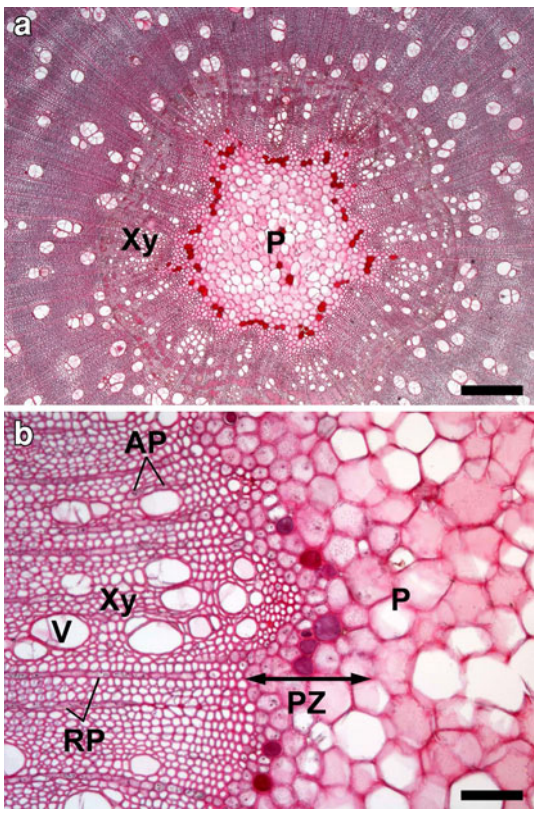
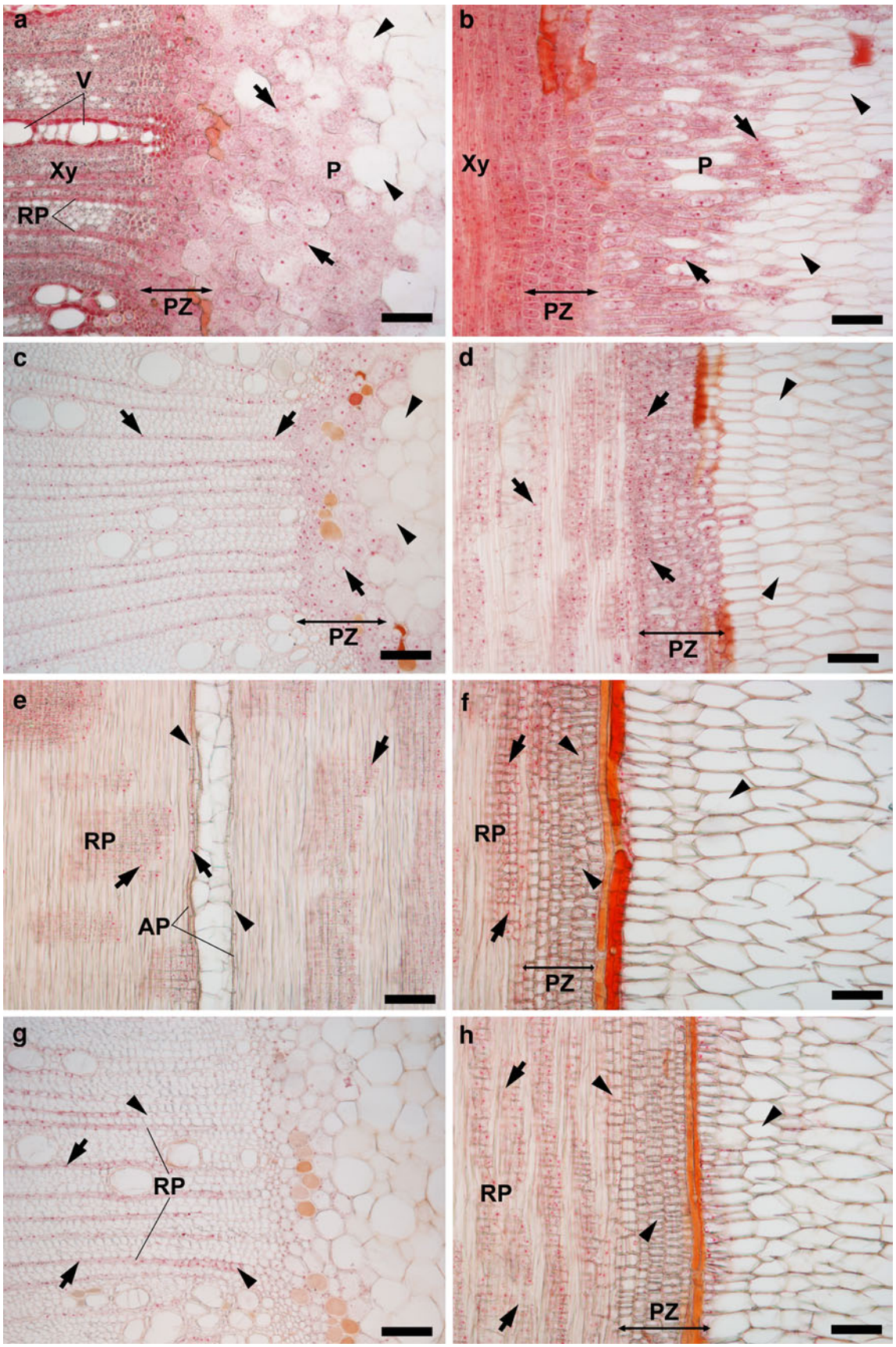


