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REVIEW ARTICLE

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Cell death of long-lived ray parenchyma cells during heartwood formation in trees

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Abstract

Cell death plays an important role in the determination of secondary xylem cell functions. Tracheary elements (TEs), such as vessel elements and tracheids, lose their organelles due to rapid autolysis after the completion of secondary wall thickening and lignification, and play an important role in water movement along the stem. In contrast, xylem axial and ray parenchyma cells (xylem parenchyma cells) remain alive for several years or longer and retain their organelles even after maturation. As a result, xylem parenchyma cells play important roles in nutrient storage, axial and radial transportation of materials, and defense responses in the stem. In addition, they are involved in the formation of heartwood, which contributes to increases in the resistance of the tree trunk to decay, as they synthesize heartwood components such as polyphenols prior to their death. The present review focuses on changes in long-lived ray parenchyma cells during heartwood formation, such as morphology and contents of organelles, gene expression, and survival rate in sapwood. This review also summarizes the differences in cell death characteristics between TEs and ray parenchyma cells. The elucidation of the cell death mechanism of ray parenchyma cells is expected to provide useful information for controlling the properties of heartwood.

Keywords Cell death, Heartwood formation, Ray parenchyma cell, Secondary xylem, Woody biomass

Introduction

Trees have a well-developed vascular cambium and continue radial growth of the stem over time [1–8]. The periclinal division of cambial cells leads to radial growth, producing secondary phloem cells outside and secondary xylem cells inside the cambium. The cambium produces substantially more secondary xylem cells than secondary phloem cells. Therefore, woody biomass, an important renewable and carbon–neutral resource, is primarily composed of secondary xylem cells.

Cell death plays an important role in the determination of secondary xylem cell functions. The differentiation of secondary xylem cells involves cell expansion or elongation, cell wall thickening (secondary wall thickening), the formation of modified structures such as pits and perforations, lignification, and cell death [4–6]. The process of differentiation is highly similar among secondary xylem cells with various functions, but the timing of cell death differs significantly. Tracheary elements (TEs), such as tracheids and vessel elements, lose their organelles due to rapid autolysis after the completion of secondary wall thickening and lignification and play an important role in water movement. In contrast, xylem axial and ray parenchyma cells (xylem parenchyma cells) remain alive for several years or longer. Even after maturation, i.e., secondary wall thickening and lignification, they retain their organelles and remain viable [4–6]. Xylem parenchyma cells form three-dimensional lattices in secondary xylem and they are connected by symplasmic networks in sapwood [9–11]. As a result, these cells play important roles in nutrient storage, axial and radial transportation of materials, and defense responses in the stem [1, 10, 12–15]. In addition, xylem parenchyma cells are involved in the formation of heartwood (Fig. 1),

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which contributes to the resistance of the tree trunk to decay, as they synthesize heartwood substances such as polyphenols prior to their death [16–23]. The elucidation of the cell death mechanism of xylem parenchyma cells is expected to provide useful information for controlling the properties of heartwood. This review introduces the cell death characteristics of long-lived xylem parenchyma cells and compares the regulatory mechanisms of cell death between xylem parenchyma cells and short-lived xylem cells, such as TEs.

Cell death of short-lived xylem cells

The cell death process of xylem cells has been mostly researched in short-lived TEs of herbaceous angiosperms, such as *Zinnia elegans* [24–27], and ample information about the cellular and molecular cell death mechanisms has been accumulated. In vitro, single mesophyll cells isolated from *Z. elegans* leaves transdifferentiated synchronously into TEs within 72 h in the presence of two plant hormones, auxin and cytokinin [25, 28]. Therefore, cell death of these TEs is thought to be a time-dependent programmed cell death (PCD). During the early stages of TE differentiation, brassinosteroid phytohormones induce the expression of genes involved in secondary wall formation and PCD [29]. Then, nucleases [30–34] and proteases [31, 35–37] accumulate in the vacuole. Autolysis of the cell organelles begins simultaneously as the accumulated autolytic enzymes are released into the cytoplasm, triggered by the collapse of the vacuole, which begins to be observed six hours after secondary wall formation [38]. Groover et al. [38] reported that nuclear DNA is fragmented during PCD in TEs.

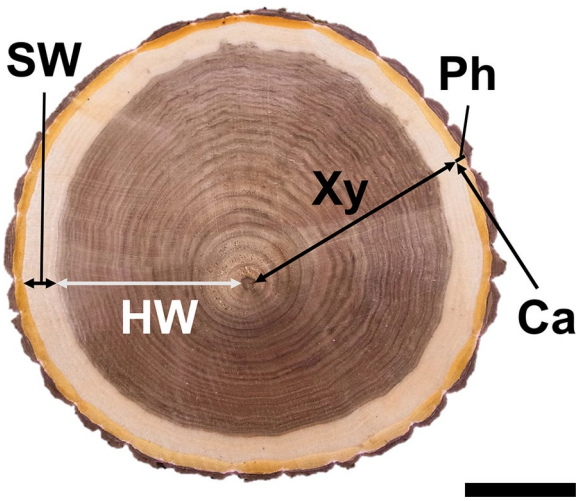

Fig. 1 Cross section of a tree trunk of *Juglans mandshurica* var. *sachalinensis*. *Ca* cambium, *HW* heartwood, *Ph* phloem, *SW* sapwood, *Xy* xylem. Scale bar = 5 cm

Similarly, we have observed nuclear DNA fragmentation during cell death in short-lived ray tracheids in *Pinus densiflora* (Fig. 2) [39, 40]. Autolysis after vacuolar collapse reportedly proceeds quickly, with nucleic acid degradation completed within 10–20 min [41]. Regarding the mechanism of vacuolar collapse, Groover and Jones [42] reported that it involves calcium ion influx into the cell, and Kuriyama [43] reported that changes in the permeability of organic anions in the vacuolar membrane cause vacuolar collapse. However, the detailed molecular mechanism has not been clarified.

