

**Structure, origin, development of interxylary
phloem, intraxylary phloem, radial sieve tube,
and their involvement in phloem network of
Aquilaria and *Gyrinops***

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Chapter 1 General introduction

1.1. Overview

Agarwood is a special type of wood containing resin, and has a long history of diversified utilizations in Asian countries such as China, Japan, India and Middle East countries including Saudi Arabia, due to its unique fragrance and medicinal value. The formation of agarwood resin is a stress response of plants to external stimuli (Chen *et al.* 2018; López-Sampson & Page 2018), and this complex process is closely related to the long-distance transportation, rapid redistribution, and efficient transformation of photosynthetic products, growth hormones, signaling molecules, and other nutritional substances in the plant's phloem tissues (Liu *et al.* 2019). Non-structural carbohydrates provide a large amount of carbon sources for agarwood resin synthesis, which flow through the phloem network to different sinks and are metabolized to form agarwood (Li *et al.* 2022). In another words, the phloem tissues provide a critically structural and substantial foundation for the formation of agarwood resin.

The phloem tissue in woody plants usually refers to the secondary phloem, which is formed by the periodical division of vascular cambium and is responsible for the transportation, regulation and transformation of photosynthetic products and growth hormones (Lalonde *et al.* 2003; Schepper *et al.* 2013). Therefore, the major function and important feature of phloem tissue is to establish symplastic connections throughout the plant body, facilitating delivery of nutrients and various signaling molecules (Van bel 2003; Heo *et al.* 2014), which play a pivotal role in the differentiation, development, damage response, and formation of secondary metabolites in the plant body (Pang *et al.* 2008; Prislan *et al.* 2019).

In addition to the secondary phloem, there are also some taxonomic groups that produce abnormal phloem such as interxylary phloem, intraxylary phloem, and radial sieve tube (Carlquist 2001; Rajput *et al.* 2022). The abnormal origin and development of these phloem tissues are specific features in the structure of agarwood-producing species. Clarifying the structural and functional characteristics of these abnormal phloem tissues is of great importance for understanding their physiological functions relevant to phloem anastomoses, regulating the growth and proliferation of agarwood-producing species, as well as inducing the formation of secondary metabolites.

It is very interesting to consider how the transport pathways of photosynthetic products are organized in trees with such abnormal phloem tissues, and the elucidation of these complex structures is of great scientific significance. To clarify these issues, this study investigated the cellular structure, developmental process and connectivity of different abnormal phloem tissues in agarwood-producing species. The second and the third chapters respectively introduce the anatomical characteristics of the intraxylary phloem and interxylary phloem in agarwood-producing species, including their distribution, cellular composition, origin and developmental process. The fourth

chapter discusses the structural changes of interxylary phloem during the formation of wound tissues, followed by cambium regeneration when agarwood-producing species subjected to artificially peeling bark. The fifth chapter introduces the structural features, origins of radial sieve tube and describes the connections between sieve tubes through common plates. The sixth chapter focuses on the fine structures of axial and radial sieve tubes in different phloem tissues and the diversified connecting structures formed between them to develop a continuous phloem network.

1.2. What is agarwood

Agarwood is a type of wood with unique aromatic resins formed by natural phenomenon or artificial treatment on a group of tree species mainly belongs to *Aquilaria*, Thymelaeaceae, which is the best known, together with *Gyrinops*, as the principal producers of the resin-suffused agarwood and includes fifteen species of trees, native to Southeast Asia, China, Bhutan and India (Ng *et al.* 1997). In other words, agarwood is a diseased tissue corresponding to the production of oleoresin which begins to become fragrant after influenced by exogenous triggers and this aromatic resinous wood is called agarwood (Chowdhury *et al.* 2016; Chhipa *et al.* 2017). Agarwood is one of its trade names, which is also known as gaharu, eaglewood, aloeswood, agila wood, agalloch, jinkoh, chenxiang (its Chinese name literally means the dark and heavy wood that sinks due to its abundant resin content) and so on (Pojanagaroon & Kaewrak 2005; Mohamed *et al.* 2013; Chowdhury *et al.* 2016).

Agarwood is highly prized that is extremely rare and valuable for its fragrance and medicinal values. It has been widely used for incense, perfume, cosmetics, medicine and although less common, agarwood may also be carved into sculptures, beads, and boxes, these sometimes also being used for religious purposes, especially in the Asian and Middle East countries, such as China, Japan and India (Barden *et al.* 2000; López-Sampson & Page 2018; Lee & Mohamed 2016). The widespread use, scarce natural resources, and high prices, have led to the over-exploitation of agarwood-producing species at worldwide level, resulting in all the members of *Aquilaria* spp. one of the most important genera of agarwood-producing species, being listed under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and their import and export requiring relevant licenses (IUCN 2002) (CITES 2004).

Naturally, agarwood formation is often linked to the physical wounding or damage of agarwood-producing species which elicit the defense mechanism of these trees to initiate the resin production (Tan *et al.* 2019). Microscopic observation shows that the phloem tissues and their adjacent ray tissues of agarwood-producing species were the areas where resin formation began earliest and was most concentrated in distribution (Liu *et al.* 2019; Adimahavira *et al.* 2023), while other elements such as vessels and fibers usually were plugged to a lesser extent (Rao & Dayal 1992). Chemical and histochemical analysis revealed that the process of agarwood formation was accompanied by the conversion of starch grains to soluble sugars, and by the production of sesquiterpenoids, chromone derivatives and other characteristic components of

aromatic resin in various types of parenchyma cells (Yagura *et al.* 2005; Ueda *et al.* 2006; Wang *et al.* 2021). The material transformation was concentrated in the interxylary phloem, providing a structural and material basis for the formation of agarwood (Liu *et al.* 2022).

1.3. Phloem tissues of woody plants

The phloem is a specialized conducting tissue in woody plants responsible for the transport and regulation of photosynthetic products and growth hormones from source to sink organs. There are various types of phloem tissues in woody plants according to different classification criteria. Classified by location, the secondary phloem is located adjacent to the outer side of the vascular cambium, the interxylary phloem is located on the inner side of the vascular cambium, mixed with the secondary xylem, and the intraxylary phloem is located between the outer edge of pith and the primary xylem. The radial sieve tubes exist in ray tissue and are mixed with the ray parenchyma cells. According to the origin of cells, the phloem tissue of woody plants can be produced by the periodic division of vascular cambium, such as secondary phloem and some types of interxylary phloem, or by the dedifferentiation of parenchyma cells whose cell walls have not yet lignified, such as some types of intraxylary phloem and radial sieve tubes.

The most common type of phloem is the secondary (or external) phloem, which is formed by the periodic periclinal division of the vascular cambium. Regardless of the types, any phloem is composed of conducting and nonconducting parts. In angiosperm, portions of the conducting phloem are recognized by living sieve tubes with turgid companion cells, parenchyma cells and fibers. Sieve tube bearing sieve areas on its walls, concerns with longitudinal transportation throughout the tree body. Companion cell is a special type of parenchyma cell formed by the unequal division of the same mother cell as sieve tube (Evert 2006). One to several companion cells usually coexist with sieve tube, typically appearing in the corners of the sieve tube (IAWA Committee 2016). The intricate cell-cell interactions between sieve tube elements (SE) and its companion cells (CC) compose the control centers of the phloem monitoring the long-distance transport of substances (Oparka & Turgeon 1999).

Physiological functions of different phloem tissues include but are not limited to the following: as one of the principal conductive tissues in vascular plants, phloem tissue has a major role in the translocation of photosynthates from the source to the sink. Besides photosynthates, it also exchanges several other compounds including secondary metabolites and signaling molecules (Rajput *et al.* 2022). When plants are subjected to exogenous invasions such as mechanical injury, fungal infection, or pest infestation, the phloem tissues close their sieve tubes, prevents intercellular communications through the deposition of callose (Nedukha 2015) and initiates the synthesis of secondary metabolites that have repellent effects on bacteria and insects, avoiding the deep spread of invasion (Tan *et al.* 2019). Meanwhile, unlignified parenchyma cells promote callus formation and cambium regeneration through redifferentiation (Pang *et al.* 2008). In addition, various types of phloem tissues embedded in the secondary xylem generally contain quite volume of parenchyma cells,

which have special significance for some plants with climbing habits that require flexible trunks (Carlquist 2013).

1.4. Abnormal phloem tissues

In addition to the most popular secondary phloem, there are abnormal phloem tissues located in the primary and secondary plant body such as the intraxylary phloem presenting between the pith and the primary xylem, and the interxylary phloem embedding within or intermitting with the secondary xylem (IAWA Committee 2016). Differentiation of these abnormal phloem tissues is either related to the variants of a single vascular cambium, to the continuous division of successive cambia (Yang *et al.* 2020), to the redifferentiation of parenchyma cells (Patil *et al.* 2011) or to a combination of all of them. Abnormal phloem tissues can be formed earlier, later, or simultaneously with the secondary phloem. Although lacking consistency, the formation of abnormal phloem tissues has its unique chronological series in different species (Carlquist 2002). Their constituent cells are similar with those of secondary phloem (Rajput *et al.* 2018), but there are often fluctuations in the ratio of sieve tube-companion cells (SE-CCs) groups, parenchyma cell or fiber (Luo *et al.* 2018; Luo *et al.* 2019). The characteristics of abnormal phloem tissues are summarized as follows.

1.4.1. Interxylary phloem

The occurrence of interxylary phloem tissues has been reported in 21 families, but they occur in relatively limited families in woody angiosperms (Carlquist 2013; Rajput *et al.* 2022). They are lens, island or band shaped phloem tissues discretely embedded in the secondary xylem or distributed alternately with the secondary xylem. According to the origin and ontogeny of interxylary phloem tissues, they are classified into: 1) Combretum subtypes; 2) Strychnos subtypes; 3); Azima subtypes; and 4) Calycopteris subtypes (Rajput *et al.* 2022). Interxylary phloem tissues are strands consisting of several axial sieve tubes and companion cells surround by a sheath of parenchyma cells, which sometimes include sclerenchyma cells (Carlquist 2013). Although the types of cells that make up the phloem tissues are similar, there are still fluctuations in the size and quantity of their functional cells, especially sieve tubes, between the interxylary phloem and other phloem tissues (Den Outer & Veenendaal 1995). This feature is attributed to their respective roles in storage and transportation functions (Quintanar-Castillo *et al.* 2024).

According to present understanding of interxylary phloem tissues, their physiological functions may include: 1) photosynthetic products conduction rather than storage based on the less starch content of their axial parenchyma cells; 2) extensive and rapid conduction to large photosynthate sinks based on the phenomenon that a number of species with interxylary phloem tissues have large fruit size and mass-flowering pattern; 3) phloem pathway multiplication and longevity based on the cambial activities of unlignified cells replacing the un-conductive sieve tubes; 4)

establishment of a three-dimensional network of phloem tissues in the secondary xylem benefiting the storage and retrieval of photosynthates throughout the stem; 5) phloem pathway preservation based on the protection provided by the surrounding lignified xylem cells; 6) rapid and efficient damage responses such as the signal molecular transportation and aromatic resin synthesis. However, many researchers pointed out that majority of the species that develop interxylary phloem tissues are lack of economic importance, which led to few of attention to their anatomical structures and physiological functions (Carlquist 2013).

1.4.2. Intraxylary phloem

Based on the investigations about samples from Acanthaceae, Convolvulaceae, Gentianaceae, Loganiaceae, Stylidiaceae, Thymeleaceae and so on, there is an intrinsic relation between the occurrence of interxylary phloem and the formation of intraxylary phloem (Patil & Rajput 2008; Carlquist 2013; Lawand *et al.* 2023; Patil *et al.* 2024). Thus, intraxylary phloem tissues may be a kind of “precursor” for interxylary phloem formation in some given species. Although the development of intraxylary phloem, interxylary phloem and the secondary (or external) phloem can occur simultaneously or sequentially, the time of their occurrence is characteristic for each species (Rajput *et al.* 2022). According to their occurrence, the intraxylary phloem tissues can be divided into three types, which are derived from the procambium (Patil *et al.* 2009), the mature pith parenchyma cells or the intraxylary phloem cambium (internal cambium, medullary cambium) (Patil *et al.* 2009; Terrazas *et al.* 2011). The latter two types belong to the secondary intraxylary phloem.

Structural composition of intraxylary phloem tissues remains similar with those of interxylary phloem tissues and external phloem tissues, possessing sieve tubes, companion cells, phloem parenchyma cells, and fibers (Zózimo *et al.* 2011). The axial sieve tubes of intraxylary protophloem tissues can be several times smaller than the pith parenchyma cells and those of secondary phloem tissues (Gondaliya & Rajput 2016; Rajput *et al.* 2018), or can be longer than those in the regular phloem (Patil *et al.* 2009; Patil *et al.* 2014; Rajput *et al.* 2014). Structurally, the axial sieve tubes in intraxylary phloem tissues are alike to those in the secondary phloem tissues, connected through the highly differentiated simple or compound sieve plates at their end walls (Rajput *et al.* 2022). Potential functions of intraxylary phloem proposed by the previous researchers include: 1) assistance in the maintenance of apical dominance; 2) spatial division in the translocation of photosynthates in the stem; 3) localized redistribution of photosynthate to branches; and 4) a higher amount and a safety way for photosynthates translocation (Zózimo *et al.* 2011; Rajput *et al.* 2018).

1.4.3. Radial sieve tube

Radial (ray) sieve tubes are radially arranged sieve tubes differentiated in the phloem ray tissues and/or xylem ray tissues of some angiosperms (Lev-Yadun & Aloni 1991; Rajput & Rao 1997; Rajput 2004b). Their occurrence shows no correlation with

plant morphology, which can be observed in vines (Pace *et al.* 2015), shrubs or trees (Rajput *et al.* 2022), but radial sieve tubes are usually found in the species which also have interxylary phloem (included phloem) tissues (den Outer & Van Veenendaal 1981; Lev-Yadun & Aloni 1991). Though the radial sieve tubes can distribute in the uniseriate and/or multiseriate rays, and can appear at the center or edges of ray tissues, their distributions are characteristic for different species (Rajput & Rao 1997; Rajput 2004a; Rajput 2004b). In a ray tissue, there may be only one radial sieve tube element, or there may be several of them, which are either clustered or dispersed in distribution (Rajput *et al.* 2022).

Similar with the axial sieve tube, radial sieve tube is also associated with one to several companion cells, and the SE-CCs groups are surrounded by ray parenchyma cells with lignified or unlignified cell walls (Sharma *et al.* 1980; den Outer & Van Veenendaal 1981). Radial sieve tubes are also connected by simple or compound sieve plates at their end walls with calloses and slimes (P-protein) depositing on them and cytoplasmic strands passing through the sieve pores, but radial sieve tubes do not develop sieve areas on their lateral walls (Rajput & Rao 1997). The development of well-differentiated sieve plates and their frequent interconnect with axial sieve tubes forming an anastomosing network indicate that radial sieve tubes are probably involved in the rapid transport of photosynthates and quick response to injury (Aloni *et al.* 1990).

1.5. Objective of the thesis

Numerous researches indicate that the formation of agarwood resin is closely related to the structure and physiological activities of the phloem tissue in agarwood-producing species. However, there are limited reports on the anatomical structures and functions of abnormal phloem tissues of agarwood-producing species and some types of them have never been studied. For the better conservation, cultivation and utilization of the valuable species, it is worthwhile to elucidate the anatomical characteristics of the phloem tissues, especially the abnormally developed interxylary phloem, intraxylary phloem, and radial sieve tube element in the agarwood-producing species, as well as their origins, development processes, connection modes, and physiological functions of these phloem tissues.

Chapter 1 provides an overview of the agarwood-producing species, the interactions between agarwood resin formation and their phloem tissues, and various types of phloem tissues in woody plants. Additionally, the objective and the outline of the study were introduced.

Chapter 2 focuses on the anatomical characteristics of intraxylary phloem tissue, including its origin, development process, and cellular composition. The difference in the contributions of three types of phloem tissues in young shoots to their physiological functions are compared in terms of their differentiation time and qualitative number of sieve tubes.

Chapter 3 focuses on the anatomical characteristics, origin, and development process of interxylary phloem tissue, and compares the anatomical similarities and differences between interxylary phloem and secondary phloem tissues.

Chapter 4 focuses on the functions of interxylary phloem in wound healing and cambium recovery of agarwood-producing species under abnormal growth conditions caused by girdling bark.

Chapter 5 focuses on the distribution, quantity, origin and structure of radial sieve tubes in agarwood-producing species, and discusses the functions of connections between radial and axial sieve tubes in forming a constant phloem network.

Chapter 6 focuses on the fine structures developing for the phloem anastomoses in axial and radial sieve tubes, as well as the structures and functions of different types of connections in forming a continuous phloem network in the secondary xylem of agarwood-producing species.

Chapter 7 summarizes the general conclusions.

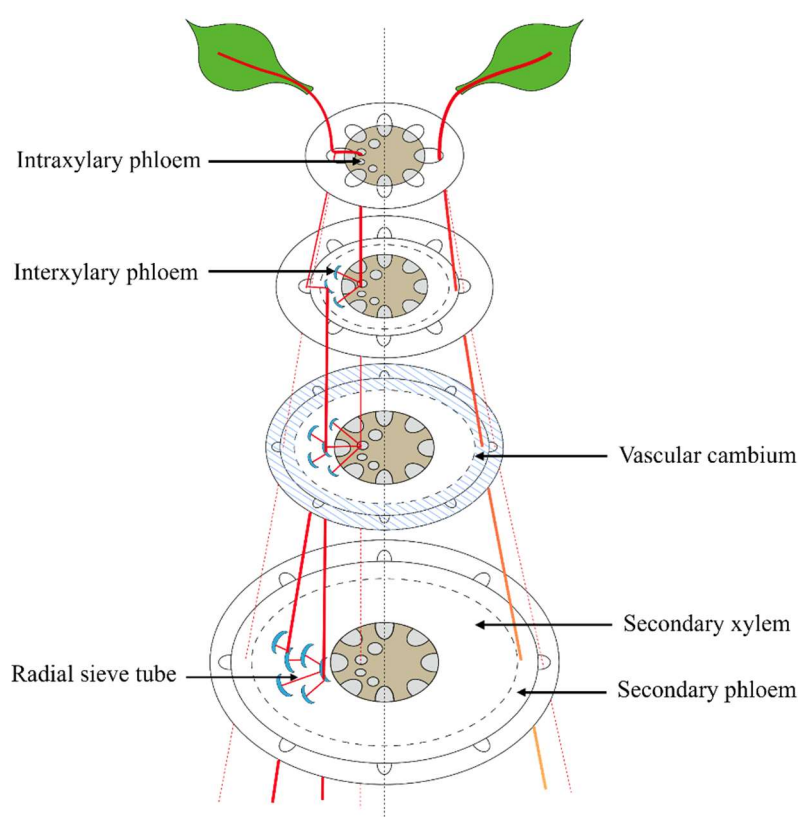


Figure 1.1. Diagrammatic representation of the relationship between intraxylary and interxylary phloem differentiation and the phloem network during xylem development in *Aquilaria* and *Gyrinops*. The left part of the diagrammatic sketch shows the phloem tissues in *Aquilaria* and *Gyrinops*, while the right part of the sketch shows the phloem tissue in common species.

Chapter 2 Structure, origin and development of intraxylary phloem

2.1. Introduction

Agarwood has been highly coveted in different cultures and countries for the past thousand years because of the distinctive fragrance of its resinous wood. *Aquilaria* and *Gyrinops*, two genera both belonging to the Thymelaeaceae, are well known for producing scented wood. Numerous researches have shown that the formation of aromatic resins is closely related to the phloem tissue of agarwood-producing species, making it one of the most concentrated parts of resin distribution (Rao & Dayal 1992; Mohamed *et al.* 2013; Chong *et al.* 2014; Liu *et al.* 2019; Liu *et al.* 2022). Therefore, elucidating the structural characteristics of the phloem tissues in agarwood-producing species is the prerequisite for understanding their physiological functions, and it is also of great significance for aromatic resins production.

In addition to the secondary phloem located at the outer side of cambium, agarwood-producing species show dispersed distribution of interxylary phloem (IP) within the secondary xylem. More recently, it has been suggested that IP tissues in mature *A. sinensis* played a predominant role in the translocation of photosynthates (Luo *et al.* 2018). However, the development of IP tissues in young shoots of agarwood is chronologically delayed, meaning that it occurs later than the development of the secondary xylem and can only be observed starting from the samples of the fifth or the sixth internodes (Luo *et al.* 2020). So, before the differentiation of the IP, which phloem tissue is mainly responsible for the transportation of photosynthetic products, hormones, and other nutrients? This is still an unresolved issue.

While studying the origin and development of IP in young shoots of *A. sinensis*, the presence of intraxylary phloem at the outer margin of the pith was accidentally detected. The finding corresponded with the definition of intraxylary phloem as phloem strands present at the periphery of the pith (Carlquist 2013; Angyalossy *et al.* 2016). Available literature provided a list of the families and genera in which intraxylary phloem characteristically occurs (Metcalf & Chalk 1983). In some of the Thymelaeaceae genera listed, both intraxylary and IP tissues were present. However, characteristics of intraxylary phloem in *Aquilaria*, the representative genus for agarwood production, has not been well documented yet. The present investigation set out to elucidate the origin, structure, and development of intraxylary phloem in young shoots of *A. sinensis*.

2.2. Materials and Methods

2.2.1. Materials

Young shoots were collected in August 2017 from the branches of a greenhouse-cultivated ten-year-old *Aquilaria sinensis* tree in the Southwest Forestry University, Kunming city, Yunnan province, China. Small branch with a diameter of about 3 cm was collected from another ten-year-old *A. sinensis* tree growing in plantation of Zhongshan city, Guangdong province, China.

2.2.2. Optical and fluorescence microscopic observation

For double staining and aniline blue staining to distinguish the time series and origin of phloem tissues, suitably trimmed samples taken from the first to the fourteenth internodes were immediately fixed in 4 % formaldehyde solution and dehydrated using a series of solvent mixtures constituted of *t*-butyl alcohol, ethanol, and deionized water. Following this, the samples were immersed three times in 100 % *t*-butyl alcohol and then processed using routine paraffin embedding. Transverse sections 7~8 μm in thickness were obtained with a rotary microtome (Leica Co Ltd, RM2145) and were stained with a combination of 1 % safranin and 0.5 % fast green. Some of the sections were stained with 0.005 % (w/v) aniline blue aqueous solution for 30 min under room temperature (Currier & Strugger 1956).

2.2.3. FE-SEM observation

For FE-SEM observation, three groups of samples were prepared as follows: 1) 7~8 μm -thick sections that had been cut from different internodes of young shoots were immersed in 0.1 % (v/v) sodium hypochlorite for 5 min at room temperature in order to extract cytoplasmic materials and were then immersed in a series of *t*-butyl alcohol dehydration concentrations, and then freeze-dried. 2) 10 μm -thick sections that had been microtome-sectioned from the small branch (3 cm in diameter) and pre-fixed using 4 % formaldehyde were prepared following the same treatment procedure as used for the first group samples. 3) 1 mm-thick small disks that had been cut from the corresponding internodes of other shoots and from the same branch pre-fixed using 50 % ethanol were cleaned for one hour using the same sodium hypochlorite, followed by the same FE-SEM sample dehydration procedure.

Afterwards, all specimens were coated with platinum at 30 mA for 80 s by means of an Ion Sputtering Apparatus (JEOL Co. Ltd, JEC-3000FC) and examined by FE-SEM (JEOL Co Ltd, JSM-7800F) at 2.0 kV accelerating voltage with a 9~10 mm working distance.

To precisely determine the developmental characteristics of sieve tube elements, the same section in which the locations of sieve tubes had been confirmed by means of

aniline-blue staining was also examined by use of the FE-SEM. The sections used for aniline-blue staining were washed with deionized water and dehydrated by means of a series of *t*-butyl alcohol concentrations. Then, the sections were freeze-dried and coated with platinum, following the same techniques as mentioned before.