Fig. 2 **a** A light micrograph of a transverse section of LH-1 sample, stained with safranin, showing the center of a branch of *Robinia pseudoacacia* L. var. *inermis*. **b** Higher-magnification view of pith and xylem. The left side of the latter micrograph corresponds to the outer side of the branch. *AP* Axial parenchyma cell surrounding vessel elements, *P* pith, *PZ* perimedullary zone (with *double-headed arrow*), *RP* ray parenchyma cell, *V* vessel element, *Xy* xylem. *Bars* 400 μm in **a** and 100 μm in **b**

In NA samples, only current year's xylem was evident. In this region, we observed nuclei that were stained with acetocarmine in both axial and ray parenchyma cells among the xylem and perimedullary cells (Fig. 3a, b). By contrast, nuclei had disappeared from many large parenchyma cells in the inner region of the pith (arrowheads in Fig. 3a, b). In LH samples, there were four annual rings in the xylem. In LH-1 samples, we detected nuclei in both axial and ray parenchyma cells among the xylem and perimedullary cells (Fig. 3c, d). In LH-2 and 3 samples, differences in the timing of the disappearance of nuclei (cell death) were apparent toward the basal region of branches among axial parenchyma cells, ray parenchyma cells and perimedullary cells. Cell death occurred first in axial parenchyma cells of xylem close to the pith (Fig. 3e). Then, the disappearance of nuclei became apparent in perimedullary cells (Fig. 3f). Finally, cell death became apparent in ray parenchyma cells in the xylem close to the pith (Fig. 3g, h). In IH samples, there were no nuclei in the xylem close to the pith or in the pith cells that surrounded the center of branches (data not shown). These results indicate that the timing of cell death differed among the various types of parenchyma cells around the pith region in branches of *Robinia pseudoacacia* L. var. *inermis*.