The detailed molecular PCD mechanisms in TEs have been elucidated in studies using *Arabidopsis thaliana*. The NAC domain transcription factors Vascular-related NAC-domain6 (VND6) and VND7 regulate the expression of genes involved in secondary wall formation and PCD of TEs and are master transcription factors for TE differentiation [44]. In addition to transcriptome analysis results, results from drug treatment experiments, such as the inhibition of both secondary wall formation and cell death by trypsin inhibitors [42], suggest that secondary wall formation and PCD progress in a coordinated manner during TE differentiation. The papain-like cysteine proteases xylem cysteine peptidase1 (XCP1) and XCP2 [45, 46], a cysteine protease involved in vacuolar protein maturation termed vacuolar processing enzyme (VPE) [47, 48], metacaspase9 (AtMC9), a type of metacaspase, a cysteine protease with a similar structure to caspases that play a central role in PCD in animals [49–51], and the nucleolytic enzyme bifunctional nuclease1 (BFN1) [52, 53] are involved in the PCD of TEs.

In wood fibers of *Populus*, nuclear DNA fragmentation and autophagosome-mediated autolysis of cellular contents occur prior to vacuolar rupture [54]. The life duration of wood fibers is approximately one month [55]. Gene expression of *XCP2*, *VPE*, and metacaspases, which are also involved in the PCD of TEs, increases during the cell death process in wood fibers in *Populus tremula* × *tremuloides* [51, 54, 56, 57]. Despite the common gene expression patterns, the differences in the autolysis process suggest that the cell death mechanisms differ between wood fibers and TEs.

For details on the cell death of TEs and wood fibers, which are short-lived xylem cells, refer to the reviews by Fukuda [24, 25], Bollhöner et al. [55], and Escamez and Tuominen [58].

Cell death of long-lived ray parenchyma cells

Xylem parenchyma cells play important roles in the storage and transport of materials for many years, after which they execute cell death processes and lose their organelles via autolysis. According to the International Association of Wood Anatomists (IAWA), the inner