2.2.4. TEM observation

For TEM observation, following 4 % formaldehyde fixation, samples collected from the fourteenth internode were cut into small pieces approximately $2 \times 2 \times 2$ mm in size and then dipped into slush nitrogen. The frozen pieces were then treated at -78 °C for 16 h with a second fixative consisting of 2 % glutaraldehyde and 1 % osmium tetroxide in acetone. The samples were kept in the fixative at -28 °C for another 5 h, followed by 4 °C for 45 min, and were allowed to come to room temperature over a period of at least 30 min. Samples were substituted with acetone three times and embedded in Spurr's resin following the usual procedure. Ultrathin sections with a thickness of 70 nm were cut by an ultra-microtome (Leica Co Ltd, Ultracut E) and were stained with 2.5 % uranyl acetate for 30 min at 50 °C and then with lead citrate for 7 min at room temperature. After washing and drying, the sections were observed using a transmission electron microscope (JEOL Co Ltd, JEM-1400).

2.2.5. Statistical analysis of the quantity of sieve tubes

Statistical analysis of fluorescence dots in the external, IP and intraxylary phloem tissues was carried out by means of the four steps described below, using specimens chosen from the first, the second and the eleventh internodes to evaluate the quantity of sieve tubes. These internodes were selected to compare the number of fluorescence dots in external, IP and intraxylary phloem before the formation of secondary xylem, during the formation of secondary xylem before the development of IP and during the formation of secondary xylem after the development of IP.

Step 1: five serial transverse sections were prepared from paraffin embedded blocks by means of rotary microtomy and stained with aniline blue aqueous solution. Step 2: a number of fluorescence photos were taken using a ten times and a twenty times objective lens. Step 3: the density of fluorescence dots and the area of external phloem between cambium and outermost external phloem fibres were measured by means of Image J. Further, the areas of IP and intraxylary phloem strands were measured by means of the same Image J. Step 4: the number of fluorescence dots in intraxylary, IP and external phloem was obtained in the first, the second and the eleventh internodes respectively, by calculating the data collected in step 3.

2.3. Results

The occurrence and development of intraxylary phloem were studied by analyzing microtome sections obtained from different internodes – from the first to the fourteenth – of more than twenty young shoots and from a mature branch of three centimeters in diameter. Furthermore, the higher magnification images obtained by means of FE-SEM and TEM facilitated the analysis of the fine structural features of intraxylary phloem, including sieve tubes, sieve plates and pores. Intraxylary phloem development in young shoots of *Aquilaria* was classified into five stages based on the different structure features that were observed by means of light microscopy after double staining, fluorescence microscopy following aniline blue staining, and FE-SEM. There was time lag in the developmental stages among individual shoots, so that the processes were described substantially on the represent averages.

2.3.1. Origin of intraxylary phloem

A transverse section obtained at the very tip of the first internode (i.e., under the shoot apex) in a young shoot of *A. sinensis* shows a typical structure made up of epidermis, cortex, the precursor of primary vascular tissue and pith (Figure 2.1a). Within the primary tissue, external phloem fibres have just differentiated and appeared as tiny clusters. In addition, tracheary elements of primary xylem with lignified walls have differentiated (arrows in Figure 2.1a), but were not fully produced at the periphery of the pith. Neither intraxylary phloem strands nor vascular cambium has yet been differentiated.

Compared to Figure 2.1a, Figure 2.1b shows a slightly advanced stage. External phloem fibres with thick cell walls were further differentiated into bundles and vascular cambium was on its way to forming a complete cylinder (arrowheads in Figure 2.1b). The area inside the primary xylem cells was completely occupied by large ground parenchyma cells. So far, no intraxylary phloem was initiated, but some parenchyma cells on the inner side of the primary xylem showed cell division (long arrows in Figure 2.1b). Thus, differentiation of intraxylary phloem was initiated immediately after primary xylem cells developed at the periphery of the pith in the tip of the first internode.

In the middle of the first internode, immature patches of intraxylary phloem consisting of approximately five to seven component cells were formed at the pith periphery. Aniline blue staining revealed the occurrence of sieve tubes emitting strong or weak fluorescence among a tiny cluster of immature intraxylary phloem cells in the first internode (Figure 2.1c). Figure 2.1d taken by FE-SEM, shows a sieve plate of one of the weak fluorescence sieve tubes. Individual sieve pores were minute, usually less than 0.1 μm , and appeared as donut-shaped structures. It was not clear whether the pores were already open, and the sizes of the pores were barely ascertained. The FE-SEM image of Figure 2.1e was also taken from the sieve tubes that presented in the same phloem bundle, but emitted strong fluorescence. Even in the sieve tube's nascent state, the figure shows the connection between sieve pores and the tubular slimes that were parts of the cytoplasmic membrane.

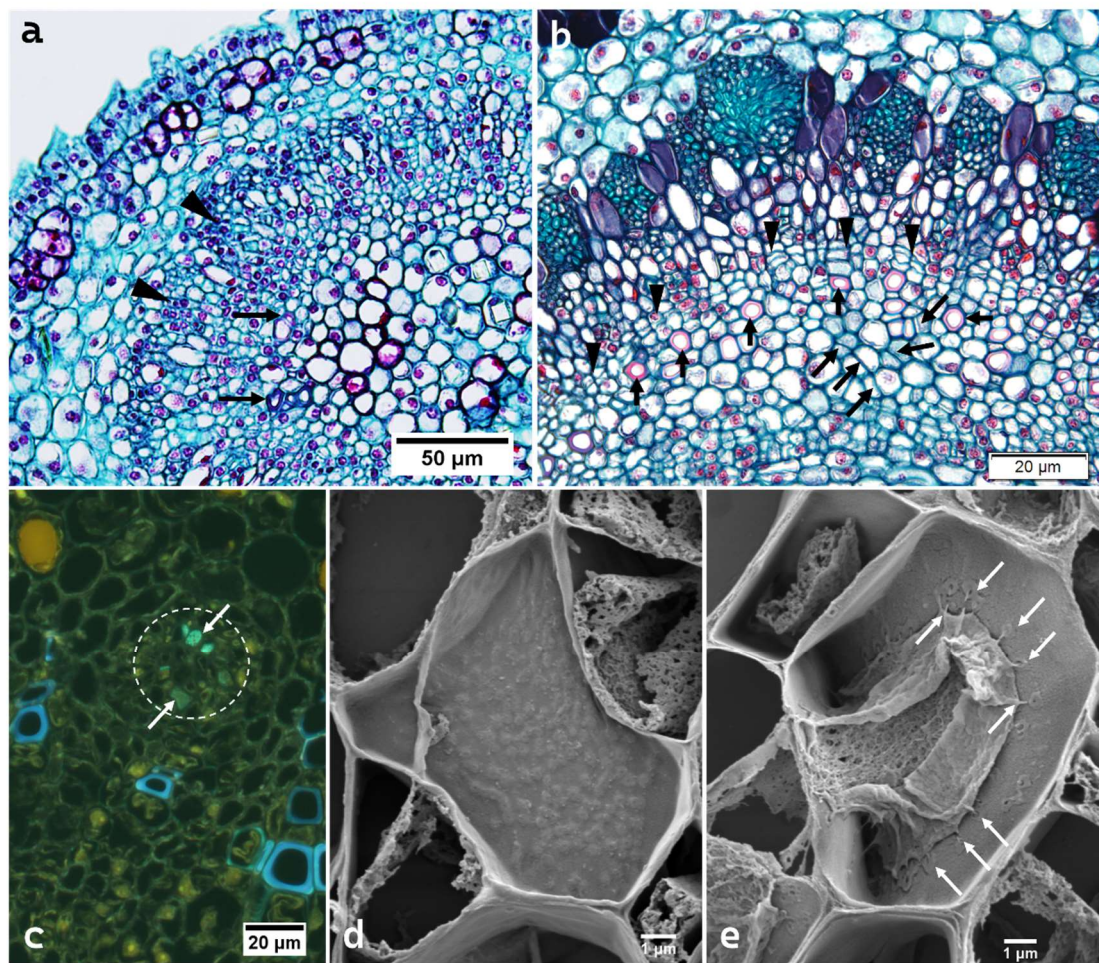


Figure 2.1. Initiation of intraxylary phloem tissue and incipient sieve tubes.

a: Cross section taken below the shoot apex shows epidermis, cortex, primary vascular tissues and pith. Arrowheads: immature external phloem fibres; arrows: primary xylem cells.

b: Cross section taken near the tip of the first internode shows intraxylary phloem differentiation from the pith parenchyma cells (long arrows). Incomplete ring of vascular cambium is shown by a series of arrowheads. Short arrows: lignified primary xylem cells.

c: Fluorescence image shows sieve tubes emitting strong or weak fluorescence in a tiny intraxylary phloem.

d and e: SEM images of sieve plates in the first internode show yet fully opened nascent sieve pores with weak fluorescence (d) and pores with strong fluorescence connected by filamentous slimes (arrows in e).

2.3.2. Development and multiplication of intraxylary phloem

In the advanced stage of shoot growth, small patches of immature intraxylary phloem strands were produced by the division of pith parenchyma cells inside the primary xylem (short arrow in Figure 2.2). These patches were composed of ten or more component cells, 4~5 μm in diameter, which quickly formed a circular arrangement at the periphery of the pith (circles in Figure 2.2a). Figure 2.2c shows an enlarged FE-SEM image of developing sieve plates with narrower sieve pores. This phenomenon was very common in the growing intraxylary phloem in the middle part of the first

internode. At least four sieve tubes, each with a sieve plate, were seen in a single intraxylary phloem strand. A higher magnification image of one of the sieve plates shows more distinct donut-like structures of the individual pores (inset in Figure 2.2c).

Following the differentiation of primary xylem, an increasing number of sieve tube clusters were easily detected in intraxylary phloem strands. The parenchyma cells diminished in size from the pith outwards toward the primary xylem and, therefore the transition between these two regions was gradual. On the other hand, some parenchyma cells located along the inner side of the primary xylem or surrounding the early formed phloem tissues divided repeatedly and became differentiating centers for newly establishing intraxylary phloem strands (long arrows and circles in Figure 2.2a).

The number of fluorescence dots corresponding to the callose deposition of individual sieve tubes was counted to compare the quantity of sieve tubes in intraxylary and external phloem tissues in the first internode where the secondary xylem was not yet developed. As is shown in Table 2.1, the average number of the dots in intraxylary and external phloem was $233 \pm 37/\text{mm}^2$ and $45 \pm 11/\text{mm}^2$ respectively. The average surface areas of intraxylary and external phloem at this stage were $148, 452 \mu\text{m}^2$ and $420, 556 \mu\text{m}^2$ respectively (Table 2.2). The average number of the dots in the surface area of the intraxylary phloem was 35, a figure twice as much as that seen in external phloem (Table 2.3).

Sclerenchyma cells such as fibres were rarely observed in the intraxylary phloem strands at this stage. In some cases, the fibres were solitary or located between sieve tube elements. Prior to the fast proliferation of intraxylary phloem cells, well established and discrete bundles of external phloem fibres separated by densely stained ray-like parenchyma cells were conspicuous in the transverse sections.

As primary growth proceeded, newly differentiated intraxylary phloem strands continually emerged in the first internode. At this stage, it was very common that no more than one to four diffuse sieve tube elements were embedded in the background parenchyma cells within intraxylary phloem bundles. However, there seemed to be no obvious relationship between the number and the distribution of sieve tube elements and the different sizes of phloem strands. At this early stage, the boundary between intraxylary phloem and primary xylem was not clearly delineated. It was impossible to distinguish the primary xylem parenchyma from the parenchyma cells in the intraxylary phloem strands.

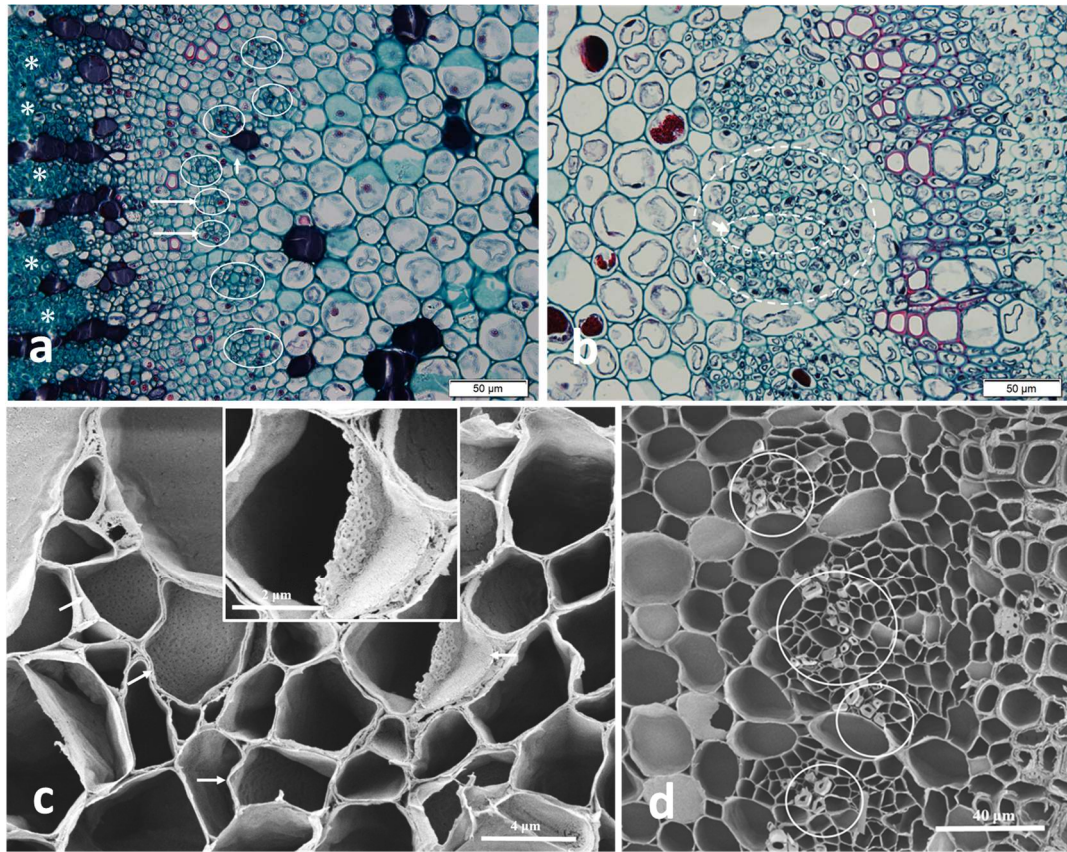


Figure 2.2. Cell division and proliferation of intraxylary phloem tissue.

a: Cross section of small strands of intraxylary phloem in the first internode. Long arrows: newly establishing intraxylary phloem strands; white circles: expanding intraxylary phloem strands; short arrow: division of a parenchyma cell; asterisks: external phloem fibres.

b: Cross section of intraxylary phloem in the second internode. Previously produced phloem strands have fused into a larger phloem strand (dotted circle). The arrow and dotted ellipse show the area where two previously produced phloem strands connect.

c: Cross section of intraxylary phloem in the first internode taken by FE-SEM shows sieve plates with pores surrounded by donut-like, possibly callose depositions (inset). Arrows: sieve tubes.

d: Cross section of intraxylary phloem in the third internode taken by FE-SEM. A discontinuous belt of sclerenchyma cells (circles) has appeared at the boundary between intraxylary phloem and pith.

2.3.3. Proliferation of intraxylary phloem

A more advanced stage of shoot growth, which was accompanied by the development of secondary xylem and IP, was observed in the second and the third internodes. Some previously formed intraxylary phloem strands fused and developed into large obvious phloem strands. In Figure 2.2b, one of the ray-like cells indicated by the arrow has just finished cell division. The other three ray-like cells (dotted ellipse in Figure 2.2b) were about to start cell division, resulting in the fusion of two phloem strands into one. Because of the cellular fusion, it was very common to find, in a more advanced stage of shoot growth, many sieve tubes in a single intraxylary phloem strand.

The number of fluorescence dots was also counted in the second internode where the secondary xylem had been formed before the development of IP. As is shown in Table 2.1, the average numbers of the dots in intraxylary and external phloem tissues

were 222 ± 36 /mm² and 41 ± 6 /mm² respectively. The average surface areas of intraxylary and external phloem tissues at this stage were 257, 223 μm^2 and 539, 803 μm^2 respectively (Table 2.2). The average number of the dots in the surface area of intraxylary phloem was 57, a figure two times more than that seen in the external phloem (Table 2.3).

A transverse section of the third internode taken by FE-SEM revealed that several discontinuous bundles of sclerenchyma cells were developed along the boundary between the pith and intraxylary phloem or inserted into the larger phloem strands, thus delimiting the integrated phloem area into small portions (Figure 2.2d). Depending on the size of phloem strands, these bundles occupied approximately one-fifth to one-fourth of the total intraxylary phloem area (Figures 2.2d and 2.3a). As intraxylary phloem development progressed, more sclerenchyma cells emerged in the adaxial part of the phloem strands. Individual intraxylary phloem strands (dotted rectangle in Figure 2.3a) were delimited by ray-like cells and belts of sclerenchyma cells.

Table 2.1. Densities of fluorescence dots / mm² in three phloem tissues located in three internodes.

No. of internodes	Intraxylary phloem	Interxylary phloem	External phloem
1 st internode	233 $\pm$ 37	—*	45 $\pm$ 11
2 nd internode	222 $\pm$ 36	—*	41 $\pm$ 6
11 th internode	246 $\pm$ 31	92 $\pm$ 10	16 $\pm$ 3

* Absence of interxylary phloem.

Table 2.2. Average surface areas of three phloem tissues (μm^2) located in three internodes.

No. of internodes	Intraxylary phloem	Interxylary phloem	External phloem
1 st internode	148, 452	—*	420,556
2 nd internode	257, 223	—*	539, 803
11 th internode	582,926	2,810,143	3, 864, 353

* Absence of interxylary phloem.

Table 2.3. Average numbers of fluorescence dots in the transverse sections of three phloem tissues.

No. of internodes	Intraxylary phloem	Interxylary phloem	External phloem
1 st internode	35	—*	19
2 nd internode	57	—*	22
11 th internode	143	259	62

* Absence of interxylary phloem.

2.3.4. Maturation of intraxylary phloem

Another significant observation regarding to young shoot tissue was the occurrence of large clusters of radially arranged sieve tubes that were composed of two to seven elements, which were continuously produced from the sixth to the fourteenth internodes (Figure 2.3a). It was noted that sieve plates stained with aniline blue were of two different shapes: circular and linear. The former corresponded to a frontal view of the plates (arrows in Figures 2.3a and 2.3b), while the latter corresponded to a lateral view (arrowheads in Figures 2.3a and 2.3b). In longitudinal section, sieve plates located in the end walls of sieve tubes were of the latter type, while those in the side walls of sieve tubes were of the former type (Figure 2.3b).

In the more mature internodes, sieve pores were wider, and even sieve pores larger than 0.5 μm were quite common. The proliferation of intraxylary phloem was gradually reduced after the development of secondary xylem, but the previously produced sieve tubes retained the structural features of conducting phloem (Angyalossy *et al.* 2016) from the first to the fourteenth internodes.

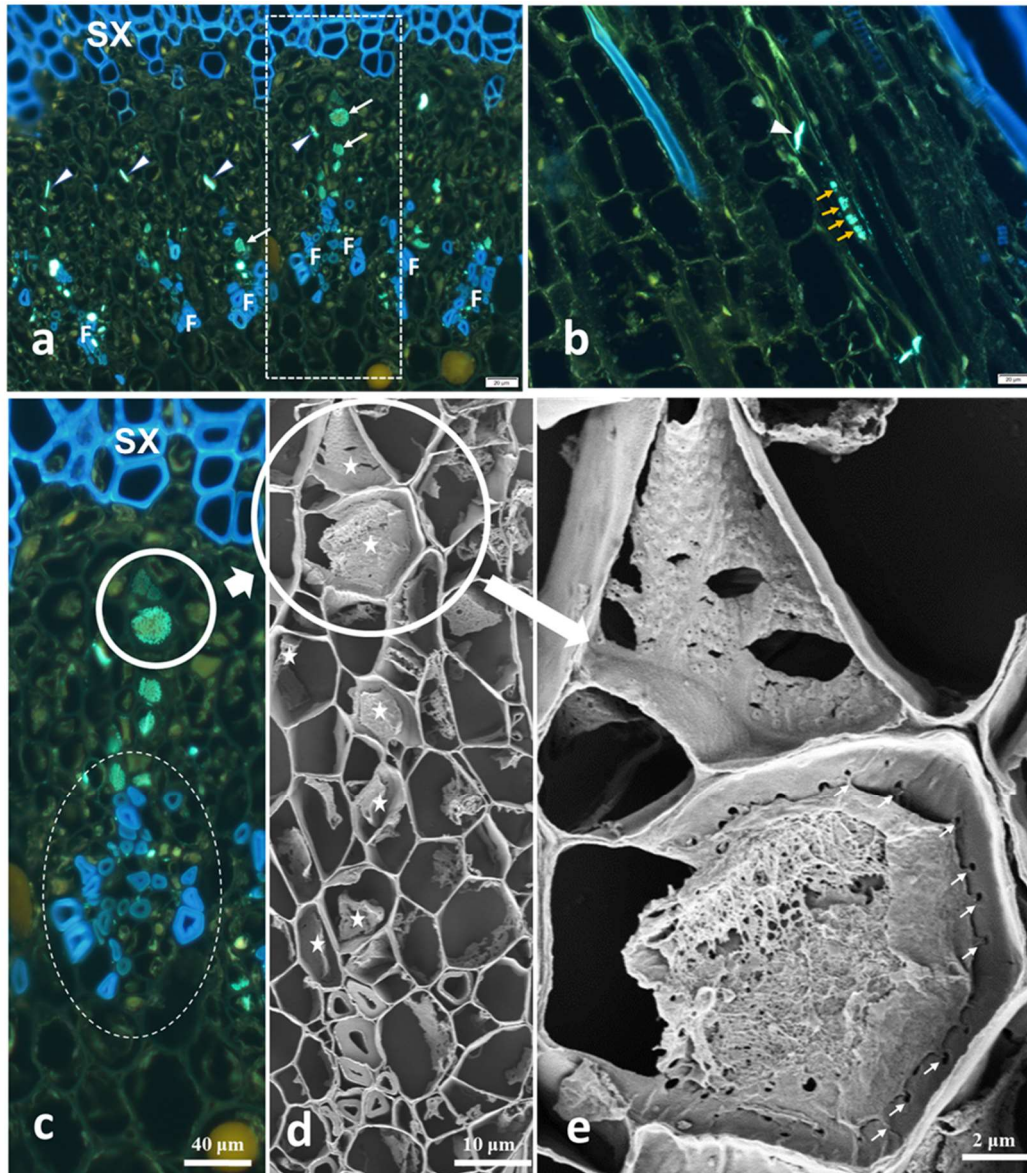


Figure 2.3. Characteristics of sieve tubes in intraxylary phloem of the sixth internode.
a: Cross section of aniline blue stained intraxylary phloem. Arrowheads and arrows point to sieve plates in cross-cut face and in frontal view. SX: secondary xylem; F: sclerenchyma fibres.
b: Radial section of intraxylary phloem. Arrowhead points to sieve plate in the cross-cut end wall of a sieve tube, while arrows indicate frontal view of sieve plates in the sieve tube sidewall.
c: Enlargement of the dotted square of a. The dotted ellipse outlines a large cluster of sclerenchyma fibres. SX: secondary xylem.
d: FE-SEM image taken at the same position as c. Stars correspond to the bright dots of sieve plates in c. The circle is an enlarged SEM image of that in c.
e: FE-SEM image with a higher magnification of the sieve plates shown in the circle of d. Arrows indicate tubular slimes passing through sieve pores.

Figure 2.3c is an enlargement of the dotted square in Figure 2.3a that demonstrated bright fluorescence of callose staining in a cluster of seven sieve plates. Figure 2.3d is a FE-SEM image of the same arrangement of the seven sieve plates indicated by stars. Two types of fluorescence, one strong and one weak, were emitted from two different sieve plates in the circle shown in Figure 2.3c and at a higher magnification in Figure 2.3d. When the two sieve plates were checked carefully, strong fluorescence was

emitted from thick sieve plates with distinct sieve pores connected or passed through by filamentous slimes as shown in the lower sieve plate in the circle of Figures 2.3c and 2.3d and enlarged in Figure 2.3e, while as seen in the upper side in the circle of Figure 2.3d, weak fluorescence was given off by a nascent sieve plate correspondingly shown in the circle of Figure 2.3c.