To evaluate the storage role of parenchyma cells around the pith, we examined the number and distribution of starch grains, after staining with iodine–potassium iodide (Fig. 4). In NA samples, there were many starch grains in axial and ray parenchyma cells among the xylem and perimedullary cells (Fig. 4a, b). In this region, there were also many starch grains in most xylem fibers, and we considered such fibers to be living wood fibers. By contrast, many of the large parenchyma cells in the inner region of the pith contained no starch grains at all. In LH-1 samples, large parenchyma cells in the inner part of the pith lacked starch grains (arrowheads in Fig. 4c, d). By contrast, there were many starch grains in the axial and ray parenchyma cells in xylem and perimedullary cells (Fig. 4c, d). In this region, no starch grains were detected in the wood fibers. In LH-2 and 3 samples, the disappearance of starch grain became evident toward the basal region of branches in these three types of parenchyma cell. Starch grain disappeared first from axial parenchyma cells in xylem close to the pith (Fig. 4e). Then, perimedullary cells lost their starch grains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◀ **Fig. 3** Light micrographs of transverse and radial sections, stained with acetocarmine, showing nuclei in parenchyma cells around the center of branches of *Robinia pseudoacacia* L. var. *inermis*. **a** A transverse section from near the apical region of a branch (NA sample). **b** A radial section from near the apical region of a branch (NA sample). **c** A transverse section of upper region of tissue with four annual rings in xylem that lacked dark-colored heartwood (LH-1 sample). **d** A radial section of upper region of tissue with four annual rings in xylem that lacked dark-colored heartwood (LH-1 sample). **e** A radial section of tissue in upper region of LH-2 sample. **f** A radial section of tissue in lower region of LH-2 sample. **g** A transverse section of tissue in LH-3 sample. **h** A radial section of tissue at the same position as in **g**. *Arrows* indicate nuclei. *Arrowheads* indicate cells without nuclei. The *left side* of the micrographs corresponds to the outer side of the branch. *AP* Axial parenchyma cell, *P* pith, *PZ* perimedullary zone (with *double-headed arrow*), *RP* ray parenchyma cell, *V* vessel element, *Xy* xylem. *Bars* 100 μm

(Fig. 4f) and, finally, starch grains disappeared from ray parenchyma cells close to the pith (Fig. 4g, h). In IH samples, there were no starch grains in the pith and the xylem cells close to the pith (data not shown). The order of disappearance of starch corresponded to that of cell death among the various types of parenchyma cells around the pith region in branches of *Robinia pseudoacacia* L. var. *inermis*.