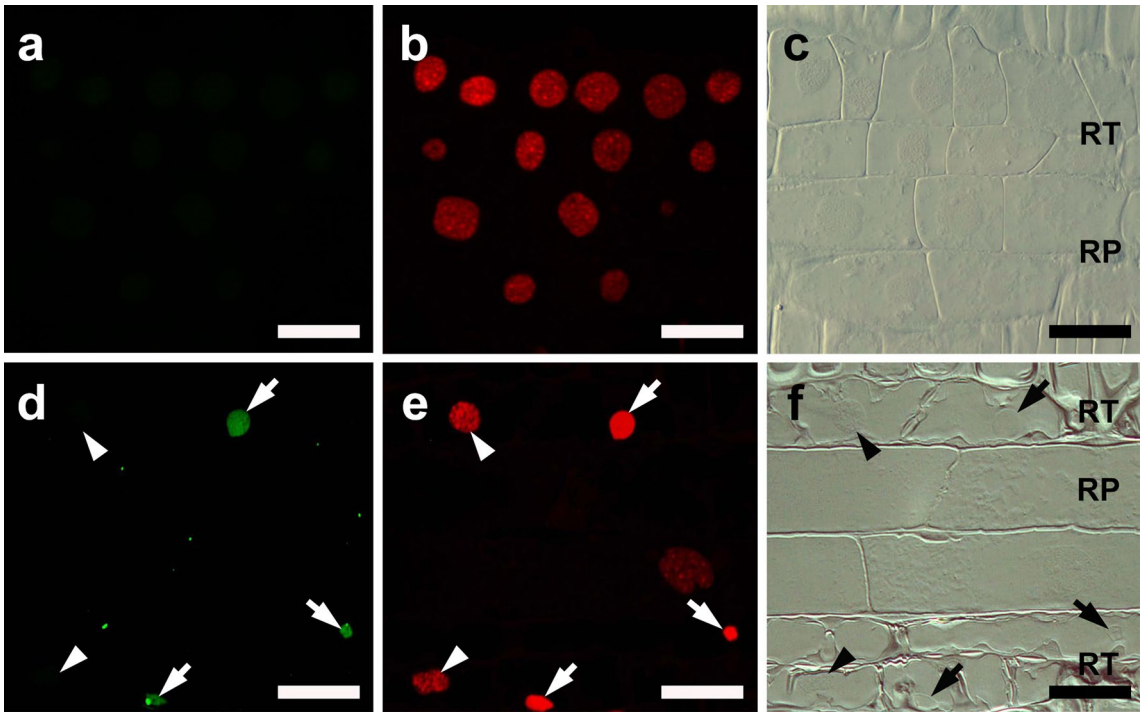

Fig. 2 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay of tissue near the cambium (**a**–**c**) and differentiating ray tracheids (**d**–**f**) to detect nuclear DNA fragmentation in *Pinus densiflora*. The left panels show TUNEL fluorescence, the middle panels show propidium iodide fluorescence, and the right panels are differential interference contrast images. Arrows indicate nuclei with DNA fragmentation in ray tracheids. Arrowheads indicate nuclei without DNA fragmentation. The left side of the micrographs corresponds to the outer side of the tree. *RP* ray parenchyma cell, *RT* ray tracheid. Scale bars = 30 µm. Figures adapted from Nakaba et al. [39]

layers of wood, which contain no living cells, are defined as heartwood, whereas the outer layers containing living cells are termed sapwood [59]. The innermost part of the sapwood, which is intermediate between sapwood and heartwood in terms of color and other general characteristics, is termed intermediate wood [59]. It is important to note that regions with these characteristics are sometimes referred to as "transition zone" or "white zone", which are defined differently by different researchers. Hillis [18] referred to the narrower of these regions as "transition zone" and the wider as "intermediate wood". Nobuchi and Harada [60] referred to the area of the inner sapwood that appears white to the naked eyes in green condition as "white zone", while the narrower, slightly colored area between white zone and heartwood was referred to as "transition zone". In this review, we use the term "intermediate wood", and provide explanations of terms when authors clearly defined the terms in their articles. To avoid confusion, we recommend authors to mention the definitions of sapwood, intermediate wood, and heartwood in each article, e.g., Nakada and Fukatsu [61].

Before the death of xylem parenchyma cells, they synthesize heartwood substances as a result of metabolic changes. These heartwood substances, which play important roles in the resistance to decay, are released from xylem parenchyma cells and diffused into surrounding tissues [62]. Heartwood color, odor, and durability are important parameters for wood utilization, and the mechanism of heartwood formation has long been of interest [18]. Understanding the process of cell death during the transition from sapwood to heartwood is important for understanding the mechanisms of cell death of xylem parenchyma cells. Cambial cells and their derivatives have been suggested to provide a suitable model system for in situ studies of cytodifferentiation in secondary tissues because their differentiation can be followed in a radial direction [63–68]. In xylem parenchyma, axial parenchyma cells are sporadically distributed in the radial direction, whereas ray parenchyma cells are continuously arranged in the radial direction, allowing analysis of continuous changes to some extent. Therefore, ray parenchyma cells have been used to analyze changes from the start of differentiation to the death of xylem parenchyma cells. Hereafter, we first introduce the morphology of ray

parenchyma cells and then the changes in cell contents and morphology related to their death.

Morphology of ray parenchyma cells and structure of rays

The long-lived ray parenchyma cells are unique to trees and are arranged radially, forming rays. The structure of the rays differs between conifers and broad-leaved trees [69, 70]. In conifers, they generally exist of a single row of cells in the axial direction (uniseriate ray) when viewed in the tangential section [69], and ray parenchyma cells are long and strip-shaped in the radial direction. Ray parenchyma cells are directly connected with adjacent longitudinal tracheids via cross-field pits. There are two types of coniferous trees: those in which the rays consist of ray parenchyma cells alone and those in which the rays consist of ray parenchyma cells and ray tracheids. Ray tracheids are generally located at the upper and lower ends of rays and undergo cell death quickly after the completion of secondary wall formation [71, 72]. Due to differences in cell wall organization, pit structure and distribution of lignin, ray parenchyma cells in conifers were classified into five types, namely, Sciadopitys, Cryptomeria, Diploxylon, Haploxylon, and Abies types [73]. According to cell wall organization, ray parenchyma cells can be divided into two types: those composed of primary walls alone (Sciadopitys type and Cryptomeria type) and those composed of primary and secondary walls (Diploxylon, Haploxylon, and Abies types). Among the species in which ray parenchyma cells form only a primary wall, there are two types: those in which cell wall thickening and lignification occur in the outermost sapwood (Cryptomeria type) and those in which lignification occurs in intermediate wood (Sciadopitys type) [73]. In *Pinus* species, ray parenchyma cells form primary and secondary walls and can be classified into three types based on their maturation process: (1) secondary wall thickening and lignification occur in the outermost sapwood, (2) secondary wall thickening and lignification occur in intermediate wood, and (3) lignification occurs without secondary wall thickening in intermediate wood [74]. Furthermore, in *Pinus* species, ray parenchyma cells with different types of maturation coexist in the same ray [74].