Large sieve tube clusters that included more than four elements per strand of intraxylary phloem were already very common in the fourteenth internode (dotted ellipses in Figure 2.4a). In contrast, callose staining in the external phloem in the same internode was still rarely observed (Figure 2.4b). When the area demarcated by the square was enlarged, typical but weak callose staining in two sieve tubes could be found (inset in Figure 2.4b).

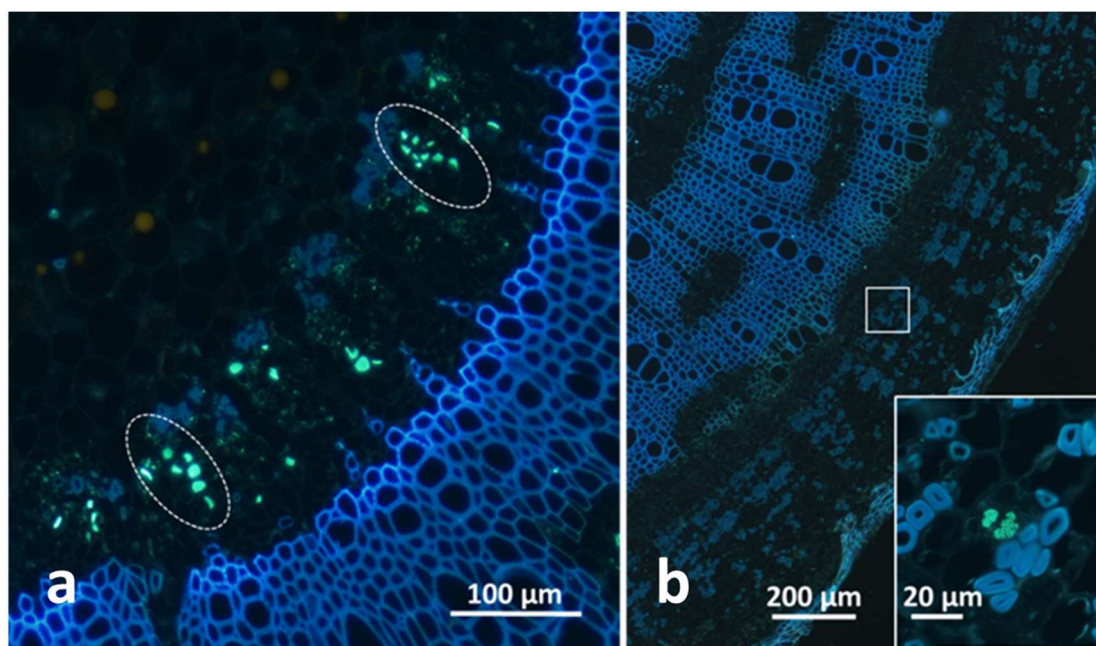


Figure 2.4. Aniline blue staining of intraxylary phloem and secondary phloem in the fourteenth internode reveals difference in the frequency of their sieve tubes.

a: Cross section of aniline blue stained intraxylary phloem. Large sieve tube clusters (dotted ellipses) containing six to eight sieve tube elements are very popular.

b: Cross section of aniline blue stained external phloem. Callose staining in sieve tubes is rarely observed except those in the inset (an enlargement of the square).

The number of fluorescence dots was counted to compare the quantity of sieve tubes in the intraxylary, IP and external phloem tissues in the eleventh internode, where the secondary xylem and multiple layers of IP strands were already formed. As was shown in Table 2.1, the average numbers of the dots in intraxylary, IP and external phloem tissues were $246 \pm 31 / \text{mm}^2$, $92 \pm 10 / \text{mm}^2$ and $16 \pm 3 / \text{mm}^2$ respectively. The average surface areas of intraxylary, IP and external phloem tissues in this stage were $582,926 \mu\text{m}^2$, $2,810,143 \mu\text{m}^2$ and $3,864,353 \mu\text{m}^2$ respectively (Table 2.2). The average number of the dots in the surface area of the intraxylary phloem was 143, a figure approximately two times more than that seen in the external phloem. However, the greatest number of fluorescence dots was still observed in the IP tissues (Table 2.3).

Even in the fourteenth internode, the innermost intraxylary phloem had not been

deformed or obliterated (Figure 2.5a) and sieve tubes in the distal part still presented callose deposition on sieve plates and open pores. Two sieve plates in Figure 2.5b, which was an enlargement of the square in Figure 2.5a, showed sieve pores of different sizes: some pores on the left plate were close to 1 μm in diameter, while those on the right plate mostly measured less than 0.1 μm . At the same time, the donut-shaped outer margins were also of different sizes. Most previously produced sieve tube elements in this internode were characterized by a heavy accumulation of callose.

Figure 2.5c shows an ultrathin longitudinal section of a sieve tube element, viewed with TEM. The sieve plate was mostly occluded with callose, which had lower contrast. The cell wall of the sieve plate was partially exposed, and the interrupted part of the cell wall ranged in thickness from 0.5 to 0.8 μm . Almost no distinct holes or pores were detected in the sieve plate except for one instance (arrow) and no slime bodies were passing through the sieve pores. This feature might suggest that the sieve tube was about to lose its functions for nutrients transportation and regulation.

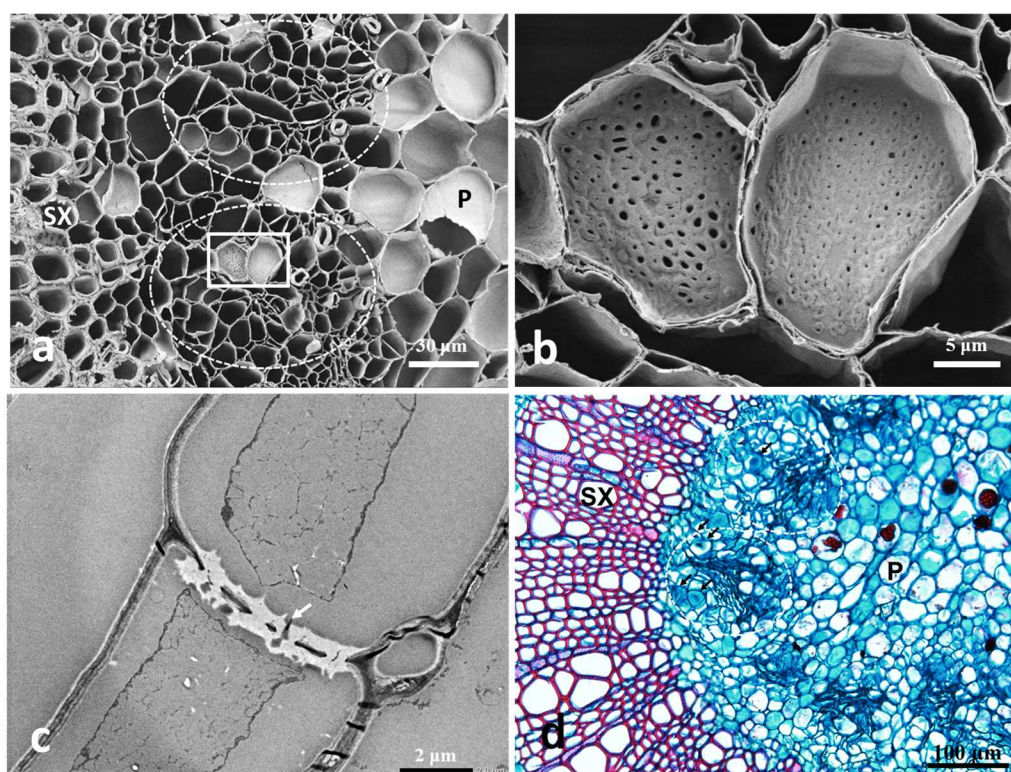


Figure 2.5. Characteristics of sieve tubes in intraxylary phloem of the fourteenth internode and small branches.

a: FE-SEM image of the fourteenth internode intraxylary phloem strands (dotted ellipses). SX: secondary xylem; P: pith.

b: Higher magnification of two sieve plates indicated by a square in a. Note that the size of pores is different between the two sieve plates.

c: TEM image of a sieve plate in intraxylary phloem of the fourteenth internode. Lower contrast area of the sieve plate is occluded with callose. A sieve pore is shown by a white arrow.

d: Cross section of intraxylary phloem (dotted ellipses) in the three-centimeter-in-diameter branch. Majority cells in the adaxial part of intraxylary phloem has been crushed. Black arrows: uncrushed sieve tubes. SX: secondary xylem; P: pith.

2.3.5. Partial senescence of intraxylary phloem

In the developmental study, the fourteenth internode was the oldest one with a diameter of approximately 4mm and no distinct crushing or deformation of intraxylary phloem was observed. Therefore, to analyze the structural changes of component cells during partial senescence of intraxylary phloem, the small branch with a diameter of three centimeters and 4~5 growth rings were checked to detect the deformed and crushed characteristics of sieve tube-companion cells (SE-CCs) groups in the phloem tissues.

A transverse section taken from a piece of the branch demonstrated the structural features of intraxylary phloem during partial senescence (Figures 2.5d and 2.6). Close to the adaxial end of intraxylary phloem strands, almost all the old intraxylary phloem cells, especially sieve tubes, were severely crushed, and sieve plates could not be seen. The sieve tubes close to the primary xylem were not crushed, but the sieve plates already appeared as a mesh-like structure. Above all, in the intraxylary phloem, majority of the sieve tubes have been deformed or crushed, but some were still turgid. Such intraxylary phloem seemed to be in a stage of partial senescence.

Figure 2.6a is a FE-SEM image that shows the crushed or deformed intraxylary phloem cells, as well as sieve tubes. In the outer side of the crushed area, two undeformed sieve tubes (arrowheads) were present. FE-SEM examination depicted the sieve plates of uncrushed sieve tubes as being occluded by dense materials. Old and unfunctional sieve tube elements with typically empty sieve pores were mostly collapsed (Figure 2.6b). The sizes of individual sieve pores were large, often over 1 μm . There appeared to be no deposition of callose. Although the structural characteristics of the sieve plates varied slightly, nearly all sieve plates were reticulated, with large and closely spaced empty pores.

The inset on the upper right of Figure 2.6c was a fluorescence microscopic image showing the bright fluorescence of callose staining on four sieve tubes (a–d) located inwards from the primary xylem, while Figure 2.6c is a FE-SEM image of the four similarly positioned sieve tubes, labeled A to D in the distal part of intraxylary phloem with smooth sieve plates covered with dense deposits. The entire sieve plates were occluded by thick deposits. Strong callose staining was detected in the dense materials of the sieve plates A to D. The sieve tube labeled E showed a mesh-like structure with empty sieve pores. The cell labeled e in the inset on the upper right of Figure 2.6c was in the same position as the sieve tube labelled E in Figure 2.6c. No callose staining in cell e was noted. Moreover, sieve tube A (inset on the upper left of Figure 2.6c) happened to show a cross-cut face, which was why, unlike those of the other sieve plates, its fluorescence microscopic image gave off a stronger brightness.

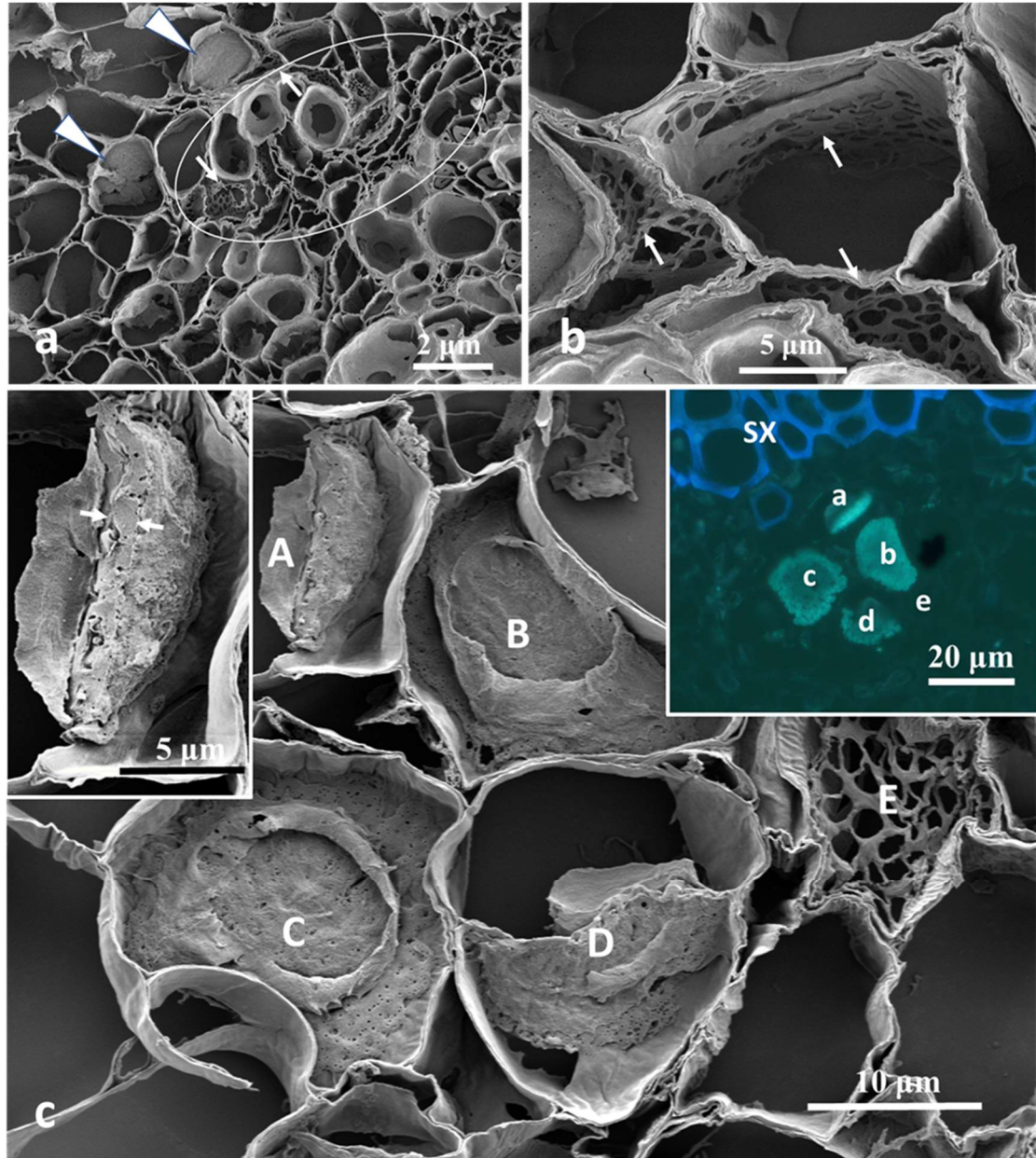


Figure 2.6. Characteristics of sieve tubes of intraxylary phloem during partial senescence.
a: Crushed part of intraxylary phloem in the branch (ellipse). Arrows: deformed sieve plates; arrowheads: undeformed sieve plates.
b: Higher magnification of adaxial part of the intraxylary phloem. Arrows: deformed and/or crushed sieve tubes with large sieve pores.
c: FE-SEM image of uncrushed sieve tubes in intraxylary phloem of the three-centimeter-in-diameter branch (A–E). Inset on the right of the figure shows five sieve tubes (a–e) after aniline blue staining, corresponding to A–E. SX: secondary xylem. Inset on the left of the figure is a higher magnification of the sieve tube A, showing the cross-cut face of its sieve plate. Two arrows show the thickness of the sieve plate. The sieve plate of E with large empty pores corresponds to e which does not show callose staining.

2.4. Discussion

Present study offered some new findings regarding the occurrence and development of intraxylary phloem in young shoots of *A. sinensis*. Intraxylary phloem was largely produced during primary growth (Evert 2006), while IP was produced during secondary growth. According to Carlquist (2013), intraxylary phloem, sometimes called internal phloem, might be a kind of “precursor” of IP that was formed in some species, and there appeared to be an intrinsic link between the occurrence of intraxylary and IP tissues in many families.

In the present study, a transition from intraxylary phloem to IP was not observed but demonstrated that intraxylary phloem was initiated by the division of parenchyma cells localized in the outer parts of the ground meristem. Moreover, after measuring the density of sieve tubes, the total surface areas of the intraxylary phloem, IP, and external phloem and calculating the total number of sieve tubes, it was found that before the differentiation of IP, there was a greater number of sieve tubes in intraxylary phloem than in external phloem. However, of all three types of phloem tissues, IP tissues were found to possess the greatest number of sieve tubes, once IP tissues were differentiated in the secondary xylem (Table 2.3). These observations suggested that, prior to the differentiation of IP, intraxylary phloem served as the predominant phloem for the various physiological functions of phloem tissue such as the translocation of photosynthates. After its differentiation, however, IP became the predominant phloem in *A. sinensis*.

2.4.1. Origin of intraxylary phloem

According to earlier studies, primary intraxylary phloem (internal protophloem) was developed from the procambial derivatives, while secondary intraxylary phloem might develop from an internal cambium (Patil *et al.* 2009; Sanghvi *et al.* 2011; Rajput & Gondaliya 2017). Occurrence of intraxylary phloem in the young shoots of *A. sinensis* did not match this pattern, because intraxylary phloem was originated from ground meristem and no obvious cambium was detected in the strands during their development. Patil and Rajput (2008) reported that the differentiation of internal phloem in the first internode of *Leptadenia reticulata* occurred prior to the differentiation of protoxylem. Furthermore, Patil *et al.* (2014) observed well established discrete strands of internal phloem in the third internode of *Solanum pseudocapsicum*, prior to the complete differentiation and lignification of protoxylem. In present observation, intraxylary phloem strands were differentiated from parenchyma cells in the ground meristem immediately following the maturation of primary xylem (Figures 2.1b and 2.2a). Parenchyma cell origin could also be found in other species and in other phloem types that developed from the redifferentiation of axial parenchyma cells (Rajput *et al.* 2008; Rajput *et al.* 2013; Moya *et al.* 2017).

In the case of *Rivea hypocriteriformis*, discrete strands of intraxylary phloem were observed in the fourth internode, but distinct sieve tubes and companion cells could only be discriminated after the seventh internode (Rajput 2016). Furthermore, in the

case of *Ipomoea hederifolia*, well developed internal protophloem appeared in the sixth internode (Patil *et al.* 2009). However, in the first internode of *A. sinensis*, while small clusters of intraxylary phloem soon appeared, differentiation of sieve tube elements occurred very early as well in primordially developing phloem strands. The number of sieve tube elements within each strand of intraxylary phloem ranged from one to four in the first internode. Developing phloem elements in more mature internodes also did not present regular patterns. Most of the linearly ordered phloem derivatives intertwined in a random manner, perhaps because of their parenchyma cell origin. These parenchyma cells could act as the sources for a continuous production of intraxylary phloem (Moya *et al.* 2017).

Based on aforementioned observations, sieve tube elements in intraxylary phloem of *A. sinensis* went through five successive structural changes until eventually losing their turgid shape and physiological function: 1) few accumulation of callose on sieve plates; 2) an increase in the size of sieve pores and the accumulation of callose around the pores; 3) amount accumulation of callose on sieve plates occluding the entire plates; followed by 4) the disappearance of almost all callose deposit; and 5) a total crushing or collapse of sieve plates and sieve tube elements.

In the early differentiating stage of intraxylary phloem, sieve tubes without fully opened sieve pores were often connected by filamentous slimes that ran through sieve pores from one sieve tube element to the next. 3D-SIM pictures of sieve tubes in tobacco resolved the fine strands as having a diameter of 103 ± 3 nm, extending to the tubular SER (sieve element reticulum), crossing sieve plate pores, and linking sieve tube elements structurally (Fitzgibbon *et al.* 2010). The observations provided further evidence for the existence of a link between sieve tube elements in the form of a membrane-like reticulum and filamentous slimes (Figure 2.1e). In this stage, aniline blue presented relatively weak and punctate staining of callose because of the small amount of deposition and the intervention from cell cytoplasm.

The corresponding FE-SEM and fluorescence microscopic pictures of the same sieve tube with tiny pores (Figures 2.3c, 2.3d and 2.3e) most likely depicted an earlier stage of its development. Callose was one of the important components of the cell plate, which arose during cell division (Pirsellova & Matusikova 2013). It has been reported that the incipient sieve plate was penetrated by densely distributed plasmodesmata that was surrounded by callose deposited beneath the plasma membrane. The callose deposition might play a vital role in preventing the further aggregation of cellulose around the plasmodesmata. (Xie & Hong 2011; Xie *et al.* 2011). After callose partially destruction, new sieve pores emerged (Barratt *et al.* 2011; Nedukha 2015).

2.4.2. Development of intraxylary phloem

As the number of internodes increasing, more sclerenchyma cells emerged in the adaxial part of intraxylary phloem strands. These densely distributed sclerenchyma cells might provide extra mechanical support to those enlarging intraxylary phloem strands. With the development of intraxylary phloem, the number of sieve tube elements in a single strand increased to seven or more, especially in the internodes that were

more mature than the sixth internode. Such fast development of sieve tube clusters in intraxylary phloem of *A. sinensis* could facilitate the rapid transport of photosynthetic products that were greatly needed during the fruiting season (Soehartono & Newton 2001; Le *et al.* 2005; Zhang *et al.* 2008). As their numbers increased, sieve tube elements in single intraxylary phloem strand frequently appeared in the form of tightly arranged lines or groups. An anastomosing sieve tube system could instantly compensate for the loss of transport capacity by providing alternative pathways composed of connecting sieve tube elements (Behnke 1998). This would be especially the case after wounding (Aloni & Peterson 1990).

Furthermore, in more mature internodes, sieve tube clusters were distinct (Figures 2.3a and 2.4a), and large sieve pores with diameter close to 1 μm were also very common (Figure 2.5b). These features might contribute to the highly efficient photosynthate conduction throughout the plant. This speculation was indirectly supported by means of the “preferential feeding by aphid” experiment in *Gomphocarpus*, which suggested that greater quantities of assimilates were transported in internal phloem than in external phloem (Botha *et al.* 1975).

During young shoots growth, the donut-like structure surrounding each sieve pore became distinct and thicker. A tapering end of cytoplasm, as seen under TEM (Figure 2.5c), and membranous strands, as seen under FE-SEM (Figures 2.1e and 2.3e), appeared to be connected or passing through sieve pores. The filamentous slimes that penetrated sieve pores and the membrane-like layer that attached to the slimes were well established during sieve tube development, which might be associated with the developing pores and further involved in the communication of signals or photosynthates through sieve plates and lateral sieve areas (Evert & Mierzwa 1989; Bird *et al.* 2003; Khan *et al.* 2007; Lee & Cui 2009).

Turnover of sieve pore callose in response to developmental and/or environmental factors was a significant feature of activated sieve tube elements, because alteration by means of three possible modifications in plasmodesmata apertures, in the sizes of the sieve pore transport pathways could modulate conductive characteristics (Zavaliev *et al.* 2011; Sevilem *et al.* 2013; Amsbury *et al.* 2018). In mature sieve tubes with open pores, areas of positive aniline blue staining looked brighter compared with the stained parts of sieve tube elements without fully open pores. Meanwhile, the existence of a relatively large quantity of callose on a sieve plate probably provided extra mechanical support to its intercellular connective parts (Galatis & Apostolakos 2010). Without a rigid lining, sieve pores might vary in size and, under pressure, might not be stable enough to maintain a sustainable flow (Barratt *et al.* 2011).

2.4.3. Partial senescence of intraxylary phloem

In all checked samples, crushed intraxylary phloem cells were detected in branches of three-centimeter diameter. Cells in the adaxial parts of intraxylary phloem strands lost their inflated shape, but some sieve tube elements with open sieve pores could still be found in the distal parts at this stage. As previously formed intraxylary phloem became unfunctional, phloem strands, mainly composed of sieve tube elements and

parenchyma cells, lost their turgidity, started to be crushed and obliterated. The pressure causing crushing was considered to arise from the development of new phloem elements as suggested by Sanghvi *et al.* (2011), or from the enlargement of parenchyma cells in the nonconductive phloem as suggested by Terrazas *et al.* (2011). A similar process has been found in *Leptadenia reticulata*, in which intraxylary protophloem tissues collapsed due to an increase in the number of adjacent parenchyma cells caused by periclinal division (Patil & Rajput 2008). Nevertheless, in some species with internal cambium, previously formed sieve tube elements already showed thick callose deposition or began to collapse prior to the origin of internal cambium (Patil *et al.* 2014).