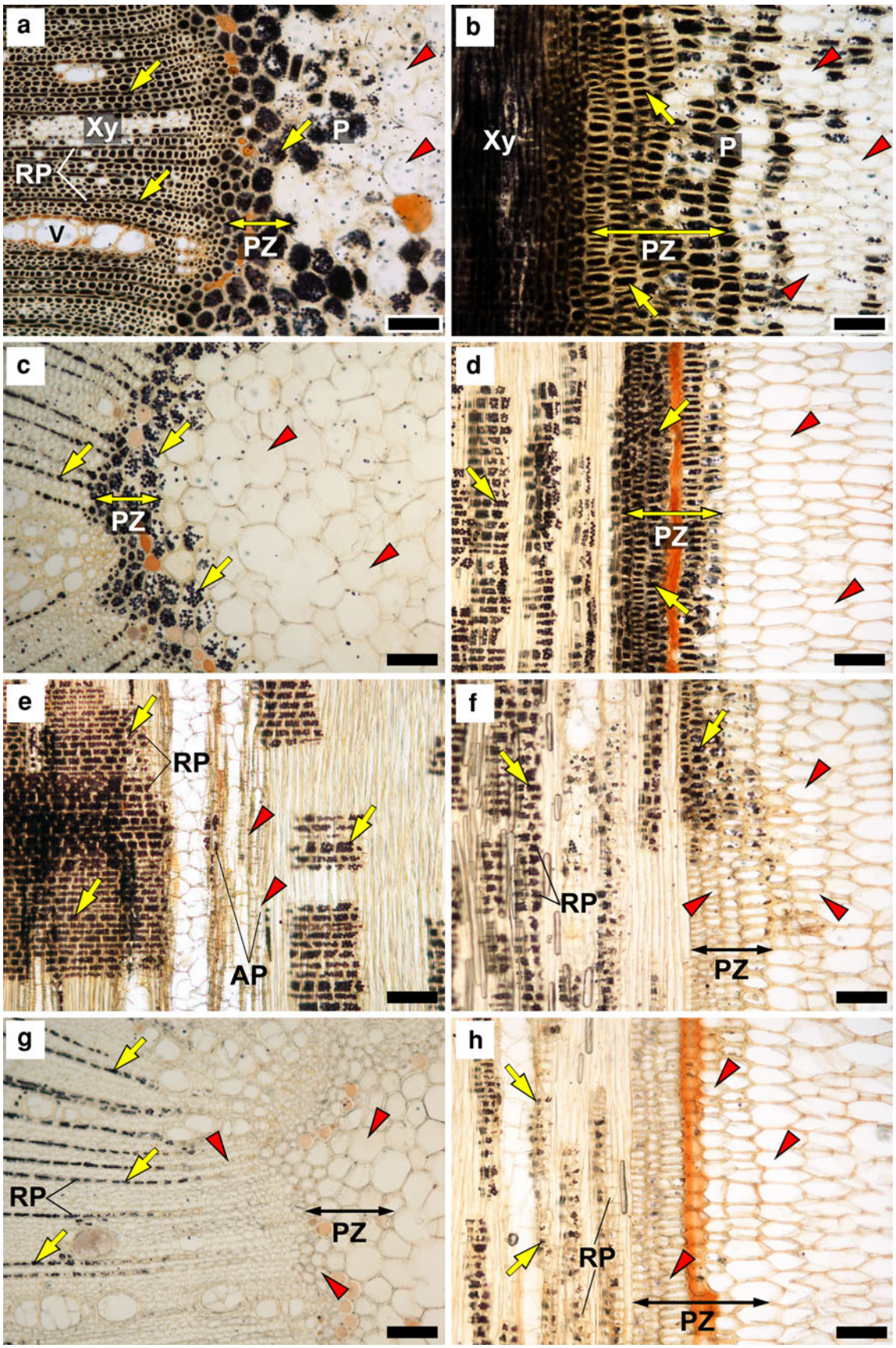
Figure 5a–c shows the variations in the autofluorescence under ultraviolet excitation (353–377 nm) of cell walls around the pith towards the basal region of branches. In NA samples, blue fluorescence, due perhaps to lignin, was emitted from all xylem and pith cells (Fig. 5a). In LH-1 samples, yellow–green fluorescence was emitted from perimedullary cells and from some of the xylem ray parenchyma cells that were located near the pith (arrows in Fig. 5b). By contrast, no variations in the autofluorescence of cell walls were evident in the axial parenchyma cells in LH-1 samples. In IH samples, yellow–green fluorescence was emitted from the pith and from all xylem cells close to the pith (Fig. 5c).

The emission spectra of autofluorescence under fluorescence excitation at 405 nm from cell walls of xylem and pith cells in the same sections shown in Fig. 5a and c are shown in Fig. 5d. The emission spectrum from the section in Fig. 5a peaked at 481.4 nm, while that in the section in Fig. 5c peaked at 500.8 nm. In addition, the relative intensity of the fluorescence from the section in Fig. 5c was higher than that from the section in Fig. 5a. These results indicate that some additional substances, which emitted autofluorescence at longer wavelengths than lignin, might have been deposited in the walls of xylem and pith cells around the center of branches. Similarly, Dünisch et al. [10] observed that some additional substances were detected in the walls of xylem cells in heartwood of *Robinia pseudoacacia* L. by UV microspectrophotometry. In species that form colored heartwood, heartwood substances are synthesized in parenchyma cells, and then, are deposited in the walls of xylem cells during heartwood formation [2–5, 9]. Therefore, variations in the autofluorescence of cell walls might reflect the deposition of heartwood substances in the cell walls.