In contrast, broad-leaved trees show considerable variation in the shape of ray parenchyma cells and the aggregation state of ray parenchyma cells within a ray [69]. Rays in broad-leaved trees consist of parenchyma cells alone and do not form ray tracheids as in conifers. In the tangential section of rays, there are single-row rays (uniseriate ray), two-row rays (biseriate ray) and multi-row rays (multiseriate ray). Among multiseriate rays, those with significantly greater height and width are termed broad rays. Based on the shape of the radial view, ray parenchyma cells are classified into three types: procumbent cells, square cells and upright cells. When a ray is composed of procumbent cells alone, it is termed homogeneous rays, and when it is partially or completely composed of upright or square cells, it is termed heterogeneous rays. In uniseriate and biseriate rays, ray parenchyma cells are divided into three types based on their contacts with axial xylem elements: contact cells, intermediate cells and isolation cells [75]. Contact cells are located predominantly within the upper and lower lines of individual rays and connect with adjacent vessel elements through pits. Intermediate cells are located within the same radial cell lines as contact cells but are not adjacent to vessel elements. Isolation cells are located within the other radial cell lines of a given ray. Even when isolation cells are adjacent to vessel elements, they are not directly connected with vessel elements through pits. In addition, ray parenchyma cells situated inside multiseriate rays which due to their position do not touch any vessels, are termed as inner cells [76]. Although studies on the differences in the timing of secondary wall thickening and lignification in ray parenchyma cells in broad-leaved trees are few, it has been reported that in *Populus* which have only uniseriate rays, secondary wall thickening occurs earlier in contact cells and intermediate cells than in isolation cells [75].

Considering these differences among various types of ray parenchyma cells is important for understanding the cell death mechanisms of ray parenchyma cells. However, limited information is available about the relationship between types of ray parenchyma cells and characteristics of the death of ray parenchyma cells. Further studies considering differences among various types of ray parenchyma cells are needed for understanding their mechanism of cell death in detail.

Changes in contents and morphology of nuclei in ray parenchyma cells

Changes in the nuclei are easy to detect because the nuclei are relatively large organelles. Therefore, nuclear changes have been observed as indicators of changes in ray parenchyma cells within sapwood. In ray parenchyma cells in intermediate wood, nuclei are smaller and have higher DNA contents than those in outer sapwood [77].

The morphological changes in nuclei in the radial direction differ between conifers and broad-leaved trees. In conifers, nuclei are spherical in ray cambial cells, become rod-shaped or ellipsoid as ray parenchyma cells elongate, and undergo condensation and disappear at the boundary between sapwood and heartwood [78, 79]. In contrast, in broad-leaved trees, nuclei are spherical in ray cambial cells, take on a spindle shape during differentiation, and finally condensate and disappear at the boundary between sapwood and heartwood after changing their

morphology to spherical again [78–80]. To quantitatively evaluate these morphological changes, the nuclear slenderness ratio [78, 79, 81, 82], nuclear irregularity index [83], and nuclear elongation index [84] have been proposed. Morphological changes in nuclei in intermediate wood can be used as an indicator of the progression of cell death in ray parenchyma cells because they correlate with cell death progression [85].

Nakada and Fukatsu [61] reported that the death of ray parenchyma cells in *Larix kaempferi* in Japan occurred from April to July based on a comparison of the locations of intermediate wood (i.e., the zone with a white color in the green condition) and nuclear disappearance. Yamamoto [74] noted that the death of ray parenchyma cells in several *Pinus* species in Japan occurred throughout the growing season, especially from July to October, based on methyl green pyronin staining and morphological analyses of the nuclei. In Taiwan, Yang [83] observed that the greatest changes in nuclear morphology in *Pinus banksiana*, *Picea mariana* and *Populus tremuloides* occurred in August, July–August, and August–October, respectively. Yang [83] concluded that heartwood formation within the stem started at the same time. We have observed significantly deformed nuclei in ray parenchyma cells in *Abies sachalinensis* from July to November in Japan (data not shown), and we speculate that cell death occurs at the same time. Although the evaluation methods differed among the previous reports, it can be assumed that the death of ray parenchyma cells occurs from spring to autumn.

Changes in other organelles and contents in ray parenchyma cells

Changes in organelles other than the nucleus have been revealed by transmission electron microscopy and other microscopic techniques, mainly in conifers such as *Cryptomeria japonica* (Fig. 3). In the outermost sapwood, abundant organelles such as mitochondria, the Golgi apparatus, endoplasmic reticulum, plastids, ribosomes, and amyloplasts exist (Fig. 3) [86–88]. Mitochondria and ribosomes are abundantly observed during the growth period, when ray parenchyma cells have high physiological activity, such as respiration and protein biosynthesis. In the inner sapwood (authors referred to relatively inner part of sapwood which located outside of intermediate wood as "inner sapwood"), the numbers of these organelles decrease [87]. In intermediate wood, ray parenchyma cells become markedly vacuolated, pressing the organelles against the periphery of the cells (Fig. 3) [87]. Osmiophilic substances, thought to be waste products, accumulate in the large vacuoles and the cytoplasm shows osmiophilic properties [62, 88]. Nobuchi and Harada [62] pointed out that vacuoles might act as autophagosomes involved in autolysis in the inner part of intermediate wood (authors used "white zone", and referred to the regions with a white color in the green condition as white zone) in *Cryptomeria japonica*. We have observed the timing of vacuolar rupture and morphological changes and nuclear disappearance during the death of ray parenchyma cells and found that the first change in vacuoles might be a dramatic decrease in storage proteins in protein-storage vacuoles. In ray parenchyma cells in intermediate wood (the regions with a white color in the green condition), not all vacuoles rupture at the same time even within the same cell, and the rupture of enlarged vacuoles might result in autolysis of the cellular contents in ray parenchyma cells in the outermost part of heartwood (Fig. 3) [89]. These observations indicate that vacuoles play an important role in the process of the death of ray parenchyma cells. Finally, ray