As a sign of cell growth, the quantity of callose deposition also fluctuated with the change in the biological activity of sieve tube elements (Krabel *et al.* 1993; Aloni & Peterson 1997; Nedukha 2015). In Bignoniaceae, the first sign of function loss appeared as a loss of protoplast in the companion cells, followed by an accumulation of definitive callose on the sieve plates (Pace *et al.* 2015). Sieve tubes in the distal part of intraxylary phloem in the branch of *A. sinensis* showed smooth plates with depositions occluding almost all the sieve pores. Because the study was able to juxtapose the FE-SEM sieve tube images with the fluorescence microscopic images of aniline blue stained (Figure 2.6c), it was reasonable to state that the depositions most likely consisted of callose. The observation was consistent with the results of many previous studies (Patil & Rajput 2008; Rajput *et al.* 2009; Patil *et al.* 2011; Patil *et al.* 2014; Rajput 2016; Moya *et al.* 2017), and might indicate that sieve tubes became decrepit and gradually lost their transport function.

Furthermore, partially senescent sieve tubes in the inside part of intraxylary phloem strands had either a heavy accumulation of callose or nearly empty sieve pores. Both were important features of decrepit sieve tubes, implying that they were deprived of the ability to adjust their transport channels due to the degenerated structure of sieve plates. The absence of callose in unfunctional sieve tubes and the sealing of sieve plates by heavy accumulation of callose could be observed in other species as well (Patil *et al.* 2011). Diminishment of nearly all the callose deposition, followed by the deformation and collapse of sieve plates and sieve tubes were clearly seen in *A. sinensis*. The same features have been reported during the senescence of intraxylary phloem strands in *Coccinia indica* (Patil *et al.* 2011).

In contrast to the cellulose which could combine into highly crystalline structures, callose is disordered making up amorphous helical structures (Wang *et al.* 2016; Przekora *et al.* 2016), but it has been proposed that like cellulose, callose could play a mechanical role in cells because of its gelling properties (Parre & Geitmann 2005), helping them to cope with tension and contraction (Galatis & Apostolakis 2010; Nedukha 2015). The observation of *Solanum chacoense* pollen tubes proved that dissolution of callose lead to a decrease in cell wall stiffness (Parre & Geitmann 2005). Consequently, the complete collapse of unfunctional sieve tubes might well result from callose destruction, which lowered the cell's resistance to the pressure exerted by later added phloem derivatives (Patil & Rajput 2008; Rajput *et al.* 2009; Sanghvi *et al.* 2011).

2.5. Summary

Agarwood-producing species such as *Aquilaria* spp. and *Gyrinops* spp. (Thymelaeaceae) produce IP in their secondary xylem and intraxylary phloem at the periphery of the pith, facing the primary xylem. In this part, young shoots of *A. sinensis* were selected to characterize the development and structure of their intraxylary phloem.

It was initiated by the division of parenchyma cells localized in the outer parts of pith, immediately following the maturation of the first formed primary xylem. Its nascent sieve plates bore donut-like structures, the individual pores of which were so small (less than 0.1 μm) that they were hardly visible under FE-SEM.

Intraxylary phloem developed into mature tissue by means of the continuous division and proliferation of parenchyma cells. During the shoots' active growth period, the sieve pore sizes were 0.1~1 μm , with filamentous slimes passing through them. In the maturation stage, large clusters of sieve tubes continued to be differentiated in the intraxylary phloem.

In the partial senescence stage observed in a three-centimeter-diameter branch, intraxylary phloem cells in the adaxial part became crushed, and partial sieve plates had pores over 1~2 μm in diameter without any callose deposition.

Before the differentiation of IP in the first and the second internodes, callose staining detected more than twice as many sieve tubes in intraxylary phloem than in external phloem. However, after differentiation of IP in the eleventh internode, more sieve tubes were found in IP than in intraxylary and external phloem. This suggests that prior to the initiation of IP, intraxylary phloem acts as the principal phloem. After its differentiation, IP takes over the role of principal phloem. IP thus acts as the predominant phloem in the translocation of photosynthates in *A. sinensis*.

Chapter 3 Structure, origin and development of interxylary phloem

3.1. Introduction

A perusal of the literature indicates that interxylary phloem (IP) can be seen in various families of eudicots (Chalk & Chattaway 1937; van Veenendaal & den Outer 1993; den Outer & van Veenendaal 1995; Inside Wood 2004-onwards; Patil & Rajput 2008; Rajput *et al.* 2009; 2013; Carlquist 1930; 2010; 2013; Angyalossy *et al.* 2016). Among the many species that possess IP, any species of *Aquilaria* and *Gyrinops* is notable, because it can produce scented wood and be economically valuable (Lee & Mohamed 2016; Wang *et al.* 2021). Based on aforesaid advantages, it has become a target of illegal logging and, as a result, has been CITES-listed and its import and export are regulated (Gasson *et al.* 2011).

Until now, the studies of IP have mainly focused on descriptive anatomy, characterizing the differences in the morphology and distribution of IP among genera or species (Gasson 2011; Rao & Dayal 1992), or clarifying the relationship between IP and aromatic resin deposition (Mohamed *et al.* 2013; Siburian *et al.* 2013; Faizal *et al.* 2017). Moreover, the descriptions of IP are mostly based on optical microscopic sections, with rare information on the separated phloem bundles and their constituent cells. Any data pertaining to the length of IP strands has not been reported in previous literature. In addition, according to Angyalossy *et al.* (2016), IP may be produced in three different ways. The IP of agarwood-producing species belongs to the diffuse type, but the details of its origination and differentiation process, as well as its structural changes during aging, have not been fully analyzed.

In view of the tight relationship between IP of agarwood-producing species and their response to exogenous stimuli, the production of aromatic resins, elucidating the differences between IP and common secondary phloem, understanding the physiological functions of IP, as suggested by Carlquist (2013), are promising topics for exploration. Staining the IP and the external phloem with fluorescent aniline blue which specifically stains callose (Aloni *et al.* 1990; Aloni & Peterson 1997), and combining the observations of fluorescence microscopy with SEM were proved to be efficient in detecting the positions of sieve tubes. Furthermore, it has been suggested that the presence of callose is indicative of functioning sieve tubes (Aloni & Peterson 1991), which would provide convincing evidence for elaborating the contributions of different phloem tissues in *Aquilaria*.

3.2. Materials and Methods

3.2.1. Materials

Wood blocks with bark attached were collected in May, July, and September during the growth season from sixteen-year-old *Aquilaria sinensis* trees (30~35 cm in diameter at breast height) growing in Xianhu Botanical Garden, Shengzheng city, Guangdong province, China. As shown in Figure 3.1, another five wood blocks ranging from bark to pith were collected from a ten-year-old *A. sinensis* tree growing in a plantation in Zhongshan city, Guangdong province, China. They were taken in April from a branch wood 1.5 m above ground level. These wood blocks with 1 cm² in square and 1.5 cm in length, were fixed in formalin-aceto-alcohol (FAA) solution or 4 % formalin immediately after having been collected from the living trees.

3.2.2. Optical and fluorescence microscopic observation

After fixation, the wood blocks were washed with deionized water and transferred to a gradient concentration of PEG (MW 2000 or MW 1500). Cross, radial, and tangential sections of 15 µm in thickness were cut with a microtome from the PEG-treated wood blocks and stained with 1 % safranin followed by 0.5 % fast green or astra blue. Permanent slides were made after series dehydration and mounting in synthetic resin (Bioleite, Okenshoji Co Ltd., Japan), then observed under a bright field microscope to investigate the characteristics and development of IP.

Furthermore, cross and radial sections obtained from wood blocks that included bark and cambium were stained with 0.005 % aniline blue aqueous solution for 30 min at room temperature. The sections were observed under a fluorescence microscope (Olympus Co Ltd., BX51 or Nikon Co. Ltd. Eclipse 80i) to detect the presence of callose (Currier & Strugger 1956).

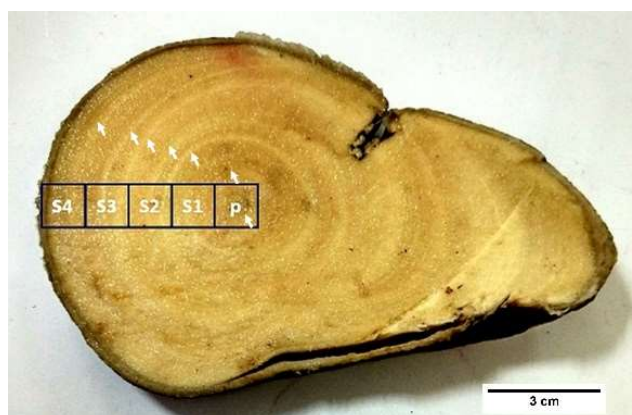


Figure 3.1. Five wood blocks from pith to bark were selected from this trunk for double staining and callose detecting. Seven growth rings can be observed in the disc (arrows). P: pith; S4: wood block with bark attached and the latest xylem tissue; S1, S2, S3: three wood blocks between “P” and “S4”.

3.2.3. Maceration of interxylary phloem and bark tissue

IP strands were isolated from the secondary xylem by means of the Franklin maceration method (Franklin 1945). The size of each specimen selected for maceration was approximately 3 mm × 3 mm square and 5~7 cm long. To remove air bubbles, the specimens were put in a test tube which was filled with water, heated to 100 °C, and boiled. Subsequently, the boiled water was replaced with a mixture of glacial acetic acid and 30 % hydrogen peroxide (1:1 v/v). The test tube, containing specimens and solution mixture, was warmed in a water bath at 100 °C for 45~60 min. The test tube was then cooled and centrifuged at 5000 rpm. The supernatant was replaced with deionized water, and the test tube was centrifuged again. The supernatant was removed by means of a pipette, a small amount of deionized water was added, and then all the contents of the test tube were transferred to a petri dish. The IP strands were carefully picked out. The process was repeated more than ten times to collect enough IP strands for further testing. Several IP strands were further macerated to separate their constitutive elements.

Bark tissue was macerated following the same process to obtain dimensional details of constitutive elements and to determine the presence or absence of sieve tube in the external phloem. In the case of bark tissue, the bark sample was reduced in advance to small pieces: freshly peeled bark was cut by a double-edged razor blade into three tangentially contiguous specimens 1 mm wide and 5 mm long.

After maceration, ten to twenty slides of IP and external phloem were prepared and were scrutinized under bright field microscopy.

3.2.4. SEM observation

Cross and radial sections were put on glass slides and dehydrated by pipetting with a series of alcohol concentrations, followed by transfer into 100 % *t*-butyl alcohol. The sections were overlaid with cover glass and dried at room temperature or in an oven at 60 °C. Dried sections were placed on a specimen carrier commonly used for SEM observation and coated with gold by means of an ion sputtering apparatus (Hitachi Co Ltd., E-1010). Cross and radial sections were observed by SEM (Hitachi Co. Ltd., TM-1000 and Hitachi Co. Ltd., S-3000N) to investigate the component cells of IP and the difference in cell wall thickness between secondary xylem and IP.

In order to confirm the presence or absence of sieve tube in bark tissue, the most well-preserved PEG-treated wood blocks showing no crushing or deformation of cambium were selected for section preparation. A total of twenty serial cross sections with a thickness of 15 µm were made and sputter coated with gold after drying following the same process for SEM observation.

3.3. Results

3.3.1. General characteristics of interxylary phloem

The average dimension of fifty IP strands in cross section was $481 \pm 225 \mu\text{m}$ in tangential diameter and $139 \pm 21 \mu\text{m}$ in radial diameter. IP strands were sometimes tangentially elongated in cross section (up to 1.5 mm), suggesting the merging of individual IP strands in tangential networks. Very small IP strands grouped in a cluster were rarely observed, except for the annual rings near pith as seen in the ellipses of Figure 3.2.

The IP strands were composed of fusiform parenchyma cells, strand parenchyma cells, axial sieve tubes, companion cells, fibres, and ray parenchyma cells. The presence of large elongated crystals (styloids) was always observed in the axial parenchyma cells. Apart from fibres, other elements of mature IP in *A. sinensis* demonstrated unlignified after double staining with safranin, fast green or astra blue. The cross-sectional SEM image clearly shows that the walls of IP cells were thinner than those of the surrounding xylem tissues. Especially when continuous ray tissues passed through IP strands, significant fluctuation in cell wall thickness of ray elements was usually very distinct (arrows in Figure 3.3).

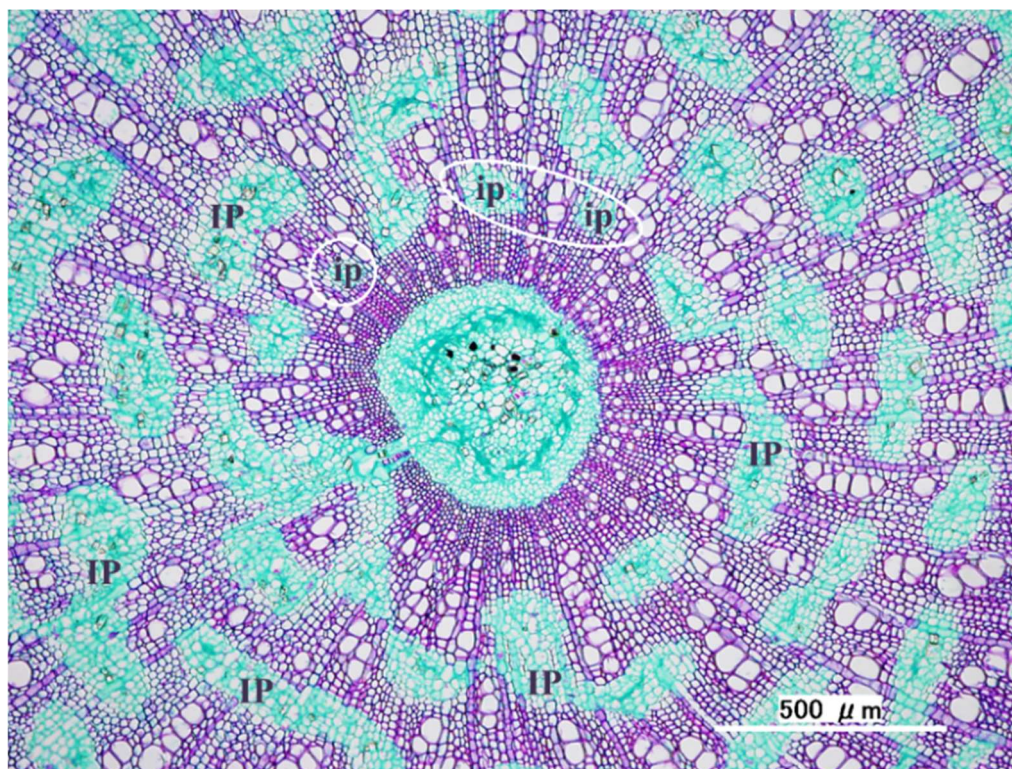


Figure 3.2. Distribution of common and small interxylary phloem strands. IP: common interxylary phloem; ip: small interxylary phloem.

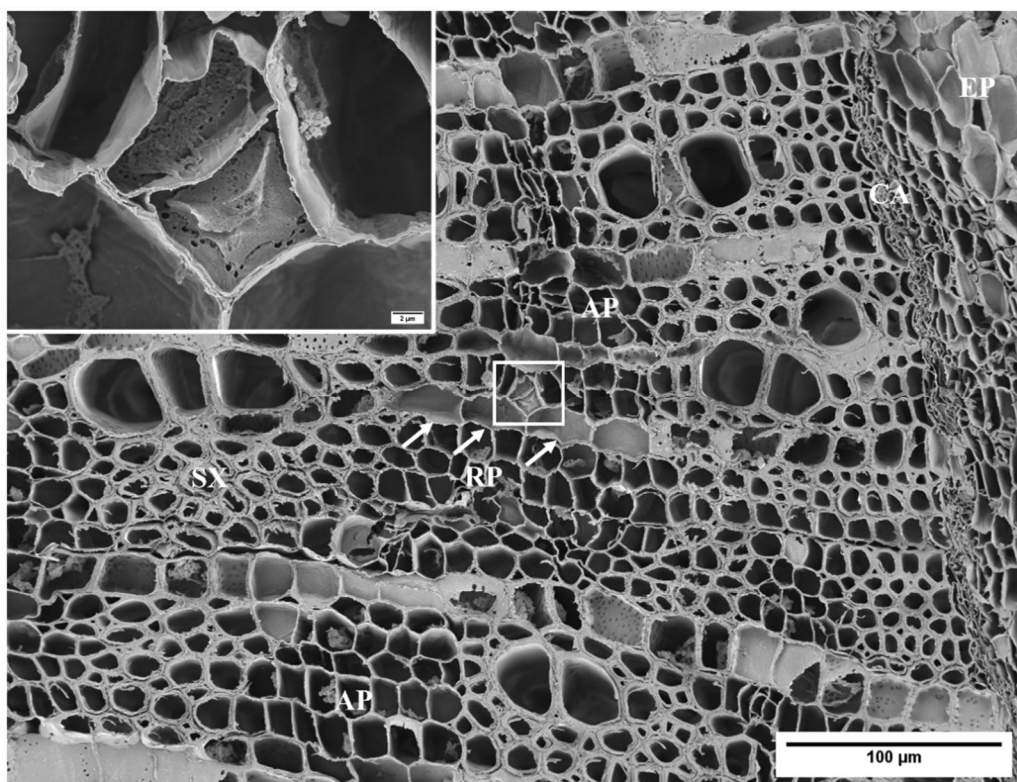


Figure 3.3. SEM image shows a cross-sectional view of secondary xylem and interxylary phloem in *A. sinensis*. AP: axial parenchyma cells in IP; EP: external phloem; SX: secondary xylem; arrows and RP: thin-walled ray parenchyma cells passing IP; square and insert: sieve tubes.

Axial sieve tubes were mainly located in the island like IP strands and several of them were often closely arranged along the tangential direction. Diameter of mature sieve tube was mostly about 20~35 μm , based on SEM observation. Their length varied from 90 μm to more than 300 μm . However, axial sieve tubes with a length of 150~250 μm accounted for majority of the samples examined. It should be noted that some of sieve tubes connected with radial sieve tubes had a large inclination to vertical direction. Since the angle between these sieve tubes and vertical direction was about 45°, it was difficult to define them accurately whether they were axial or radial sieve tubes. They were usually shorter in length, even shorter than the typical radial sieve tubes, therefore the description of the length of axial sieve tube mentioned here did not include such highly inclined elements.

Axial sieve tubes were connected by the sieve plates at their ends to form long distance sieve tube strands in IP tissues. In many cases, they showed simple sieve plates, and sometimes compound sieve plates (Figure 3.4). The angles between simple sieve plates and cell axis were usually larger, in other words, simple sieve plates were closer to the horizontal direction (Figures 3.4a and 3.4b), while compound sieve plate usually had a very steep angle with cell axis (Figures 3.4c and 3.4d). Two or more companion cells were found lying along the sieve tube, but their distribution did not follow a certain pattern (Figure 3.4a). One to several fibres appeared close to the sieve tube-companion cells (SE-CCs) functional groups, which were furtherly enclosed by several layers of unligified parenchyma cells.

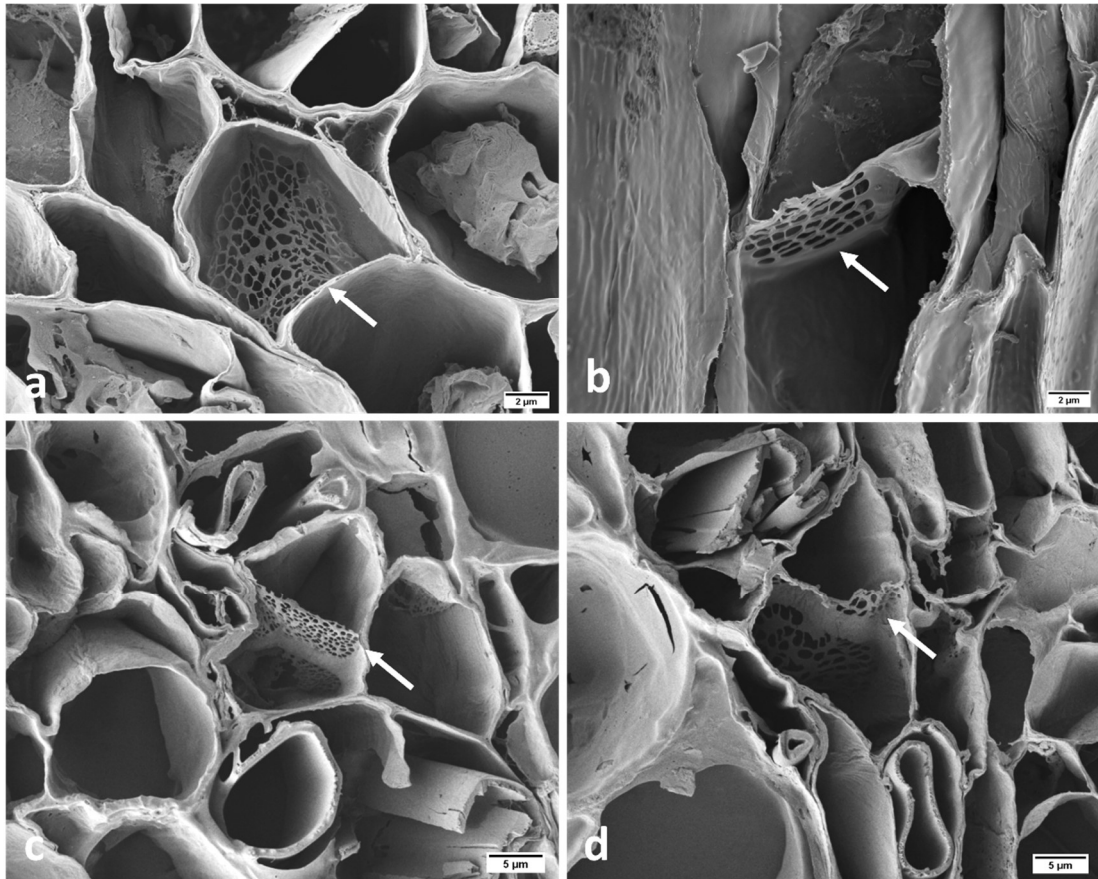


Figure 3.4. SEM images show simple and compound sieve plates of axial sieve tubes in interxylary phloem. a, c, d are transverse sectional images and b is longitudinal sectional image. a and b show front and side view of simple sieve plates. Both are almost perpendicular to the cell axis (arrows). c and d show compound sieve plates with very steep angles to the cell axis (arrows).

3.3.2. Origin and development of interxylary phloem

The origin and development of IP was studied in cross sections from more than twenty wood blocks collected during the active growth season. The development of IP was classified into five different stages as follows:

1) Inward phloem formation of vascular cambium was initiated by the local broadening of the cambial zone (stage 1). In this stage, differentiation of component cells of IP was not yet initiated. (Figure 3.5a).