In LH-1 samples, no dark-colored heartwood was visible to the naked eye. However, variations in the autofluorescence of cell walls were visualized by fluorescence microscopy (Fig. 5b). Yellow–green fluorescence of cell walls was observed only in some of the xylem ray parenchyma cells that were located near the pith and perimedullary cells. This observation indicates that these additional substances were synthesized not only in xylem ray parenchyma cells but also in perimedullary cells in *Robinia pseudoacacia* L. var. *inermis*. We also observed the disappearance of starch grains from xylem ray parenchyma cells and perimedullary cells around the pith in LH-2 and 3 samples during cell death (Fig. 4f-h). It has been suggested that stored starch is used for the synthesis of heartwood substances [5]. Therefore, the disappearance of starch from xylem ray parenchyma cells and perimedullary cells might be involved in the synthesis of heartwood substances.

In addition, the disappearance of nuclei and starch grains from perimedullary cells occurred earlier than that from xylem ray parenchyma cells (Figs. 3f–h, 4f–h). Moreover, in LH-1 samples, all perimedullary cells showed yellow–green fluorescence, while no variations in the autofluorescence of cell walls were evident in some xylem ray parenchyma cells that were located near the pith (arrowheads in Fig. 5b). Therefore, it seems possible that the synthesis of heartwood substances might start in perimedullary cells in the pith region. High-level expression of genes that are related to the synthesis of heartwood substances was observed in xylem parenchyma cells at the sapwood-heartwood transition zone in *Robinia pseudoacacia* L [23, 24]. Similar genes might be strongly expressed in perimedullary cells during heartwood formation.

By contrast to the above observations, there were no clear variations in the autofluorescence of cell walls of axial parenchyma cells in LH-1 samples (Fig. 5b). However, we did observe the disappearance of starch grains from axial parenchyma cells during cell death (Fig. 4e). It has been suggested that, in *Cryptomeria japonica*, axial and ray parenchyma cells in xylem might synthesize and accumulate different kinds of heartwood substances [13, 25]. Nobuchi and Harada [26] reported differences in terms of the ultrastructural changes during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◄ **Fig. 4** Light micrographs of transverse and radial sections, stained with iodine–potassium iodide, showing starch grains in parenchyma cells around the center of branches of *Robinia pseudoacacia* L. var. *inermis*. **a** A transverse section from near the apical region of a branch (NA sample). **b** A radial section from near the apical region of a branch (NA sample). **c** A transverse section of upper region of tissue with four annual rings in xylem that lacked dark-colored heartwood (LH-1 sample). **d** A radial section of upper region of tissue with four annual rings in xylem that lacked dark-colored heartwood (LH-1 sample). **e** A radial section of tissue in upper region of LH-2 sample. **f** A radial section of tissue in lower region of LH-2 sample. **g** A transverse section of tissue in LH-3 sample. **h** A radial section from the same position as the section in **g**. *Arrows* indicate starch grains. *Arrowheads* indicate cells without starch grains. The *left side* of the micrographs corresponds to the outer side of the branch. *AP* Axial parenchyma cell, *P* pith, *PZ* perimedullary zone (with *double-headed arrow*), *RP* ray parenchyma cell, *V* vessel element, *Xy* xylem. *Bars* 100 μm

heartwood formation between axial and ray parenchyma cells in xylem. These observations together suggest that axial parenchyma cells might play a different role in heartwood formation from that of ray parenchyma cells and perimedullary cells in *Robinia pseudoacacia* L. var. *inermis*.

In conclusion, the initiation of heartwood formation occurred within 4 years in the pith regions of branches in *Robinia pseudoacacia* L. var. *inermis*. It appears that not only xylem ray parenchyma cells but also perimedullary cells in the pith might be involved in the synthesis of heartwood substances. Further analysis of perimedullary cells should provide useful information about the early stages of the formation of heartwood.