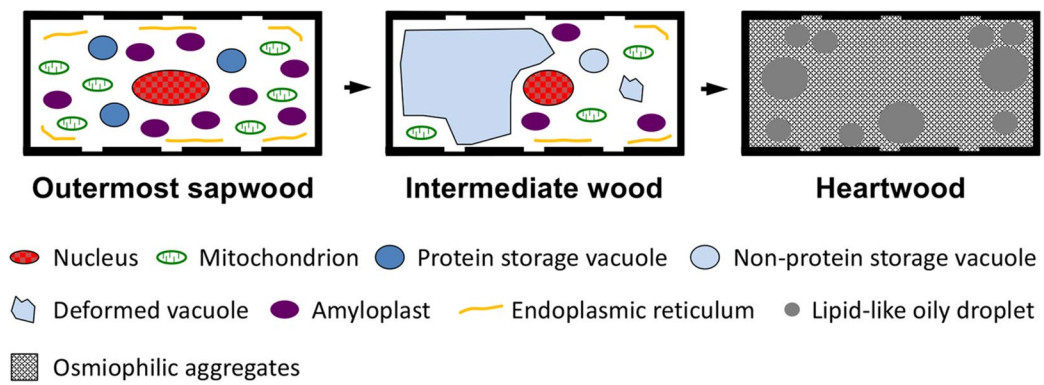

Fig. 3 Schematic diagram of the changes that occur in organelles during cell death of ray parenchyma cells in *Cryptomeria japonica*. In the outermost sapwood, there are abundant organelles. In the inner sapwood, the number of these organelles decreases. In intermediate wood, ray parenchyma cells become markedly vacuolated, and the amount of storage proteins in protein-storage vacuoles dramatically decrease. In heartwood, there are no organelles or starch grains as storage materials

parenchyma cells in heartwood contain no organelles or starch grains as storage materials, although residues of organelles and cytoplasmic substrates can be observed (Fig. 3) [62, 88]. These observations suggest that an efficient nutrient recycling system exists during the process of ray parenchyma cell death, in which autophagy might play an important role.

Changes in the survival rate of ray parenchyma cells in the radial direction

Not all ray parenchyma cells in sapwood dies simultaneously. Early death in some ray parenchyma cells has been reported. In conifers that do not form ray tracheids in rays, such as *Cryptomeria japonica* and *Abies sachalinensis*, cell death occurs earlier in ray parenchyma cells located in the upper and lower radial lines of a ray than in other radial lines (Fig. 4) [88, 90, 91]. In conifers that form ray tracheids in rays, such as *Pseudotsuga menziesii*, *Picea abies*, *Pinus densiflora* and *Pinus banksiana*, cell death occurs earlier in ray parenchyma cells that are in contact with ray tracheids than in those not in contact with ray tracheids [71, 83, 91–93]. As mentioned above, in uniseriate and biseriate rays in broad-leaved trees, ray parenchyma cells are of three types designated contact cells, intermediate cells, and isolation cells, which differ in terms of their contacts with axial xylem elements [75]. We compared the timing of cell death of these three types of ray parenchyma cells in hybrid poplar (*Populus sieboldii* × *P. grandidentata*) and found that cell death occurs earliest in contact cells, then in intermediate cells, and finally in isolation cells [75]. The timings of secondary wall thickening and lignification differ between ray parenchyma cells that die earlier and other ray parenchyma cells [68, 71, 74, 75, 90]. In addition, the amounts of starch grains, lipids, storage proteins, and heartwood substances differ between ray parenchyma cells that die earlier and the other ray parenchyma cells [71, 75, 90]. These observations indicate that positional information might be an important factor in the regulation of the timing of cell death, differentiation, and the function of ray parenchyma cells in conifers and broad-leaved trees that form uniseriate rays.

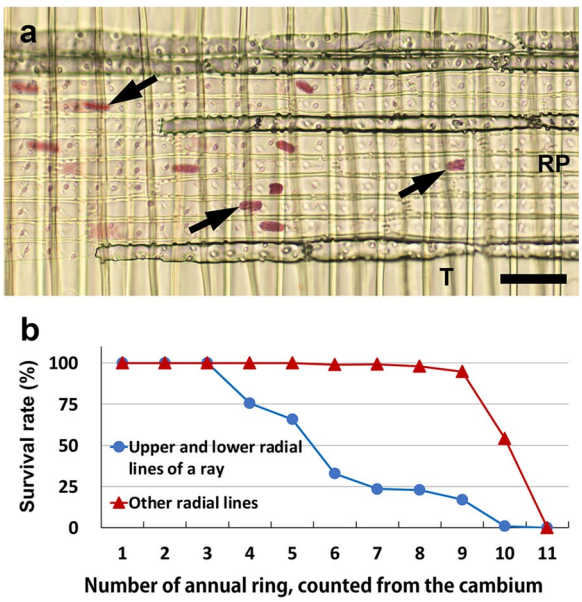

Fig. 4 Early death of ray parenchyma cells located in upper and lower cell lines of rays in *Abies sachalinensis*. **a** Light micrograph of a radial section, stained with acetocarmine, showing nuclei (arrows) in the ray parenchyma cells of the 10th annual ring from the cambium. The left side of the micrograph corresponds to the outer side of the stem. RP, ray parenchyma cell; T, tracheid. Scale bar = 50 µm. **b** Survival rates of ray parenchyma cells in August, determined from the current year's annual ring to the annual ring in which all ray parenchyma cells had lost their organelles. Each rate was calculated as the percentage of ray parenchyma cells that contained a nucleus among 100 ray parenchyma cells. **a** Adapted from Nakaba et al. [90]