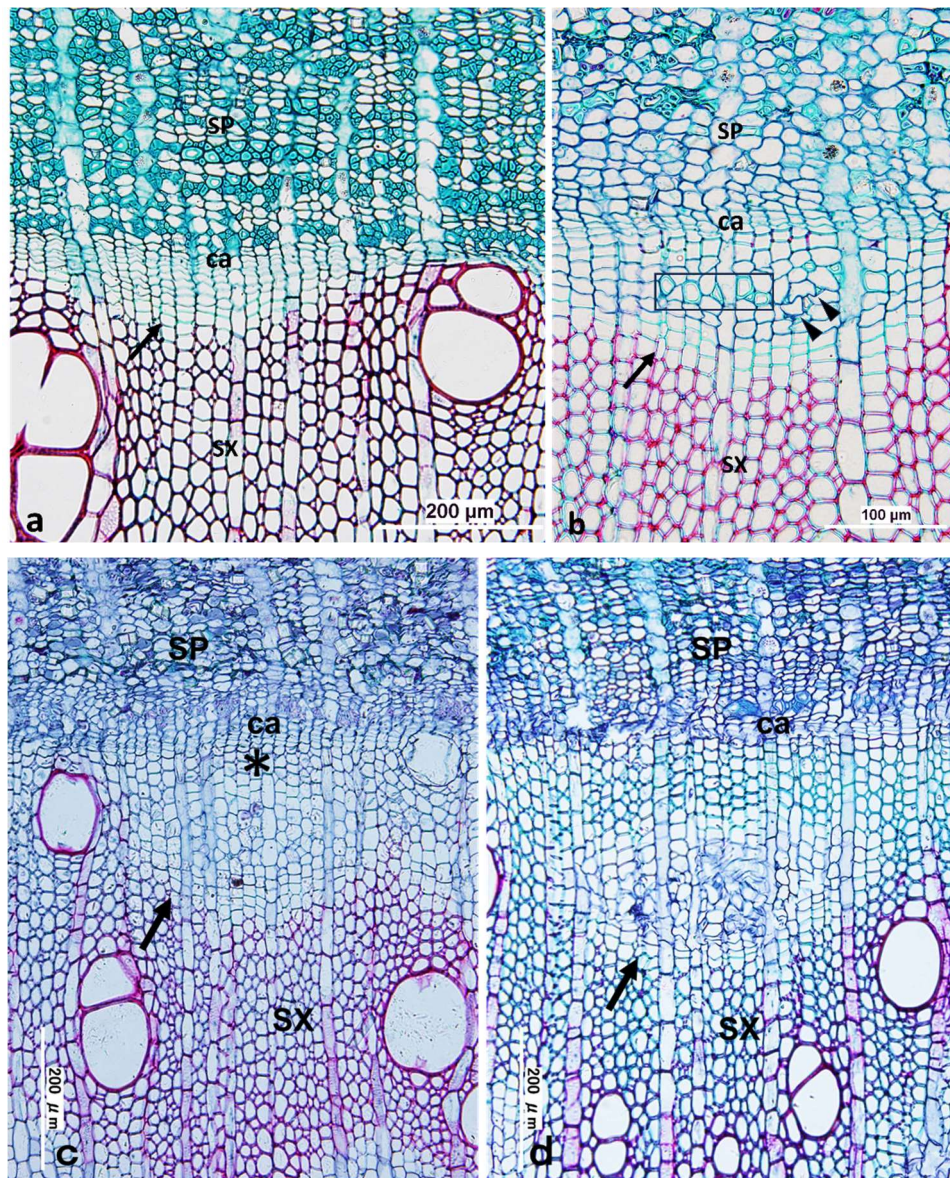
2) As inward growth of immature cambial derivatives continued, differentiation of fibres and sieve tubes became distinct (rectangle and arrowheads in Figure 3.5b). The ordered radial seriation of the component cells in center of immature IP was partially disturbed by fibre and sieve tube differentiation, which took place earlier than that of parenchyma cells. Subsequently, the general shape of IP and the radial seriation of its parenchyma cells were completed during this stage.

3) IP was constricted as immature xylem cells encroached between the cambium and immature IP (Figure 3.5c). IP was just separated from the cambium and became more mature with the SE-CCs groups easily distinguished from the surrounding cells.

The xylem and IP rays remained continuous.

4) IP completely detached from the vascular cambium (Figure 3.5d). In this stage, IP was fully surrounded by immature xylem cells, the walls of which stained positive by fast green. The cell walls of IP fibres were distinct and showed secondary wall thickening.

5) IP was surrounded by thick-walled, lignified xylem tissues and the fibres within IP developed distinctly thick walls. (Figure 3.5e).



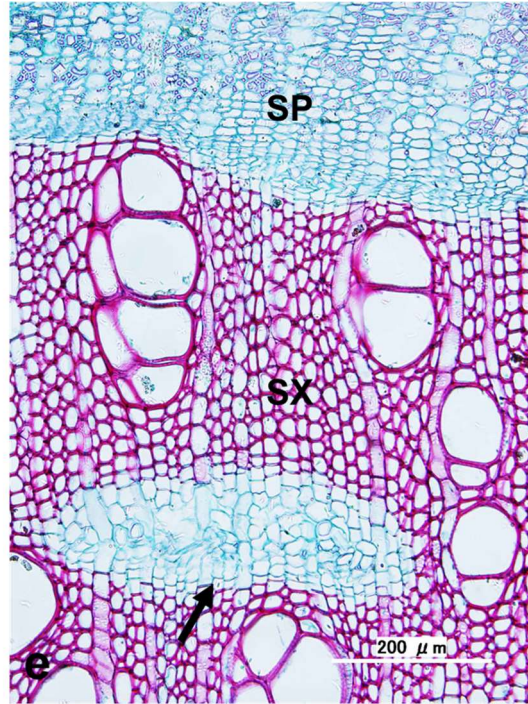


Figure 3.5. Developmental stages (1 to 5) of interxylary phloem. ca: cambium; SP: secondary phloem; SX: secondary xylem; arrows: IP strands; arrowheads: SE-CCs groups; rectangle: fibers; asterisk: the area between cambium and immature IP.

- a: Stage 1. Initiation of inward phloem formation of partial cambial zone.
- b: Stage 2. Differentiation of fibres and sieve tubes in immature IP.
- c: Stage 3. IP is constricted at a point between the cambium and immature IP (asterisk).
- d: Stage 4. IP detaches from the cambium.
- e: Stage 5. IP is surrounded by lignified xylem tissues.

After maturation of IP in stage 5 as described above, further structural changes in IP were studied from the outermost ring to the pith (cf. Figure 3.1). IP strands in both blocks S4 and S3 showed fully expanded cells without any crushing of phloem tissues (Figure 3.6a). However, obliteration of phloem tissues was initiated in the outer part of block S2 (arrows in Figure 3.6b). IP strands in the inner part of block S2 already showed crushed phloem. Crushed phloem tissues were more distinct in the IP strands of both blocks S1 and pith (Figure 3.6c). The observation indicated that the crushing of phloem tissues was initiated in the fourth growth ring from the bark, which was between the third and the fourth growth ring boundaries in Figure 3.1.

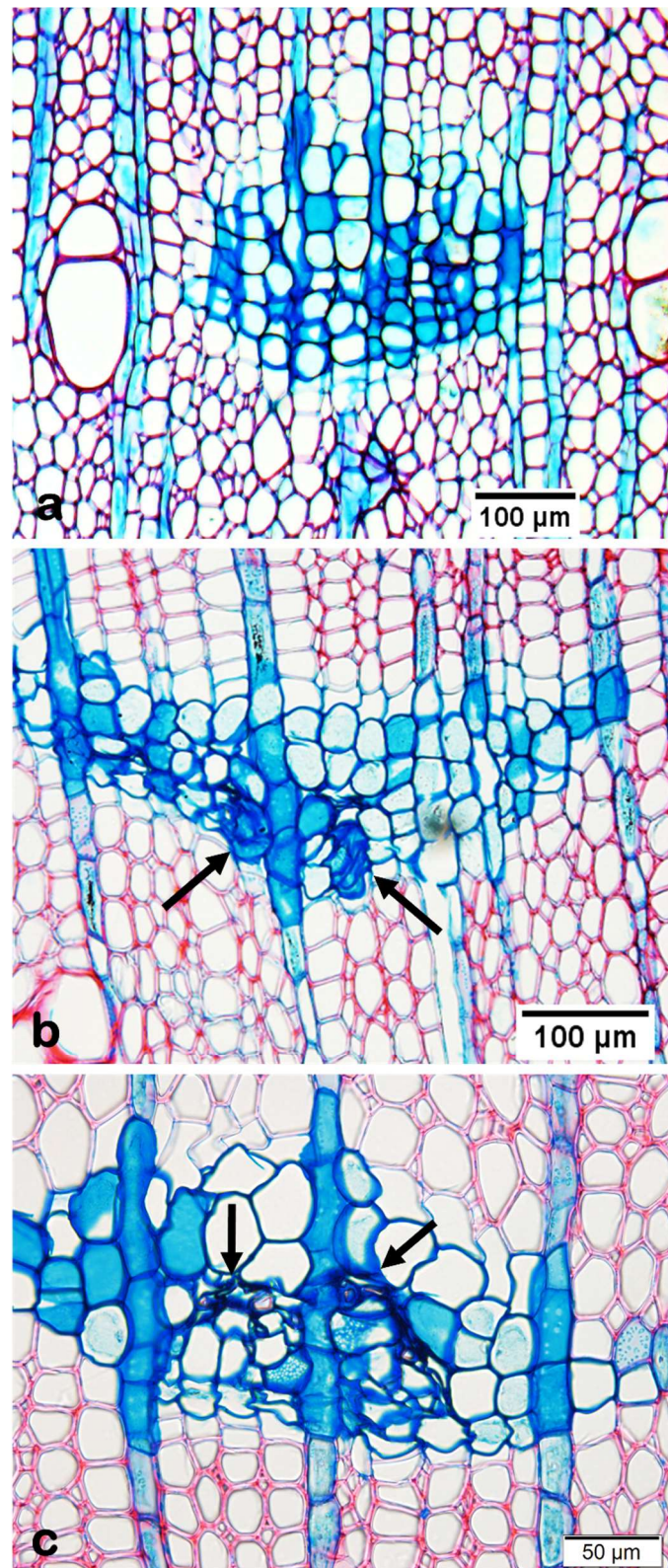


Figure 3.6. Structural changes of IP strands in different wood blocks near pith, S2, and S3 (cf. Figure 3.1).

a: Intact IP strand in block S3.

b: Partial obliteration of IP strand in S2 (arrows).

c: Obviously crushed IP strand near pith (arrows).

3.3.3. Constituent cells of interxylary phloem and bark tissue

Immediately after maceration, many xylem fibres could not separate from IP strands but attach themselves around the strands, as shown in Figure 3.7a. When the test tube was gently shaken before collecting IP strands, most xylem fibres detached from the IP strands, while the component cells of IP remained connected (Figures 3.7b and 3.7c). Two to three layers of fusiform parenchyma cells constituted the periphery of the IP strand. The strand parenchyma cells were thin-walled, short and intermixed with sieve tubes and/or IP fibres. In a magnified image of IP, a bundle of sclerotic fibres was observed in the center of IP attached with strand parenchyma cells, which contained large crystals (Figure 3.7c).

The average length and width of fifty IP strands was measured as 14 ± 4 mm and 417 ± 124 μm respectively. Further maceration of IP strands left component cells separated or partially separated, and it was useful for characterizing the cellular component of IP: axial fusiform and strand parenchyma cells, sieve tubes, companion cells, fibres, and ray parenchyma cells. The length and width of individual component cells were shown in Table 3.1.

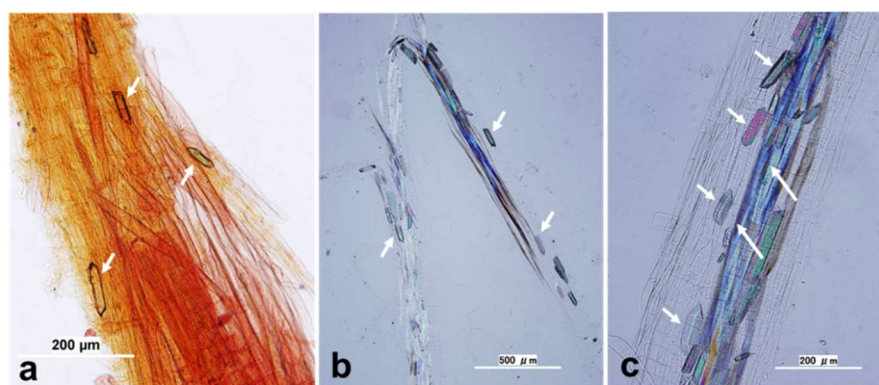


Figure 3.7. Interxylary phloem strands isolated by maceration.

a: A part of IP before washing with deionized water. Note many xylem fibers attach to IP; b and c: Photos show IP strands taken under weak polarized light in crossed Nicol with $1/4 \lambda$ plate after washing with deionized water. b: Part of isolated IP. c: Enlarged image of IP shows large crystals (short arrows) and sclerotic fibres (long arrows).

Table 3.1. Cell types and their dimensions of interxylary phloem

Cell Type	Length range (μm)	Average length $\pm$ SD (μm)	Width range (μm)	Average width $\pm$ SD (μm)
Sieve tube	125.4–361.0	215.7 ± 57.2	16.8–44.8	29.0 ± 5.3
Fusiform parenchyma cell	235.9–506.9	347.0 ± 63.0	18.9–37.0	25.8 ± 4.0
Strand parenchyma cell	58.5–141.2	85.6 ± 19.2	15.2–37.7	23.3 ± 6.3
Fibre	617.7–1755.9	1106.1 ± 250.5	12.7–35.0	22.0 ± 5.2

After maceration, IP sieve tubes often appeared in the form of a long chain. Individual sieve tube was connected to each other by sieve plates (Figure 3.8). Sieve tubes were surrounded by multiple layers of fusiform and strand parenchyma cells, and the average length of sieve tubes was shorter than that of fusiform parenchyma cells but longer than strand parenchyma cells (Table 3.1).

Bark tissue was also macerated to investigate its cellular composition and dimension, especially to detect whether sieve tube could be found in the external secondary phloem. External phloem and xylem fibres were $4185 \pm 451 \mu\text{m}$, $783 \pm 95 \mu\text{m}$ in length and $18 \pm 3 \mu\text{m}$, $33 \pm 5 \mu\text{m}$ in width, respectively. Of all the fibres, the external phloem fibres were by far the longest, while xylem fibres were the shortest, and IP fibres were of intermediate length. Xylem fibres were the widest, while external phloem fibres were the narrowest. The extremely long external phloem fibres were very thick-walled (Figure 3.9) with gradual tapers from the relatively wide bodies towards the tips. The average length of the wide-lumened bodies of the fibres was $520 \mu\text{m}$, about one eighth of the total fibre length. Appropriate ratio of length to width was one of the features that made the secondary phloem of *A. sinensis* a potential material for papermaking. However, based on the results of maceration, the presence of sieve tube was rarely detected in the secondary phloem tissues of all examined samples.

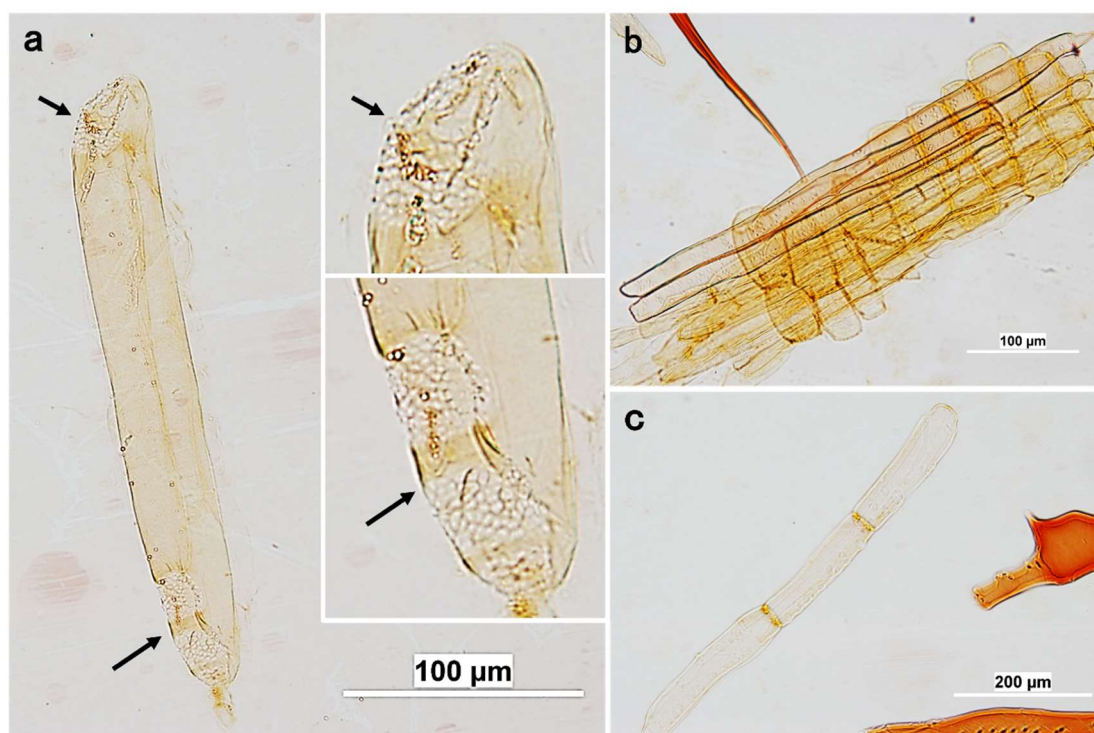


Figure 3.8. Sieve tube and parenchyma cells of IP after maceration.

- a: A sieve tube element separated from IP. Insets are higher magnification of sieve plates with assembled sieve pores at the end walls of the sieve tube element.
- b: Bundle of fusiform parenchyma cells attaches with ray parenchyma cells separated from IP.
- c: Single parenchyma cell strand separated from IP.

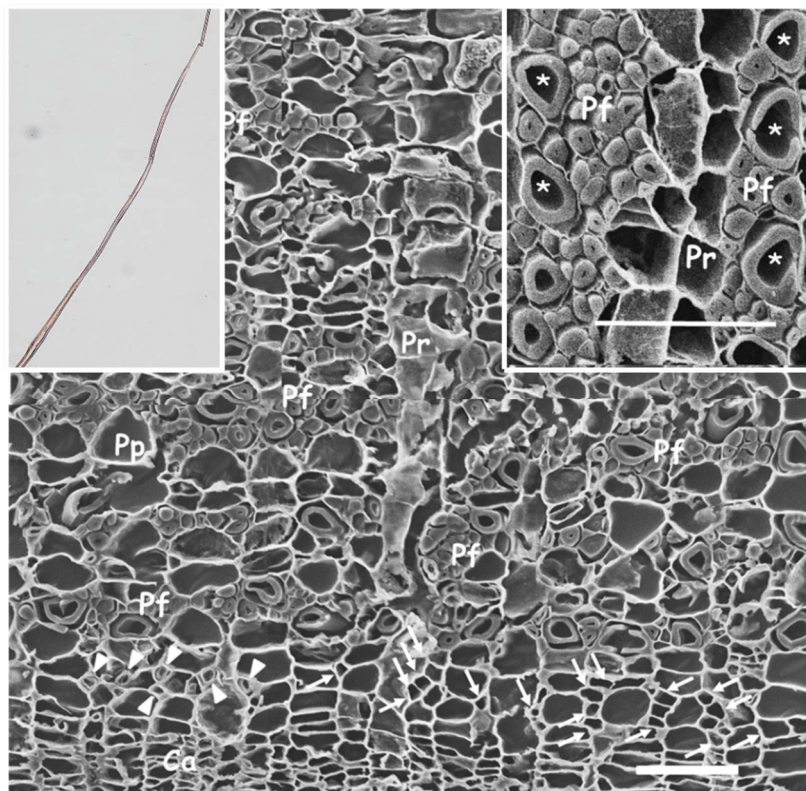


Figure 3.9. Composite SEM image shows a transverse section of cambium and external phloem in *A. sinensis*. Arrows: differentiating phloem fibres; arrowheads: immature phloem fibres; Ca: cambium; Pf: phloem fibres; Pp: phloem parenchyma cells; Pr: phloem ray cells; scale bar = 50 μ m. Upper left corner inset: macerated fiber; Upper right corner inset: higher magnification of phloem fibres; asterisk: phloem fibres with wide cell lumen; scale bar = 50 μ m.

3.3.4. Callose detection by aniline blue staining

Callose localized on sieve plates could be detected by aniline blue staining, and a positive reaction for callose indicated the presence of sieve tubes. In Figure 3.10, the lignified xylem fibres showed a bright autofluorescence, while callose plugs showed bright green fluorescence. Some stained callose resembling beads arranged in a longitudinal direction, presenting the characteristic of scalariform sieve plates. Positive staining for callose was also observed in IP strands that were localized close to the cambium (Figure 3.11a). However, in the external phloem tissues of the samples collected from the Xianhu Botanical Garden in Shenzhen city and the agarwood plantation in Zhongshan City, Guangdong Province, positive staining of callose in sieve tubes was rarely detected, indicating an obvious difference in the density of functional sieve tubes between the external phloem and IP.

Aniline blue staining of IP strands was also performed on the sections made from the wood blocks including more mature IP tissues and pith showed in Figure 3.1. Wood blocks S1 to S4 all displayed positive staining of callose in IP strands, but IP strands in the area containing the pith were rarely stained by the dye (data not shown). It might be due to the partial senescence of IP strands near the pith. Unfunctional sieve tubes in these phloem tissues finally loosed their callose and quickly crushed.

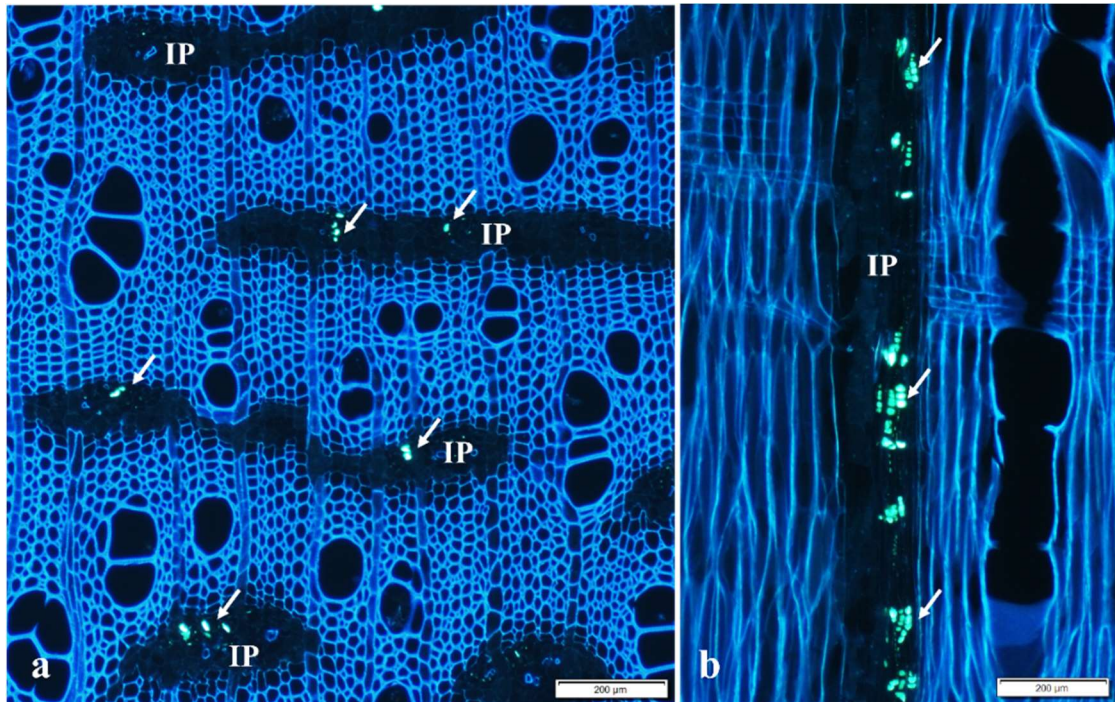


Figure 3.10. Fluorescence microscopy images show aniline blue staining of callose (arrows) in cross (a) and radial (b) sections. IP: interxylary phloem.