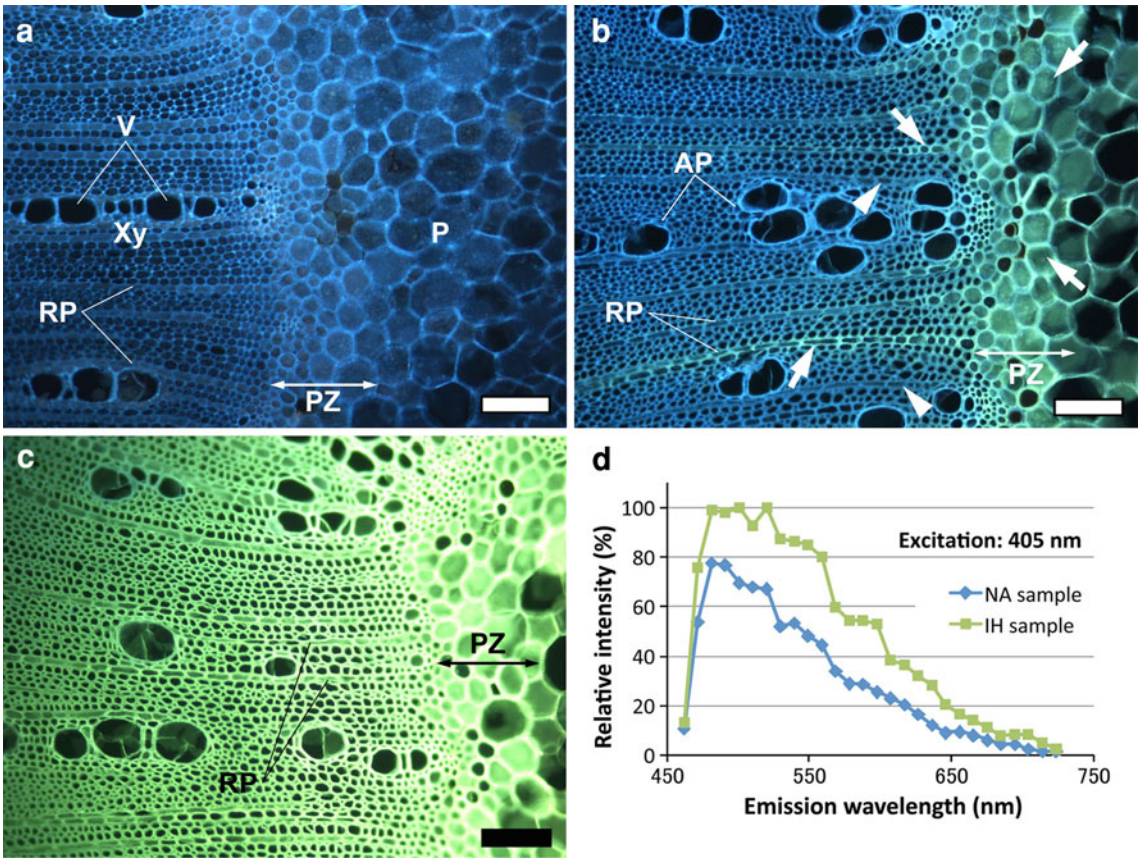

Fig. 5 Autofluorescence images obtained by fluorescence microscopy (**a**–**c**) and emission spectra obtained by confocal laser-scanning microscopy (**d**) of transverse sections of tissue around the pith of branches of *Robinia pseudoacacia* L. var. *inermis*. **a** A transverse section from near the apical region of a branch (NA sample). **b** A transverse section, from around the pith, of upper region of tissue with four annual rings in xylem that lacked dark-colored heartwood (LH-1 sample). **c** A transverse section, from around the pith, of tissue with four annual rings in xylem and dark-colored heartwood (IH sample). **d** Averaged emission spectra of autofluorescence under fluorescence excitation at 405 nm from cell walls of xylem and pith cells around the pith, namely, from sections in (**a** *blue dots* and *line*) and (**c** *green dots* and *line*) ($n = 6$ in each case). *Arrows* indicate yellow–green fluorescence. *Arrowheads* indicate ray parenchyma cells that emitted no yellow–green fluorescence. The *left side* of the micrographs corresponds to the outer side of the branch. *AP* Axial parenchyma cell surrounding vessel elements, *P* pith, *PZ* perimedullary zone (with *double-headed arrow*), *RP* ray parenchyma cell, *V* vessel element, *Xy* xylem. *Bars* 100 μm

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