The location where the death of ray parenchyma cells starts within sapwood and the percentage of ray parenchyma cells that die earlier differ among species. The concept of survival rate of ray parenchyma cells presented by Ziegler [94] is useful in evaluating such differences among species. Nobuchi et al. [91] calculated the survival rates of ray parenchyma cells in 20 conifer species and found three survival rate decline types: (1) all ray parenchyma cells from cambium to the sapwood-heartwood boundary are alive, (2) the survival rate of ray parenchyma cells starts to decline from the middle sapwood, and (3) the survival rate of ray parenchyma cells starts to decline from the outer sapwood. In addition, Nobuchi et al. [95] reported the survival rate of ray parenchyma cells in 26 broad-leaved tree species and showed that most ray parenchyma cells from the cambium to the sapwood-heartwood boundary are alive and that it is rare for a tree species to have ray parenchyma cells that start to die from outside the sapwood-heartwood boundary. However, Nobuchi et al. [95] considered only procumbent cells, not upright and square cells, in calculating these survival rates. Spicer and Holbrook [93] showed that ray parenchyma cells retained their nuclei until these abruptly disappeared at the sapwood-heartwood boundary in three broad-leaved tree species, *Acer rubrum*, *Fraxinus americana* and *Quercus rubra*. These results indicate that, in broad-leaved trees, the differences in the radial decline trends of the survival rate of ray parenchyma cells among species are small, whereas in conifers, the location within the sapwood where cell death initiates may vary from species to species. It remains unclear why the survival rate of ray parenchyma cells varies among species.

It is expected that the physiological activity of ray parenchyma cells decreases from the outer sapwood towards sapwood-heartwood boundary because the numbers of organelles decrease in the inner sapwood [62, 88]. This expectation is consistent with the drastic decreases in the amount of RNA, which reflects cellular metabolic activity [96], and the mitochondrial reduction capacity, which reflects respiratory activity [78] in the outer sapwood. Radial trends in respiratory activity (oxygen consumption) have been reported, but results were rather inconsistent, ranging from a decrease [97–102] to no change [103] or even an increase [103, 104] in respiratory activity in intermediate wood compared to outer sapwood. However, as mentioned above, the survival rate of xylem parenchyma cells decreases towards the heartwood; therefore, the survival rate should be considered in evaluating radial variation in the respiratory activity of xylem parenchyma cells in sapwood. Spicer and Holbrook [93] evaluated the respiratory activity, which was corrected based on the area fraction, and survival rate of xylem parenchyma cells in outer and inner sapwood. In *Tsuga canadensis*, the respiration rate was lower in the inner sapwood than in the outer sapwood before correction, but after correction, the rates were similar [93]. Although results may vary among species, it is important to consider the survival rate when evaluating physiological activities, such as the respiratory activity, in xylem parenchyma cells, particularly for conifers.

Comparison of disappearance pattern of nuclei between tracheids and ray parenchyma cells

Ray parenchyma cells survive for many years after the completion of secondary wall thickening and lignification. In contrast, cell death of TEs occurs immediately after the completion of secondary wall formation. The cell death program in TEs is tightly coupled with secondary wall formation [25]. These features suggest that the cell death mechanism in ray parenchyma cells might differ from that of PCD in TEs. Therefore, we compared the distribution of cell death (disappearance of nuclei) between short-lived tracheids and long-lived ray parenchyma cells in secondary xylem of conifers and found that the cell death pattern of ray parenchyma cells differs from that of tracheids. In longitudinal and ray tracheids, cell death occurs successively in a radial direction and is related to the radial distance from the cambium (Fig. 5a) [71, 72, 90]. In other words, cell death of tracheids occurs in order from the pith side to the bark side in a radial direction. These results indicate that the length of time from the start of differentiation might control the timing of cell death of tracheids. This scenario resembles the time-dependent PCD of TEs in cultured *Z. elegans* cells [24, 25, 28]. In contrast, in ray parenchyma cells, successive cell death does not occur even within a given radial cell line of a ray (Fig. 5b) [71, 90]. These results indicate that the process of cell death of ray parenchyma cells might not be fully explained by the cellular and molecular mechanisms of cell death that have been proposed for short-lived TEs [25].