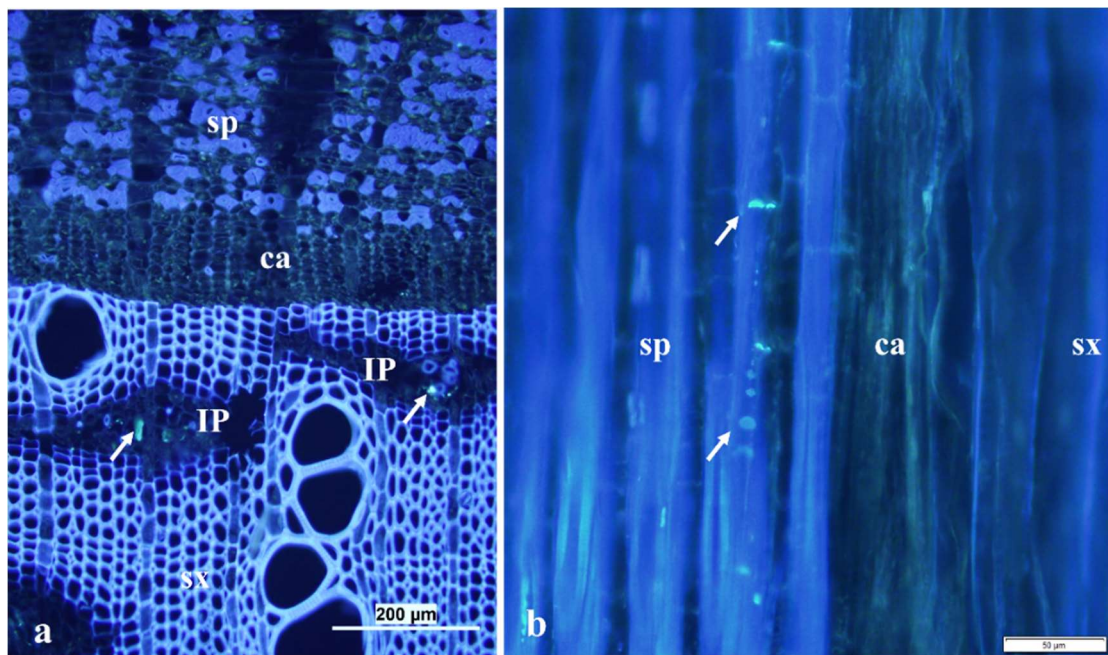


Figure 3.11. Callose stained by aniline blue in the sieve tubes of IP strands close to cambium (a) and in the sieve tubes of external phloem (b). Callose staining is rarely observed in the cross section of external secondary phloem in a, but a sieve tube strand with three elements is found in radial section of the phloem in b. Arrows: callose; ca: cambium; IP: interxylary phloem; sp: secondary phloem; sx: secondary xylem.

3.3.5. SEM observation of serial sections

To compare the anatomical characteristics between external phloem and IP, especially to determine the frequency of sieve tubes, for better understanding their functions, serial cross sections were made along the tree axis and observed by SEM. Each individual section was 15 μm in thickness, the total thickness of the twenty serial sections was 300 μm , a figure longer than the average length of the IP sieve tubes. All elements in a transverse square of 400 μm^2 were carefully examined.

The entire cross-sectional area in the external phloem (Figures 3.9 and 3.11a) was mainly occupied by phloem fibres, phloem parenchyma cells, and ray parenchyma cells. The phloem fibres with obviously thick cell walls were observed as clusters that formed islands or short tangential bands. Some phloem fibres showed large cell lumen, while others very narrow (upper right corner inset in Figure 3.9), depending on whether their long tapering tips or central wide-lumened bodies had been sectioned.

Phloem parenchyma cells were much larger in diameter than phloem fibres. When the area near the cambium was more closely scrutinized, many small cells could be seen between or adjacent to phloem parenchyma cells (arrows in Figure 3.9). These elements corresponded to the differentiating phloem fibres but not to the companion cells. The arrowheads on the left half of the cambial zone showed immature phloem fibres with the initiation of cell wall thickenings. These phloem fibres were in the further advanced stage of cell differentiation.

Compared with the IP, sieve tubes in the external secondary phloem were relatively rare, 1~2 sieve tubes were occasionally found in few of sections, which were embedded in dense parenchyma cells (Figures 3.11b and 3.12). The sieve tubes already developed open sieve pores, but their diameters were much smaller than those of the mother cells of vascular cambium area (Figure 3.12).

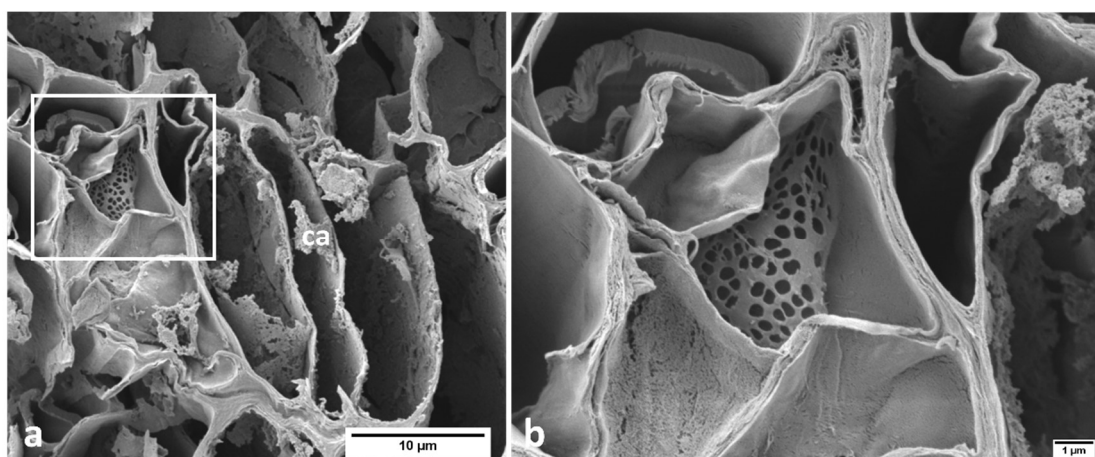


Figure 3.12. SEM photomicrographs of bark tissues in *A. sinensis*. b shows a higher magnification of the square in a. Sieve tubes are occasionally found in external phloem tissues. ca: cambium.

3.4. Discussion

3.4.1. General characteristics of interxylary phloem and external phloem

The general features such as location, shape, dimension of IP and external phloem tissues in *A. sinensis*, a species that possesses a diffuse type of IP were investigated through conventional optical microscopic, fluorescence microscopic and SEM observations. The cellular compositions and dimensions of different phloem tissues were compared through the successful maceration of whole IP strands and the bark tissue.

The IP strands were around eighteen times longer than xylem fibres. The longer the IP was, the more easily the photosynthates would be transported in the woody stem. On cross section, the IP strands show diffuse pattern and the dispersed distribution of IP in secondary xylem is also a characteristic of certain nutritional tissues in plants. The phloem strands potentially provide efficient allocation and exchange of nutrient and energy (Carlquist 1999) which are crucial for the synthesis of aromatic resins in the surrounding parenchyma cells. IP strands were always surrounded by lignified xylem tissue and, except for the thick-walled fibres, were composed of unlignified cells such as parenchyma cells, sieve tubes and companion cells. At their margin, IP strands were constituted by multiple layers of fusiform parenchyma cells, and inwardly strand parenchyma cells. The presence of large crystals (styloids) was always observed in the parenchyma cells of IP strands. This feature was very popular in a great number of species that developed IP (Carlquist & Zona 1988; Carlquist 2001), which might prevent predation of IP by insects' attack (Carlquist 2013).

Because the maceration liquid used was specific to lignin decomposition, it was presumed that the cell walls of lignified xylem fibres were decomposed by maceration liquid faster than those of non-lignified parenchyma cells. Parenchyma cell walls were composed not of lignin but of cellulosic layers. The layers were highly cross-linked and filled with xyloglucan, pectins and structural proteins (McCann & Roberts 1991; Carpita & Gibeaut 1993), which might be less susceptible to maceration liquid. Moreover, the fact that IP strands of *A. sinensis* were always surrounded by multiple layers of fusiform and strand parenchyma cells and were thus resistant to the penetration by the maceration liquid, might have facilitated the isolation of IP strands and dimensional measurement.

The structure of external phloem fibres in *A. sinensis* was one of the unique characteristics of its bark anatomy. The average length of external phloem fibre was $4185 \pm 451 \mu\text{m}$, which was extremely long compared to the average lengths of IP and xylem fibres. It has been reported that the members of Thymelaeaceae were characterized by extremely long bast fibres and the bast of various species belonging to different genera were utilized for the manufacture of paper (Pearson & Brown 1932; Cheng *et al.* 1992). In addition, the external phloem fibres possessed very narrow cell lumens except in the middle parts, which showed relatively larger diameters and whose average length only corresponded to one-eighth of the total fibres. This characteristic must be the reason why the external phloem fibres of *A. sinensis* were strong and well suited for the manufacturing of paper and related products.

3.4.2. Origin and development of interxylary phloem

The development of IP in *A. sinensis* was divided into five different stages. Among the reported species containing IP tissues, no one showed a completely consistent time-sequence and developing processes with those displayed in agarwood-producing species.

According to the origin and ontogeny of IP tissues, they were classified into: 1) *Combretum* subtypes; 2) *Strychnos* subtypes; 3); *Azima* subtypes and 4) *Calycopteris* subtypes (Rajput *et al.* 2022). *A. sinensis* with diffuse type of IP strands fell into the *Combretum* subtype. The characteristic of *Combretum* subtype was the periodic division of the vascular cambium, in which local parts of the vascular cambium alternately divided into xylem and phloem tissues centripetally at certain time intervals.

Other agarwood-producing species have also been selected to study the development of IP. Rao and Dayal (1992), who investigated the secondary xylem and the formation of ‘agar’ in *Aquilaria agallocha*, suggested that a secondary cambium was formed in the thin-walled parenchyma of IP and that it produced abnormal tertiary tissue. However, as described in this chapter’s “Development of IP” section, it has not been observed that once an island of IP had detached from the vascular cambium, further differentiation or activities of meristematic tissue such as secondary cambium still occurred in it.

The development of IP was also investigated in many other species that even did not produce aromatic resin but showed IP strands like those of *A. sinensis*. These species include *Salvadora persica* (Carlquist 2013), *Leptadenia pyrotechnica* (Gondaliya & Rajput 2016), *Strychnos millepunctata* (van Veenendaal & den Outer 1993), and *Combretum nigricans* (den Outer & van Veenendaal 1995). However, the authors of the first two papers investigated the development of IP using mature tissue, while present study followed the development of IP beginning with its initiation from the vascular cambium and concluding with its maturation within one growth ring. Thus, the development of IP in *Salvadora*, *Leptadenia* and that in *Aquilaria* could not be adequately compared.

In contrast, the latter two papers investigated the differentiation of IP from the vascular cambium. The cell derivatives in *Strychnos millepunctata* and *Combretum nigricans* also produced a diffuse type of IP that looked quite similar with that of *A. sinensis*. However, there were crucial differences among the three species. For example, complementary cambium segments were formed in the parenchyma cells at the outer border of immature IP in *S. millepunctata* and in the inward border of immature IP in *C. nigricans* but were not formed in *A. sinensis*. In addition, new phloem elements in IP strands quickly squashed the older ones in both *S. millepunctata* and *C. nigricans* but sluggishly in *A. sinensis*. At least, obvious cell crush did not present in the third growth ring from the bark (Figure 3.1). The features comprised the predominant differences between *A. sinensis* and the other two species. As a whole IP strands in *S. millepunctata* and *C. nigricans* could form inward from the conventional vascular cambium or differentiate from the complementary cambium located at IP border, a finding that only partially corresponded with the observation of *A. sinensis*.

Regarding the differentiation time, development of sieve tube elements in IP

strands of *Combretum floribunda* was initiated only after the formation of thick-walled xylem derivatives from the outer cambial segment (Rajput *et al.* 2009). In *A. sinensis*, the process was initiated soon after the immature IP had been produced from the vascular cambium. It could be confirmed by the positive signals of aniline blue staining appearing at the cambium areas.

Additionally, the arrangement and shape of IP strands in *A. sinensis* were similar with those in *Combretum apiculatum* but the former did not show overarching of cambium. IP strands of *A. sinensis* differentiated directly from cambial zone cells. Although the arrangement of IP strands in *Thunbergia alata* was quite different from *A. sinensis*, the present observations supported that the small segments of vascular cambium in *A. sinensis* produced phloem strands toward the inside for a brief period instead of xylem (Angyalossy *et al.* 2016).

3.4.3. Detection of sieve tube by fluorescence staining and SEM observation

Aniline blue staining detected the occurrence of callose in IP strands of *A. sinensis* from the latest growth ring to the seventh ring near the pith (Figure 3.1). On the other hand, IP strands of the wood blocks S4 and S3 did not show crushing of phloem cells. These findings suggested that vast majority of the sieve tubes in the IP strands of wood blocks S4 and S3 were still in charge of the transportation of photosynthetic products, whereas the sieve tubes in IP strands of blocks S2 and pith might have become nonfunctional by senescence.

In contrast, callose staining was rarely observed in the external secondary phloem, which suggested that there were sparse sieve tubes in the external phloem tissue of *A. sinensis*. den Outer and van Veenendaal (1995) also mentioned that sieve tubes were rarely found in the external secondary phloem of *Combretum nigricans*. Therefore, it was conceivable that sieve tubes had degenerated. If such was the case, the sporadic sieve tubes in the secondary phloem were highly likely to be overlooked, even with the help of light microscopy and SEM. Considering all these points, it was concluded that in *A. sinensis*, sieve tubes in IP strands could be responsible primarily for the transport of photosynthetic products.

It has been commonly observed that one to several ray tissues traversed IP strands in cross sections. The same phenomenon has already been shown or described in developing IP tissues of other species (van Veenendaal & den Outer 1993; den Outer & van Veenendaal 1995; Rajput *et al.* 2009; Patil *et al.* 2011; Moya *et al.* 2017). Therefore, it was conceivable that IP strands, together with ray tissues, should make up a three-dimensional network and play a vital role in the transport of nutrients, hormones and signaling molecules. The fine structures related to cross-linking of sieve tubes in different phloem tissues, as well as the patterns of interconnection to form the phloem network were elaborated in Chapter 6.

3.5. Summary

The structure and development of IP and external phloem tissues in *A. sinensis* were investigated using light and scanning electron microscopy. Complete IP strands were isolated, measuring 14 ± 4 mm in average length and 417 ± 124 μ m in average width. The outer margin of IP was composed of multiple layers of fusiform parenchyma cells, adjacent to which were strand parenchyma cells that wrapped around sieve tubes and companion cells located at the inner side of IP, as well as sporadic fibers around SE-CCs groups.

The developmental process of IP was divided into five stages: 1) IP started its differentiation within a small segment of a broad cambial zone, at the cost of xylem differentiation. 2) Inward growth of IP advanced, and fibres and sieve tubes differentiated. 3) IP was constricted by the encroachment of immature xylem cells between cambium and immature IP. 4) IP was isolated from the cambium and surrounded by immature, unlignified xylem tissues. 5) IP was surrounded by lignified xylem tissues, and the fibres within IP developed distinctly thick walls.

In nearly all the IP islands of a ten-year-old stem, sieve tube elements showed positive staining of callose with aniline blue. However, the staining of callose was rarely observed in the external secondary phloem of *A. sinensis* collected from two different plantations. Sieve tubes also were rarely detected by SEM observation of numerous serial cross and radial sections of the external phloem. Therefore, it was suggested that the transport of photosynthetic products in the stem of *A. sinensis* might mainly take place in the IP tissues.

Chapter 4 Experimental proof of potential activity of interxylary phloem

4.1. Introduction

In the plant kingdom, there were 400 families of seed plants (Mabberley 2008), and interxylary phloem (IP) was only observed in 21 of these families including 11 from lianas (Carlquist 2001; Angyalossy *et al.* 2012). Therefore, the occurrence of IP in seed plants was not common. Thymelaeaceae was one of these families and included agarwood-producing species. According to Carlquist (2013), understanding of the physiological significance of the specific modes of IP produced by cambial variants was a promising topic for exploration. Early studies suggested that agarwood resins could be produced in IP (or included phloem) and ray tissue (Tabata *et al.* 2003; Mohamed *et al.* 2013). More recently, Liu *et al.* (2019) strengthened this hypothesis by chasing the formation of resins at different time intervals. These studies suggested that IP might have some unforeseen functions that have not been described so far.

Because of the economic as well as the environmental importance of *Aquilaria*, the study has focused on identifying the potential functions of IP in *Aquilaria sinensis* and suggested that photosynthates were mainly transported through IP and rarely by external phloem in the bark (Luo *et al.* 2018). The previous results show that IP plays a crucial role in the growth of agarwood-producing species under normal conditions. Furthermore, if the IP performs a function similar with the external phloem, agarwood-producing species would have the ability to survive after complete girdling (100 % removal of the external phloem and cambium).

Stem girdling was performed quite often for a variety of reasons, for example, to examine the relationship between the presence of anomalous structure and the nature of wound response in lianas, to assess bark functions, to determine the capacity of trees to recover from various girdling practices, to determine the survival rate of trees for the sustainable use of their medicinal barks, and to study the functional aspects of phloem and its direct impact on tree growth (Dobbins & Fisher 1986; Li & Cui 1988; Oven & Torelli 1994; Delvaux *et al.* 2010a; 2010b; 2013; Monteiro *et al.* 2011; Pandey *et al.* 2011; De Schepper & Steppe 2011; Baldauf & dos Santos 2014; Chen *et al.* 2014; Guedje *et al.* 2016). To identify additional roles associated with IP under abnormal growth, branches of *A. sinensis* were complete girdling to investigate the structural changes and the recovery of vascular cambium and secondary tissues.

4.2. Materials and Methods

4.2.1. Materials

Agarwood trees, approximately 12 years old, cultivated in the plantation in Zhongshan city, Guangdong province, China, were used in the present work. Approximately 2-cm-long regions of 36 leafy branches (5~10 mm in diameter) were completely girdled to remove the bark, external phloem and vascular cambium (Figure 4.1a). Girdled samples were collected on the day of girdling, at one, two, three, and four weeks, and at two, four, and six months after girdling. Immediately after the samples (small pieces of branches) were obtained, they were dipped in 2.5 % glutaraldehyde or 4 % formaldehyde and then cut into appropriate size for paraffin or epoxy resin (Epon 812, nacalai tesque, inc., Japan) embedding.

4.2.2. Substitution and embedding

For paraffin embedding, samples were dehydrated through serial processes in *t*-butyl alcohol (30~100 %) and then placed in 50 % paraffin at 60 °C for 8 h, followed by 100 % paraffin twice at 60 °C (8 h each time).

For epoxy resin embedding, samples were first dehydrated through serial passages in different concentrations of ethanol, followed by two times treatments with 100 % ethanol (10 min each time) and then three times treatments with 100 % propylene oxide (10 min each time). For full penetration of the resin, samples were kept shaking during the substitution steps. Substituted samples were first left in a mixture of 1:1 propylene oxide and epoxy resin for 12 h, then a mixture of 1:2 propylene oxide and epoxy resin for another 12 h, followed by 100 % epoxy resin twice, each for 12 h. For polymerization of epoxy resin, samples were kept at 35 °C and 45 °C for one day each, and 60 °C for 1~2 days.

4.2.3. Staining and observation

Paraffin embedded samples were cut into 20- μ m-thick sections. One group of sections was double stained with 1 % safranin and 0.5 % astra blue and observed under a bright field light microscope. Another group was stained with 0.005 % aniline blue aqueous solution for 30 min at room temperature, observed with the same microscope attached with an epifluorescence apparatus, and then washed and freeze dried. Dried sections were coated using an ion sputter apparatus (JEOL JFC-1100E) at 10 mA for 5 min, and observed using a scanning electron microscope (JEOL JSM-6060) with a working distance of 20 mm and accelerating voltage of 5 kV.

Resin embedded samples were cut into 1~2 μ m semithin sections using a Reichert-Jung microtome. The sections were stained with 0.1 % toluidine blue-O and observed under a light microscope.

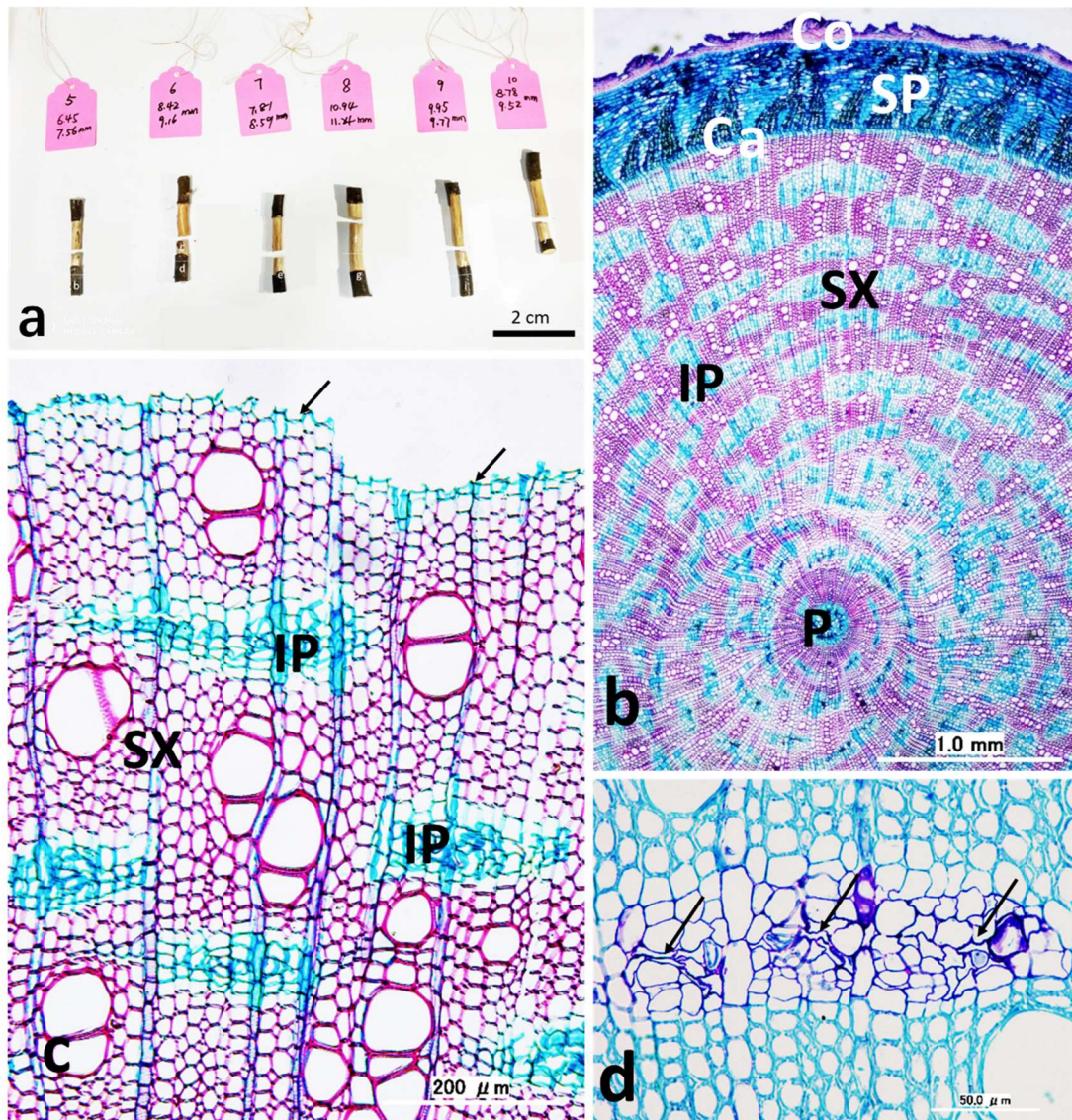


Figure 4.1. Bark peeled samples and their structural characteristics before and after girdling.
a: Examples of specimens from six branches after complete girdling.
b: Cross section of a branch right before girdling on day 1.
c: Cross section of a branch right after girdling on day 1. Arrows indicate girdled edge.
d: Cross section of IP strand in secondary xylem, one week after girdling. Phloem cells in the core part of the IP are collapsed (arrows). Ca: vascular cambium; Co: cork tissue; IP: interxylary phloem; P: pith; SP: secondary phloem; SX: secondary xylem.

4.3. Results

Redifferentiation of IP tissues was analyzed before and after peeling the bark until the regeneration of vascular cambium and secondary tissues. This study described the structural changes during the redifferentiation of IP strands and regeneration of vascular cambium, one week to six months after girdling.

4.3.1. Redifferentiation and proliferation of interxylary phloem

4.3.1.1. Redifferentiation of IP strands one week after girdling

A normal structure of secondary vascular tissues was observed in a transverse section of a branch before and after peeling the bark on day 1 (Figure 4.1b and 4.1c). IP strands were distributed roughly in concentric layers and showed intact structure without collapse of their core cells including sieve tubes, companion cells and fibres, except those close to the pith. Right after the bark attached to the cambium was removed, the girdled edge (or marginal layer of a naked branch after peeling the bark) was stained with safranin and astra blue, indicating that the bark attached to the whole cambium was successfully peeled off (arrows in Figure 4.1c).