Molecular mechanisms of cell death of ray parenchyma cells

The molecular mechanisms regulating the death of ray parenchyma cells have not been fully unraveled. Comprehensive gene expression analyses to elucidate the mechanism of heartwood formation have revealed increased expression of nucleases, proteases, transcription factors, cytoskeleton-associated protein, and desiccation-related protein [105–109]. Huang et al. [110] reported that the *KNAT3*-like homeobox transcription factor involved in

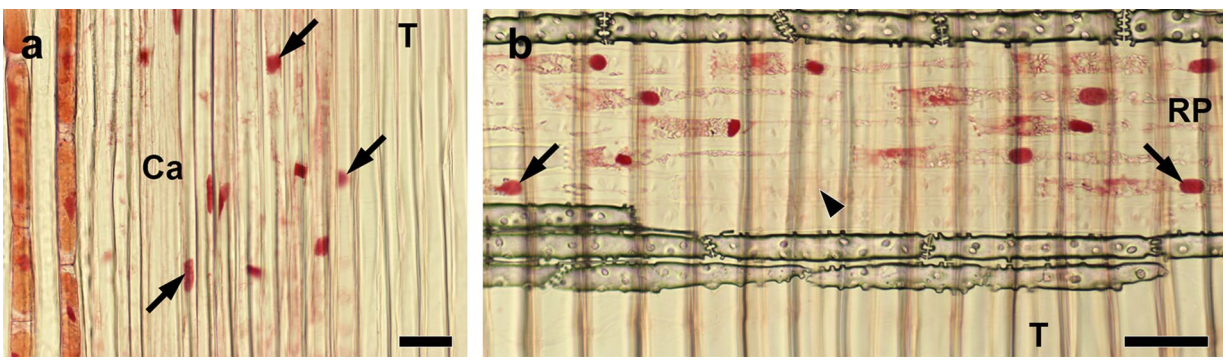

Fig. 5 Comparison of nuclear disappearance patterns between tracheids and ray parenchyma cells in *Abies sachalinensis*. **a**, **b** Light micrographs of radial sections, stained with acetocarmine, showing nuclei in differentiating tracheids near the cambium (**a**) and ray parenchyma cells of the 10th annual ring from the cambium (**b**). Arrows indicates nuclei. Arrowhead indicates a dead ray parenchyma cell. The left side of the micrographs corresponds to the outer side of the stem. *Ca* cambium, *RP* ray parenchyma cell, *T* tracheid. Scale bars = 50 µm. **a** Adapted from Nakaba et al. [90] and **b** adapted from Funada et al. [6]

cell specialization and patterning was highly expressed in inner sapwood and intermediate wood (authors used "transition zone", and referred to regions which fluoresce blue under UV light as transition zone) of black walnut (*Juglans nigra*). Using real-time PCR analysis, Moshchenskaya et al. [111] showed that *BFN* gene expression was increased in the intermediate wood (authors used "transition zone", and referred to two growth rings at the border with heartwood as transition zone) compared to the inner sapwood in *Pinus sylvestris*. We have assessed the variation in the radial direction of gene expression of the transcription factors *NAC* and *MYB*, which are associated with secondary wall formation and PCD in short-lived xylem cells, and *XCP*, a cell death marker in TEs, in hybrid poplar (*Populus tremula* × *P. alba*) ray parenchyma cells using real-time PCR analysis [112]. We found that these genes continued to be expressed in the sapwood and that their expression did not increase prior to cell death [112]. It remains unclear where the products of the above genes are localized in xylem parenchyma cells and how the cell death process proceeds. Further studies on the localization of autolytic enzymes and functional gene analyses are needed in the future.

Gene functions that may be related with the death of ray parenchyma cells are difficult to analyze in trees because it takes several years or more for heartwood formation to initiate in individual trees, which hinders a good understanding of the molecular mechanisms of cell death in ray parenchyma cells. We believe that model experiments inducing cell death with secondary metabolism similar to heartwood formation would be useful to achieve a breakthrough in understanding the cellular and molecular mechanisms of cell death in ray parenchyma cells. We have been conducting cytological analyses of cell death accompanied with secondary metabolism in ray parenchyma cells using an artificial cell-death-induction system [113]. In this system, established by Imai and Nomura [114], cell death and the biosynthesis of agatharesinol, a heartwood substance, are induced in small sapwood sticks under high-humidity conditions. Such model experimental systems are expected to be useful in the functional analysis of cell death-related genes. Combining the results of cell death analyses in intact ray parenchyma cells with those obtained using the cell-death-induction system will greatly advance our

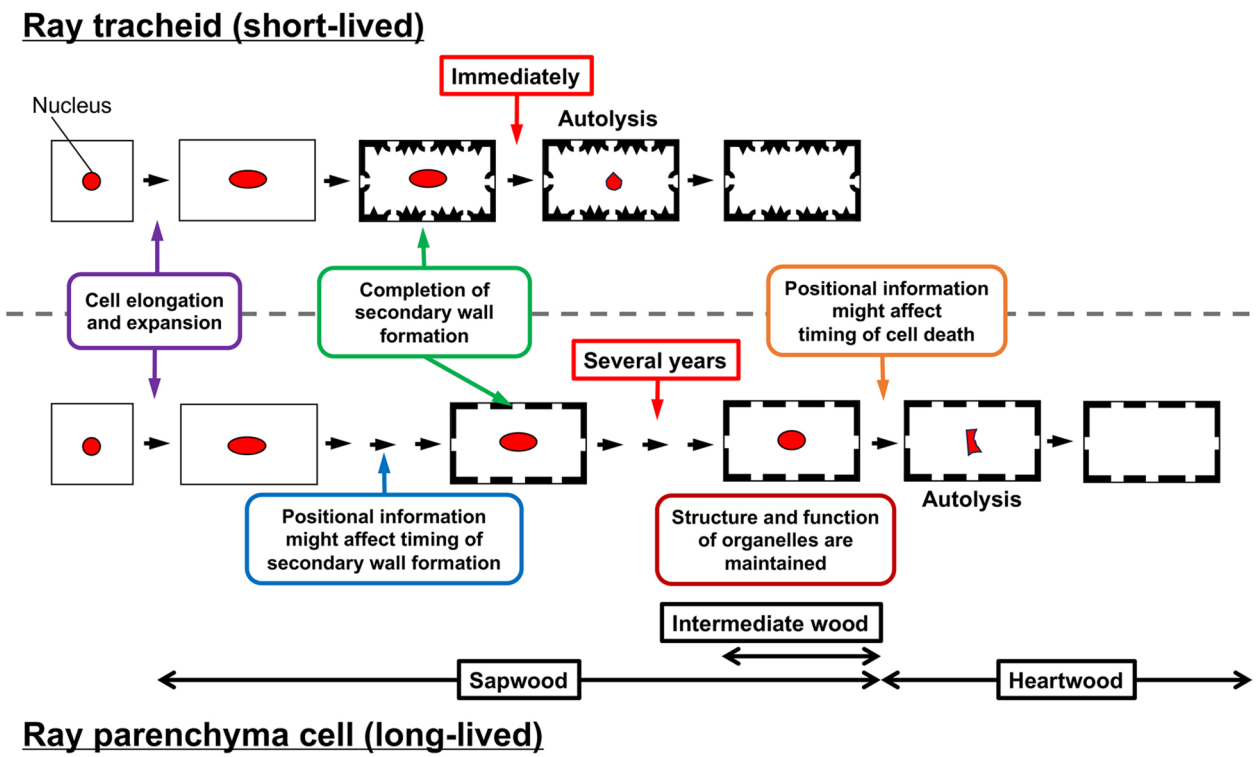

Fig. 6 Schematic diagram of cell death in short-lived ray tracheids (a tracheary element) and long-lived ray parenchyma cells in conifers. Ray tracheid: autolysis occurs immediately after completion of secondary wall formation. The length of time from the start of differentiation might control the timing of cell death. Ray parenchyma cell: autolysis occurs after several years or more after the completion of secondary wall formation. In addition, positional information might affect the timing of differentiation and cell death. The death of ray parenchyma cells in broad-leaved trees has similar characteristics to that in conifers

understanding of the molecular mechanisms of cell death in ray parenchyma cells.

Conclusions

Elucidating the mechanism of cell death in ray parenchyma cells is important for understanding the mechanism of heartwood formation, which is a unique phenomenon in trees with a large stem and long life. The process of cell death in long-lived ray parenchyma cells differs from that in short-lived xylem cells such as TEs, and is likely to differ in terms of the regulatory mechanisms as well. In particular, the existence of an interval of several years to several decades between the completion of secondary wall formation and cell death must be an important feature in explaining the regulatory mechanism of cell death in ray parenchyma cells (Fig. 6). As ray parenchyma cells are unique to trees, elucidating their cell death-regulatory mechanism might lead to a better understanding of the cell death mechanism unique to trees.

As discussed in this review, numerous cytological studies on cell death of ray parenchyma cells during heartwood formation have been conducted. It has been shown that morphological changes occur in the nuclei in sapwood, other organelles decrease in numbers from outer sapwood to intermediate wood, and the survival rates of ray parenchyma cells in the radial direction differs among species. Furthermore, their positional information might affect the timing of differentiation and the death of ray parenchyma cells (Fig. 6). However, the regulatory mechanism of cell death in ray parenchyma cells has not yet been elucidated. Further studies considering differences among various types of ray parenchyma cells are needed for understanding their mechanism of cell death in detail. In addition, in order to understand the mechanism of cell death in ray parenchyma cells, it is essential to elucidate not only the morphological changes throughout the process, but also the molecular mechanisms, such as changes in the expression patterns of cell death-related genes and their products. We believe that further cellular and molecular biological studies combining analysis of intact ray parenchyma cells with cell-death-induction model experiments will provide new insights into the mechanism of heartwood formation, which remains largely unknown. In particular, a better understanding of the molecular mechanisms will provide important evidence for the identification of cell death triggers, as it will help determine when and where cell death begins. Furthermore, a detailed molecular mechanism will allow a clear discussion of the relationship between cell death and the biosynthesis of heartwood substances.

Abbreviations

BFN	Bifunctional nuclease
MC	Metacaspase
IAWA	International Association of Wood Anatomists
PCD	Programmed cell death
TE	Tracheary element
VND	Vascular-related NAC-domain
VPE	Vacuolar processing enzyme
XCP	Xylem cysteine peptidase

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Author contributions

SN wrote the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Competing interests

The authors declare that they have no competing interests.

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