IP strands maintained a slender or oval shape, as observed in transverse sections, and appeared to be intact one week after peeling off the bark. However, the core parts of most IP strands partially deformed or collapsed, leaving short and dark lines of crushed cell walls impregnated with resinous substances (arrows in Figure 4.1d). Furthermore, IP strands located in the first to the third layers from the girdled edge initiated various deformations in shape and structure, as outlined in the following observations.

(i) Some IP strands showed hollowed structure (asterisks in Figures 4.2a and 4.2d). (ii) Many IP strands showed distinct, wavy and dark lines, often attached with resinous cells, along the tangential direction of IP body on either the abaxial or adaxial side, or both sides (arrows in Figure 4.2b). (iii) A few of IP strands extended cell chains as bridges, mainly in the tangential direction, from one or both wings of IP strands to connect to others (arrows in Figure 4.2c). Upon staining, the cell chains showed color similar with ray cells, suggesting their parenchymatous nature. Connections of more than two IP strands were frequently observed at this stage (Figure 4.3a). (iv) Ray tissues were often involved in the connections of two or more IP strands in diagonal or radial direction (arrow in Figure 4.2d). (v) Parenchyma cells of IP strands started differentiation and produced thick-walled and lignified cells mainly on the adaxial side of IP strands. (vi) Parenchyma cells on the abaxial side of IP strands produced resinous cells (Figure 4.2c). The outer part of IP strands was occupied with these cells and appeared dark reddish. Furthermore, the resinous cell layer was protected by folded cell walls in the outermost part of IP strands (Figure 4.3b).

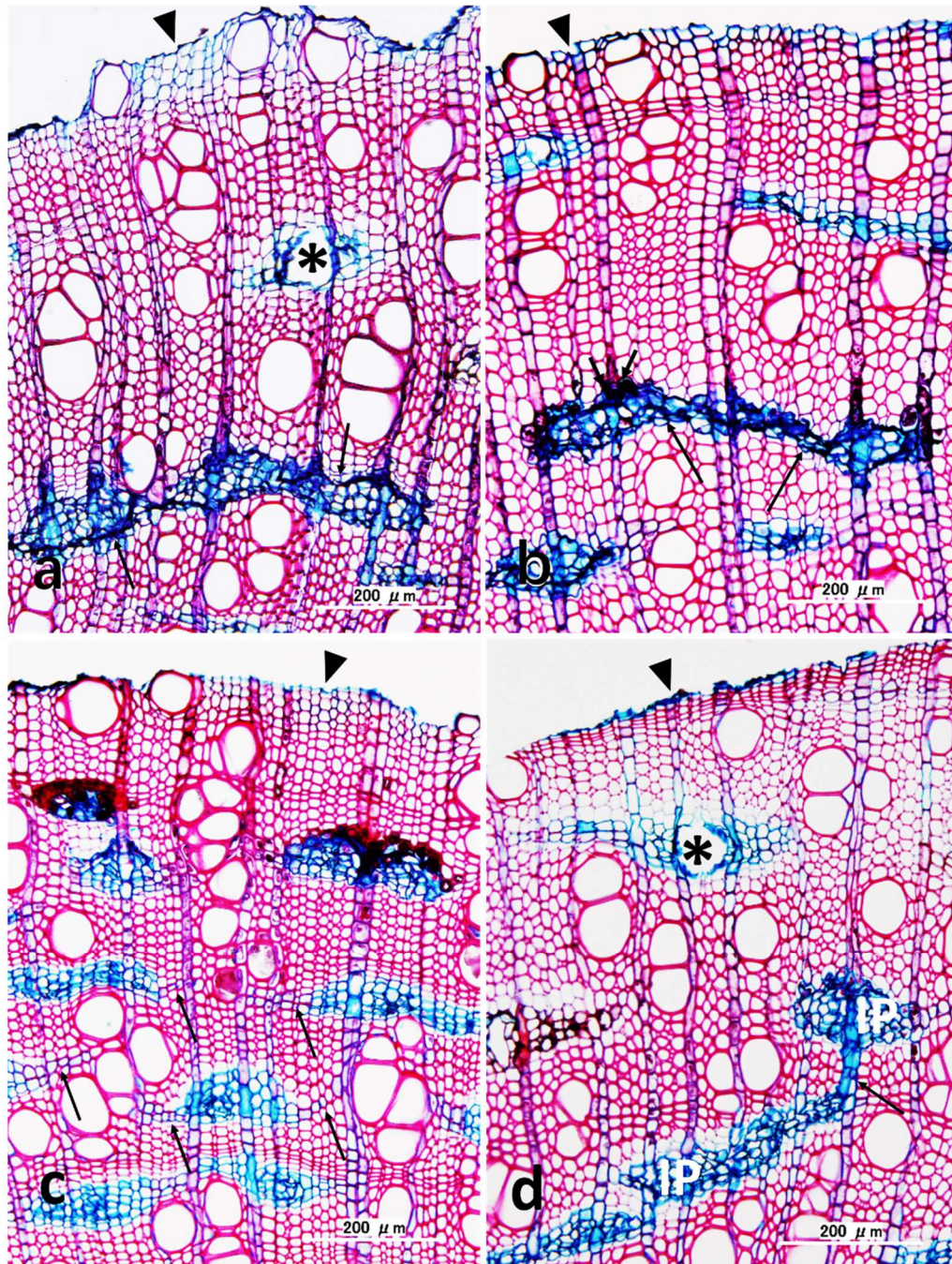


Figure 4.2. Transverse sections show deformations of IP strands, one week after girdling.
A: IP strands with hollow (asterisk) or dark lines (arrows) are shown in the first to the third layers of IP strands from the peeling edge.
B: Resinous cells (short arrows) are associated with dark lines made of crushed or deformed cells (long arrows).
C: IP strands extend cell chains (arrows) to connect to others.
D: Two IP strands are connected by ray cells (or possibly, radial sieve tubes) (arrow). Arrowheads: girdled edge; asterisks: IP strands with hollows; IP: interxylary phloem.

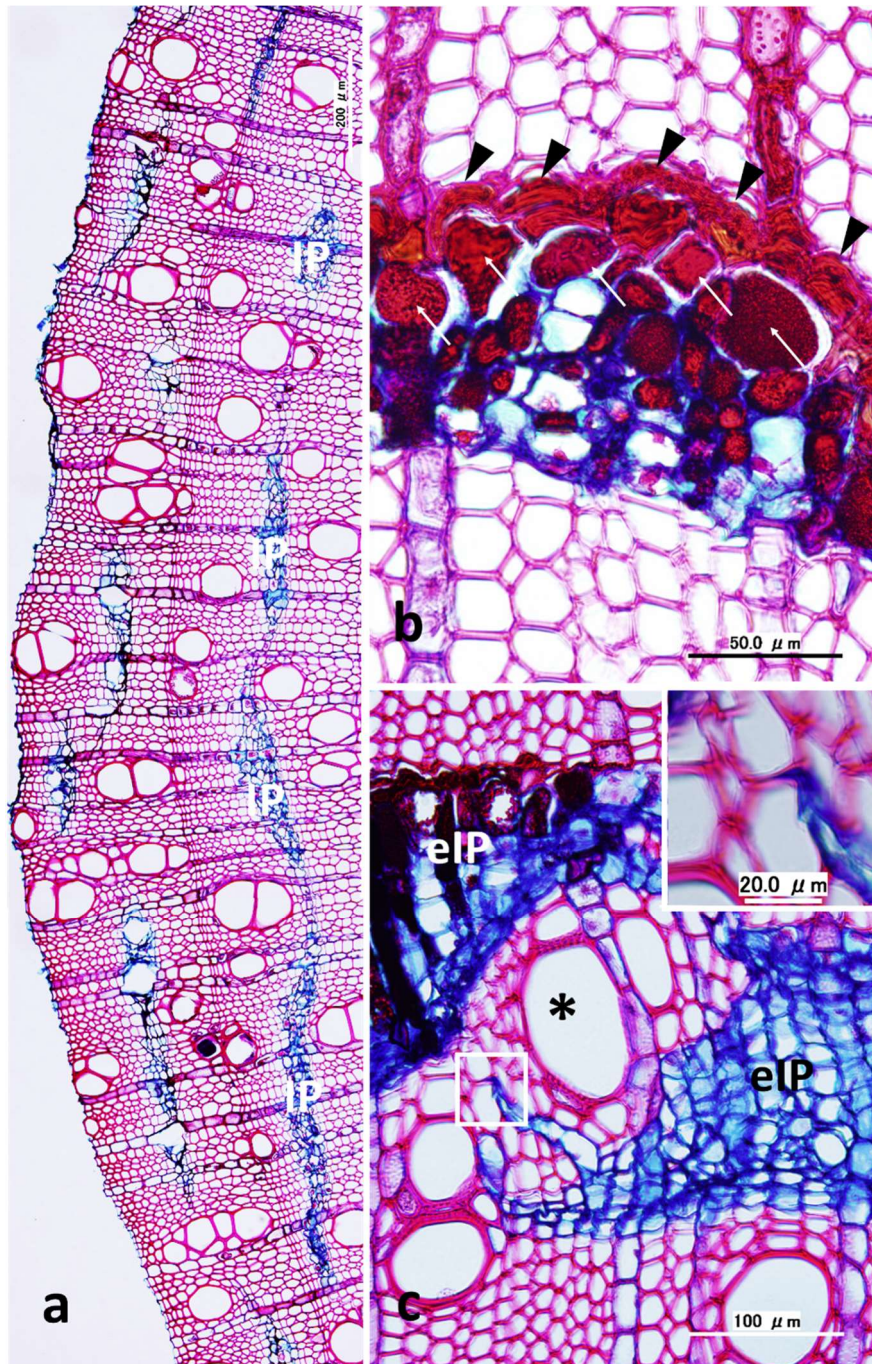


Figure 4.3. Transverse sections show cell connections and invasion of IP strands, one week (a) and two weeks (b, c) after girdling.

A: Four IP strands are connected by cell chains in the tangential direction.

B: Many resinous cells (arrows) occupy the outer margin of IP and are protected by reddish and deformed cell walls (arrowheads).

C: Two enlarged IP strands are about to capture a fragment of secondary xylem tissue (asterisk). An enlarged IP on the right extends cell chains. IP breaks up the secondary xylem by invading the intercellular layer with a tip of the cell chain. The white square indicates a point of the invasion of the cell tip. IP: interxylary phloem; eIP: enlarged interxylary phloem. Inset: Higher magnification of the white square shows the invasion of a cell tip of the enlarged IP to intercellular layer.

4.3.1.2. Redifferentiation of IP strands two weeks after girdling

Figure 4.4a shows the structure of IP strands two weeks after girdling. A basic IP became larger following the proliferation of its component parenchyma cells. This IP was named enlarged IP (eIP), which was commonly observed at this stage. Most eIP strands at this stage have already formed thick-walled, lignified tissues that would eventually become a layer of wound xylem (arrows in Figure 4.4a).

The eIP in Figure 4.3c was in an active functional state of extending three cell chains as bridges to adjacent eIP strands. As a result of the activity of the eIP, an area of secondary xylem tissue (asterisk in Figure 4.3c), including one large and three small vessels, was captured by two eIP strands. Prior to IP strands capturing secondary xylem tissue, the tips of cell chains extending from IP strands invaded the intercellular layer of the fibres that made up the secondary xylem (square and inset in Figure 4.3c). Eventually, the secondary xylem was cleaved and became smaller, while at the same time, IP strands penetrated the gaps and proliferated further. After being captured by eIP strands, a fragment of secondary xylem remained angular afterward and became part of the eIP body without further digestion or disintegration of the fragment.

Generally, the component parenchyma cells in basic and enlarged IP strands of the first week seemed to have active proliferation ability but they have not yet organized as a meristematic cell layer. However, as the enlarged IP strands bound together and grew up in size, the inner side parenchyma cells divided repeatedly and even larger clusters of IP strands developed. The clusters were referred to as confluent IP (cIP) strands and were observed frequently at this stage.

The parenchyma cells in some cIP strands seemed to have an organized meristematic nature forming a layer of 1 to 3 cells wide, as shown by small arrowheads in Figure 4.5a. At the same time, Figure 4.5a also shows the differentiation of wound xylem on the adaxial side of the cIP, and the resinous cells on the abaxial side of three to several cells wide. At this stage, differentiated cork cells stained by the dye were observed for the first time (ellipse in Figure 4.5a).

4.3.1.3. Redifferentiation of IP strands three weeks after girdling

A series of enlarged and confluent IP strands, typical at this stage of redifferentiation of IP, were shown in Figure 4.4b. The right side of the figure shows two isolated enlarged IP strands, the lower one of which was supposed to connect to a confluent IP in the center of the figure. It was assumed that the confluent IP was formed by the connections of three or more enlarged IP strands. Figure 4.4b also shows the successive stages of development from enlarged to confluent IP strands. Higher magnification of part of one confluent IP in the transverse plane was shown in Figure 4.6a as observed by SEM. The confluent IP strand revealed three cores where sieve tubes and the adjacent cells were localized to form small masses (arrows in Figure 4.6a). The square in Figure 4.6a represents one of the cores, in which a sieve tube with a thick sieve plate made of callose deposits was observed (arrow in Figure 4.6b).

During the proliferation of parenchyma cells on the adaxial side of enlarged and

confluent IP strands, not only were strands of new parenchyma cells but also strands of wound xylem were observed after differentiation (asterisks in Figures 4.4b and 4.4c). As observed by fluorescence microscopy combined with SEM, the wound xylem cells were significantly lignified, with simple perforations in end walls or occasionally in lateral walls and commonly vested pits in lateral walls (Figure 4.5b). The tracheary elements were mostly 50~60 μm in longitudinal length with a range of 20~100 μm and were commonly surrounded by bundles of regularly arranged and newly differentiated parenchyma cells having the same length as normal parenchyma cells, and frequently connected directly to inner secondary xylem.

Figure 4.5c shows callose stained by aniline blue, indicating the locations of sieve tubes. One confluent IP, distributed as a large tangential band, was found in the center of the figure and six bright spots were observed in the cIP. Almost all the basic IP strands inward from the cIP had 1 to 3 or more bright dots, but those external to the cIP did not show the presence of callose staining because these IP strands have been deformed and ceased to function (Figures 4.4 and 4.6d).

Compared to basic IP strands, the occurrence of sieve tubes in cIP strands and bIP strands was found to be much less per unit area. When 60 basic IP strands and 10 cIP strands were examined carefully, the number of fluorescent dots per IP strand was not much different between the two types, suggesting that the number of sieve tubes did not substantially increase during redifferentiation and expansion from the basic IP to the cIP.

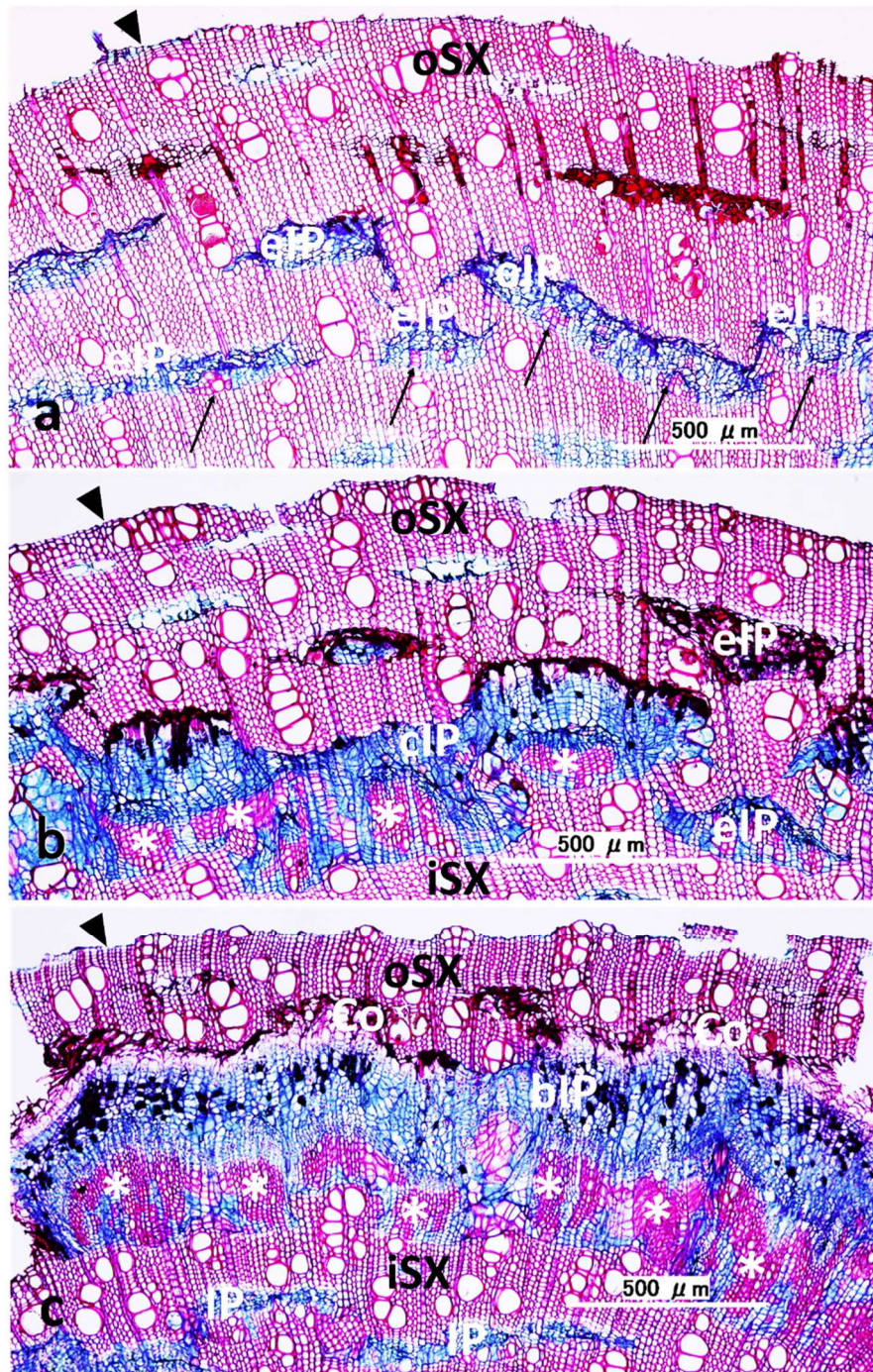


Figure 4.4. Three different stages of IP strands redifferentiation; two weeks (a), three weeks (b) and four weeks (c) after girdling.

A: The tangential arrangement of six enlarged IP strands is shown in 400~1000 μm deep from the girdled edge. Thick-walled, lignified tissues are formed (arrows).

B: A large confluent IP is going to merge with an enlarged IP on the right side of the figure.

C: A banded IP shows conspicuous development of cork tissue at the outer margin and a thick belt of wound xylem in the inner side. Asterisks: thick-walled and lignified tissues, corresponding to wound xylem; arrowheads: girdled edge; Co: cork tissue; eIP: enlarged interxylary phloem; cIP: confluent interxylary phloem; bIP: banded interxylary phloem; IP: interxylary phloem; oSX: outer secondary xylem; iSX: inner secondary xylem.

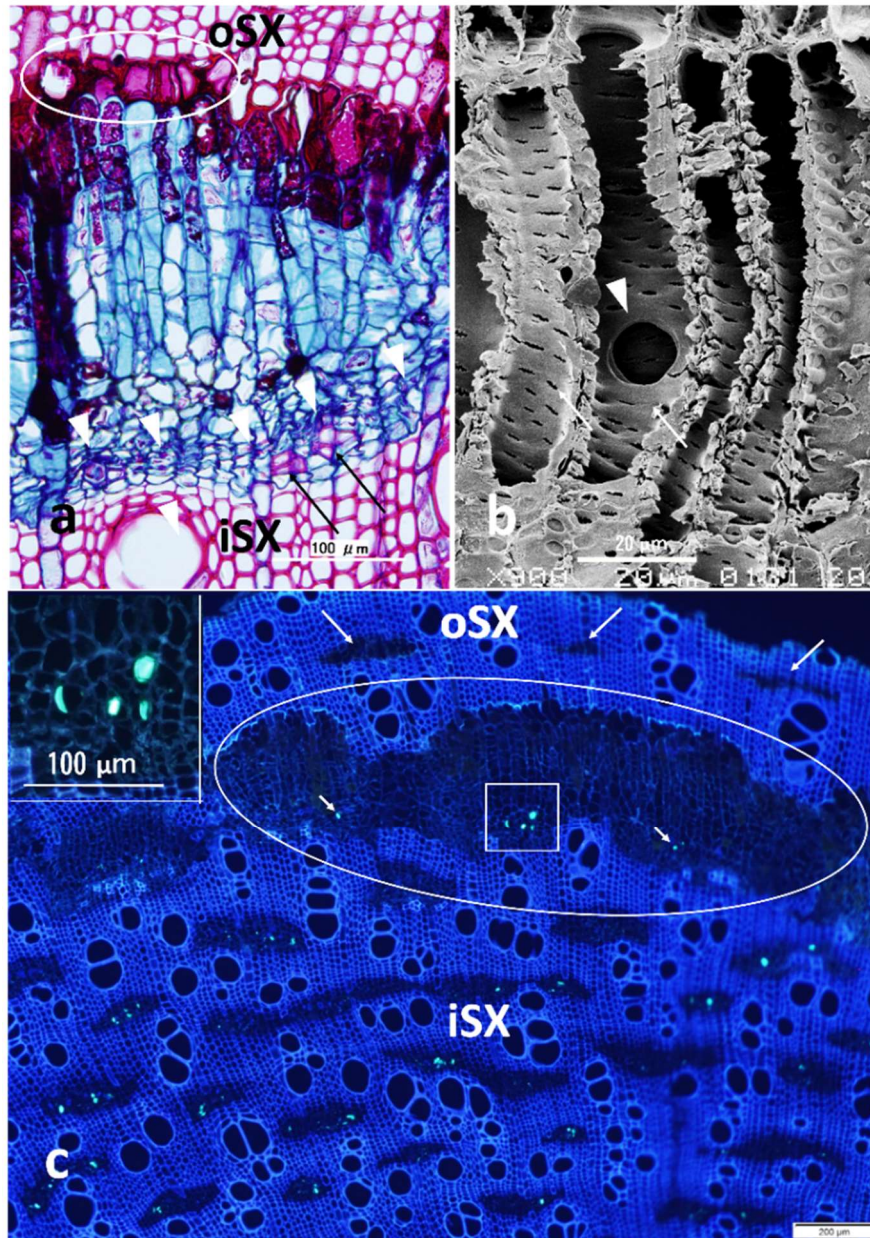


Figure 4.5. Double staining, fluorescence staining and SEM observation show cell regeneration, expansion, and coalescence of IP strands two weeks after girdling.

A: A confluent IP shows many resinous cells (ellipse) and nascent cork cells in the abaxial side. Differentiation of wound xylem is indicated by long arrows and a meristematic cell layer is shown by a series of arrowheads.

B: SEM image of wound xylem cells shows simple perforation (arrowhead) and vested pits (arrows) in the lateral wall.

c: Fluorescence microscopic image shows callose staining in a differentiating confluent IP (ellipse) and basic IP strands in the inner secondary xylem, but no staining in IP strands in the outer secondary xylem (long arrows). Confluent IP shows six bright spots (short arrows and square) where sieve tubes are located. Square: one of the cores in a confluent IP; iSX: inner secondary xylem; oSX: outer secondary xylem. Inset is a higher magnification of the square showing blue-green fluorescence of callose staining in four locations indicating sieve tubes.

4.3.2. Regeneration of complete vascular cambium and secondary tissues

4.3.2.1. Redifferentiation of IP strands four weeks after girdling

In girdled branches, the secondary xylem formed prior to girdling was divided into two parts due to the differentiation of a continuous banded IP — outer and inner secondary xylem tissues located outward and inward to the banded IP respectively, as shown in Figures 4.6a and 4.6d (named oSX and iSX). How deep from the girdled edge did the confluent or banded IP strands differentiate? To examine the exact depth, the distances between the girdled edge and the outermost as well as the innermost borders of confluent or banded IP strands were measured from 20 branches, 2~4 weeks after girdling. The confluent or banded IP strands were found to be differentiated in a narrow zone, 400~1000 μm deep from the girdled edge.

At four weeks after girdling, the predominant structural feature of IP redifferentiation was banded IP strands (Figure 4.4c). Compared to the irregular shape of confluent or short banded IP strands (Figure 4.4b), the mature banded IP in Figure 4.4c appeared smooth at the boundary between outer secondary xylem tissue and the IP, and showed a highly differentiated belt of wound xylem. In the mature banded IP, the vascular cambium was located at the paraxial side and adjacent to the wound xylem, forming a wavy pattern.

The predominant event at this stage was the regeneration of vascular cambium (Figure 4.7a). After confluent and short banded IP strands were differentiated, a mature banded IP developed almost circumferentially around the secondary xylem of the branch. The outer secondary xylem tissue corresponded to the outermost layer of the secondary xylem laid down before girdling. After bark attached to the vascular cambium was peeled off, fragments of vascular cambium or cambial zone were stained blue by astra blue and remained as a wavy line at the girdled edge or outer margin of the old secondary xylem tissue (arrows in Figure 4.7b).

A transverse section of the branch at this stage shows the following arrangement of stem tissues from outside to inside: old and outer secondary xylem, new (regenerated) periderm, new secondary phloem, new vascular cambium, wound xylem, old and inner secondary xylem, and pith. Figure 4.7c shows a higher magnification of the regenerated secondary vascular tissues. Phellogen with phelloderm (double-headed arrow) and vascular cambium have been regenerated. New secondary xylem including several vessels and one IP island was regenerated. Secondary phloem fibres were also regenerated (dotted ellipse). It was noted that the transition from the wound xylem to the regenerated secondary xylem was gradual and the boundary between the two was not clear (solid ellipse in Figure 4.7c).

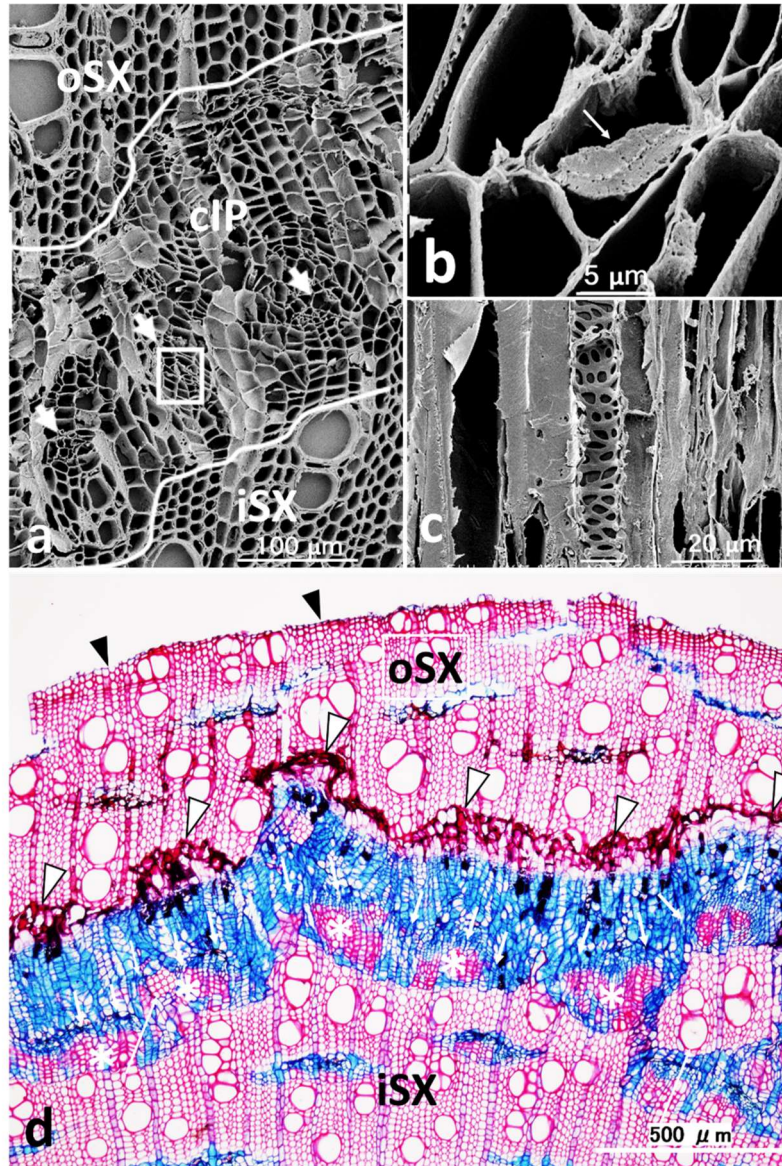


Figure 4.6. Double staining and SEM observation show cell differentiation and coalescence of IP strands three weeks after girdling.

A: SEM image of a confluent IP shows three cores where phloem cells form small clusters (arrows). Wavy lines indicate the borders between confluent IP and outer or inner secondary xylem tissues.

B: Enlargement of the square in a, rotated 90 degrees counterclockwise, shows a sieve plate (arrow) with thick callose deposits.

C: Reticulate thickening of a tracheary element in wound xylem.

d: Short banded IP shows the protective layer made of cork tissue (white arrowheads) in its outermost margin. A belt of wound xylem (asterisks) is differentiated on the adaxial side of the banded IP. Two angular fragments of secondary xylem tissue indicated by long arrows are captured by the banded IP. Discontinuous meristematic cells (short arrows) that would become a vascular bundle in the future are generated external to the wound xylem. Black arrowheads: girdled edge; bIP: banded interxylary phloem; cIP: confluent interxylary phloem; iSX: inner secondary xylem; oSX: outer secondary xylem.

Differentiation of thick cork tissue with some immature cork cells, appearing as a continuous layer 4~6 cells wide, was commonly observed at this stage (Figures 4.7a and 4.7c). A thin layer of meristematic cells, 2~4 cells wide and corresponding to

phellogen and phelloderm, was observed between the cork tissue and resinous parenchyma tissue (double-headed arrow in Figure 4.7c). Inside the phloem tissues, a highly ordered vascular cambium was present as successive layer of 3~4 cells wide.

Figures 4.7a and 4.7c corresponded to the lower and upper sides of the same branch. In the lower side of the branch, distinct strands of tracheary elements corresponding to wound xylem were produced (asterisks in Figure 4.7a). However, vascular cambium (arrowheads in Figure 4.7a) located at the paraxial side and close to the wound xylem was just beginning to differentiate secondary xylem. Therefore, no immature secondary xylem tissue could be observed except some dividing cells in the cambial zone.

In the upper side of the branch, new secondary xylem tissue with a few of vessels and particularly a new IP island that has just differentiated were formed (Figure 4.7c), providing evidence of the regeneration of vascular cambium as well as secondary tissues. Strands of wound xylem intercalated with various shaped parenchyma tissues (white asterisk) were found to be located inwardly adjacent to the vascular cambium. Two fragments of secondary xylem (black asterisks) previously captured by eIP or cIP strands were included in the banded IP, one on the outside of the vascular cambium and the other on the inside. It was worth noting that both fragments showed angular shapes, suggesting no further digestion after being captured by those IP strands.

4.3.2.2. Differentiation of regenerated cambium 2~6 months after girdling

Transverse sections of branches from 2~6 months after girdling shows the following arrangement of stem tissues from the outside to the inside: old and outer secondary xylem, new (or regenerated) periderm, new secondary phloem, new vascular cambium, new secondary xylem, wound xylem, old and inner secondary xylem, and pith (Figure 4.8a).

The recovered vascular cambium was about 4~5 cells wide (Figure 4.8b). Newly formed secondary phloem was completely comparable to the secondary phloem laid down prior to girdling with several layers of phloem fibres (ellipse in Figure 4.8b). Phellogen and phelloderm adjacent to the outside of the secondary phloem were composed of approximately 6~7 layers of elements.

Regenerated secondary xylem, including approximately 7~10 layers of IP strands, was simultaneously produced by the division of the complete ring of renewable vascular cambium. However, diameter of the vessels in regenerated secondary xylem was slightly smaller than that of vessels in the secondary xylem laid down prior to girdling. Distribution density of vessels in the new xylem was also lower than that of vessels in the old xylem. These anatomical changes were consistent with the observation of the vessels produced after wounding by peeling the bark of poplar stem (Frankenstein & Schmitt 2006), which suggested that although the new cambium restarted bidirectional cell division, its ability to produce xylem was different. Additionally, majority of ray tissues in the secondary xylem before girdling were uniseriate, but the number of biseriate rays increased a lot in the new secondary xylem.

The wound xylem was detached from the regenerated vascular cambium and

moved inward far from the cambium, resulting in a disorganized array (dotted ellipse in Figure 4.8a). Commonly, some of the eIP or cIP strands would be left losing the opportunities to join the main banded IP in the process of proliferation. The fragments of secondary xylem tissue (asterisk in Figure 4.8c) that have been captured by such eIP and cIP strands were forced to move inward along with the wound xylem during the regeneration of secondary xylem tissue and became a part of tissue debris (dotted ellipse in Figures 4.8a and 4.8c), but such fragments of the secondary xylem consistently kept the same angular shapes as when they were captured by eIP or cIP strands.

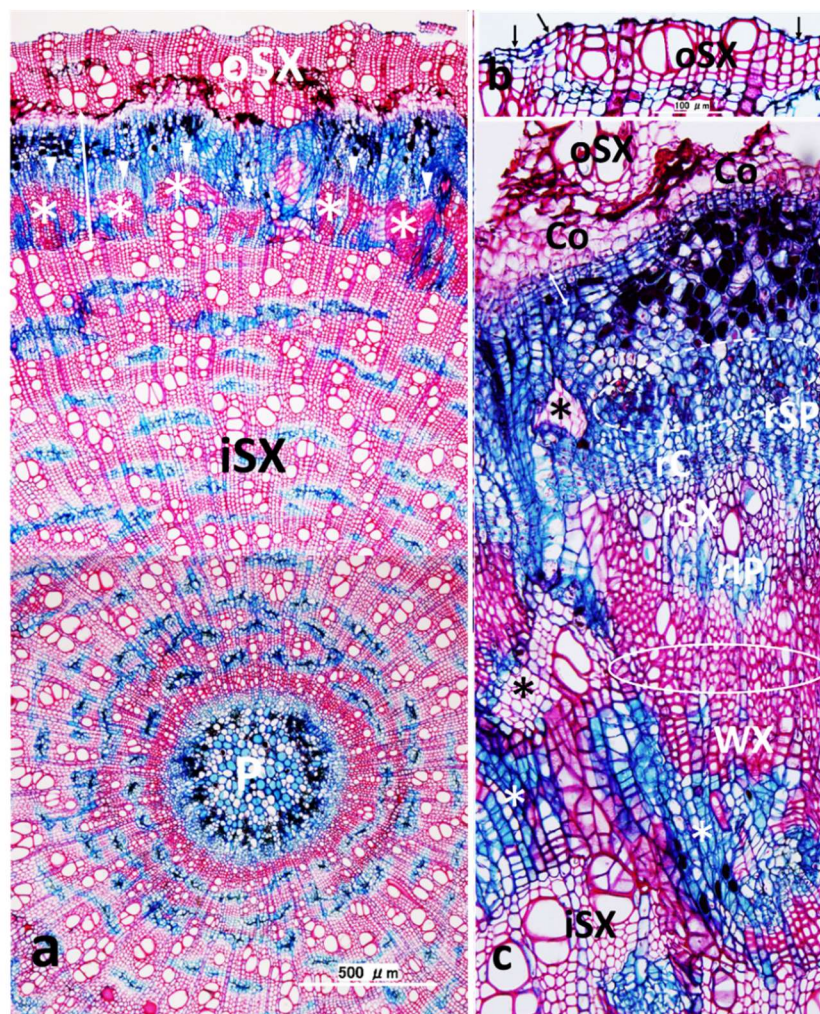


Figure 4.7. Completed ring of vascular cambium is going to be regenerated from banded IP strands and the secondary tissues differentiate from the new cambium.

A: A part of the almost complete ring of a banded IP (double-headed arrow) that is regenerated four weeks after girdling. Arrowheads show the location of the regenerated vascular cambium. The wound xylem is shown by asterisks.

B: Higher magnification of girdled edge (arrows) in outer secondary xylem.

c: Higher magnification of a banded IP opposite to panel (a) in the same microtome section. A solid ellipse indicates the transition zone between regenerated secondary xylem and wound xylem. Regenerated secondary phloem fibres are shown in the dotted ellipse. Two fragments of secondary xylem captured by former banded IP are shown by black asterisks. Double-headed arrow: the layer of phellogen and phelloderm; rC: regenerated cambium; Co: cork tissue; rIP: regenerated interxylary phloem; P: pith; WX: wound xylem; iSX: inner secondary xylem; oSX: outer secondary xylem; rSX: regenerated secondary xylem; rSP: regenerated secondary phloem.

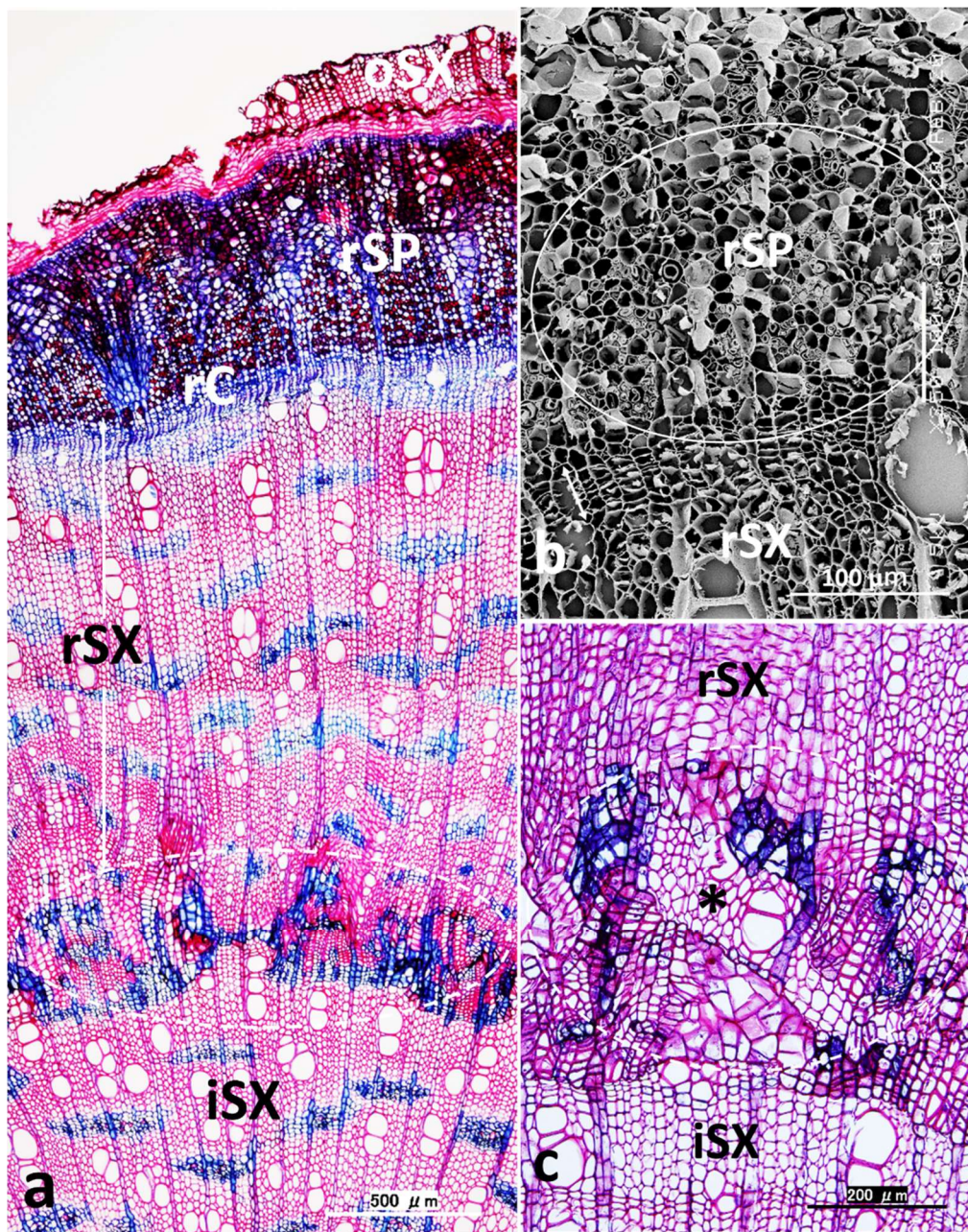


Figure 4.8. Completed ring of vascular cambium is fully developed and the secondary tissues are continuously produced by the new cambium four months after girdling.

A: A complete circular ring of vascular tissue is regenerated. Wound xylem tissue associated with parenchyma strands (dotted ellipse) has been transferred inward by the formation of new secondary xylem, and it shows a dispersed pattern.

B: SEM image shows regenerated secondary phloem (ellipse) and vascular cambium (double-headed arrow) with 4~5 cells wide, indicating full recovery of secondary vascular tissues.

C: Cross section shows tissue debris (dotted ellipse), including wound xylem, a fragment of IP, a fragment of secondary xylem and parenchyma cells, already transferred inward to the regenerated secondary xylem. A fragment of old secondary xylem (asterisk) is observed without any change of its shape. rC: regenerated cambium; rSP: regenerated secondary phloem; rSX: regenerated secondary xylem; iSX: inner secondary xylem; oSX: outer secondary xylem.

4.4. Discussion

This study aimed to clarify the role played by IP of agarwood-producing species under abnormal growth condition, through the regeneration of vascular cambium, followed by the recovery of secondary xylem and phloem. To this end, the changes of IP-deformations and reconstruction of secondary vascular tissues were tracked step by step for six months after completely girdling the branches of *A. sinensis*. During the regeneration process of secondary vascular tissues, the following four major events, as related to the response of IP tissues to stress and their functional activities are discussed.

4.4.1. Deformation of interxylary phloem during wound stress

Stem girdling has been used to study the functional aspects of phloem and its direct impact on tree growth. Generally, regeneration of bark was from the proliferation of callus tissue that differentiated following the division of cambium on the wound margins (Chano *et al.* 2015) or of immature xylem cells at the stage of primary wall formation (Stobbe *et al.* 2002; Chen *et al.* 2014), or of parenchyma cells, the main component of ray tissue (Li & Cui 1988; Zhang *et al.* 2011). While callus has long been regarded as a dedifferentiated and pluripotent cell mass (Zhang *et al.* 2011), findings in *Eucommia ulmoides* Oliv. have suggested that tissue or cell regeneration did not need complete dedifferentiation to a pluripotent state (as callus), and that new sieve elements could originate from the trans-differentiation of immature axial xylem cells (Pang *et al.* 2008).

Bark regeneration appears to be related to the size of debarking area. Small-scale debarking led to the regeneration of periderm and wound cambium starting from surface callus, followed by phloem derived from wound cambium (Stobbe *et al.* 2002). After large-scale girdling, sieve elements and regenerated cambium directly developed from differentiating xylem cells rather than from callus (Pang *et al.* 2008; Zhang *et al.* 2011; Chen *et al.* 2014).

In the present experiments, cambium and immature xylem cells were successfully removed at the time of girdling, and no proliferation of immature xylem cells was found at the wound surface. Ray tissues densely distributed in the secondary xylem of *A. sinensis*, were exposed to the air after girdling, but no callus tissue formed from the ray tissues. Therefore, it was not cambium, immature xylem or ray tissues, but the IP tissue that responded to the stress due to girdling in *A. sinensis*. However, no detailed studies have been carried out on the time course of the redifferentiation of IP strands after complete girdling in *A. sinensis*.

According to the earlier work, IP strands located in the three outermost growth rings were alive without any collapse of sieve tubes in the ten years old *Aquilaria* tree (Luo *et al.* 2018). In the girdling specimens used in this study, the IP strands were distributed over the entire secondary xylem in 15~20 layers with an almost concentric pattern. At this point, the central part of the IP strands, distributed in the third to the fifth layers from the pith, has partially collapsed, but the other IP strands were still functional.

Whether it was a trunk or branch, peeling the bark resulted in serious stress such as water loss and pressure change in the xylem, which could act as a stress signal that was communicated within different parts of a tree (Thompson & Holbrook 2003; De Schepper & Steppe 2011). In *A. sinensis*, soft and fragile IP strands in the secondary xylem sensed the abnormal stress in the first week after girdling and immediately responded through various structural deformations.

Partial collapse of component cells of all IP strands in the secondary xylem was a common response. Specifically, hollowed IP strands were often observed in a narrow zone from the first to the third IP layers from the girdled edge. This was the initial and crucial response that was closely related to the further development of IP strands and the regeneration of vascular tissue at a later stage. Cells that made up the IP strands were broken down possibly triggered by the pressure change, resulting in the formation of hollowed IP, which was a sign of the disintegration of IP strands for protecting the peripheral layer of secondary xylem tissue that was adjacent to the girdled edge.

Cell chains extending from one IP strand to others, and redifferentiation of parenchyma cells on both adaxial and abaxial sides of IP strands were efficient ways for quick proliferation of the IP strands. These events happened due to the sudden loss of water and pressure change (Thompson & Holbrook 2003; De Schepper & Steppe 2011) and resulted in the recovery and enhancement of alternative conductive paths consisting of inner side IP strands. In *Quercus robur* L., with either complete or partial girdling of bark, lateral sugar transportation was enhanced after wounding, by a change in the lateral nutrient transport path (De Schepper *et al.* 2013), which was known as the leakage-retrieval mechanism (Ayre *et al.* 2003; van Bel 2003; Gould *et al.* 2004).

In *A. sinensis*, radial sieve tubes were the links between dispersed IP strands, which formed a 3D network of sieve tubes (Luo *et al.* 2020). In this species, girdling was virtually partial peeling, and bark removal did not interrupt the phloem transport pathway. In the case of an active change in the original translocation pathway, the amount of energy delivered by assimilates would temporarily increase to boost the structural changes (De Schepper *et al.* 2013), which could make the plant quickly trigger cell division and callus formation.

A prominent structural deformation of IP strands was the increase in resinous cells along with the dark resinous lines, associated with a folded and resinous cell wall layer. This deformation of IP strands occurred in the second week after girdling, when sporadic cork cells were produced at the outer margin of IP strands, which predicted the location where a periderm would be formed in the later stage. Several studies have suggested that suberization started very early in younger tissues compared to the mature stem. For example, in twigs of *Picea abies*, *Thuja prientalis* and *Metasequoia glyptostroboides*, suberization started around day 10 after wounding; in seedlings of *Pinus sylvestris*, suberization started around day 8 after wounding; and in *Abies alba*, suberin was detectable in some lignified cells by day 16 after wounding (Rittinger *et al.* 1987; Wahlstrom & Johansson 1992; Oven & Torelli 1994).

4.4.2. Intensive differentiation of interxylary phloem

Another prominent structural deformation of IP strands started with the cell junction followed by the fusion of two or more IP strands, mainly by tangential or diagonal bridges of cell chains and infrequently by radial bridges of ray tissues, which facilitated IP strands to develop quickly into enlarged, confluent and further banded strands. Furthermore, when IP strands and their derivatives such as enlarged, confluent and banded IP strands increased their mass, they had to occupy more space. To make the space for expansion, IP strands needed to break the secondary xylem, which consisted of thick-walled and lignified cells, and kept growing by two different processes.

The first process involved breaking the secondary xylem tissue by invasion or intrusive growth of IP-cell tips through intercellular layers and capturing pieces of the secondary tissues, including xylem fibres, vessels and rays. The second process involved the proliferation of component parenchyma cells of IP strands on both the abaxial and adaxial sides to produce potential sources of phloem tissue on the abaxial side of IP strands and wound xylem tissue on the adaxial side, possibly for the intensification and protection of the fragile tissues like parenchyma and meristematic cells. While these two processes occurred almost simultaneously, the enlarged, confluent and banded IP strands became larger by penetrating the cracks in the broken tissues to eventually make room for a continuous ring of regenerated secondary tissues.

During the continuous expansion, there is no evidence to suggest that the secondary xylem patches captured by eIP or cIP strands would be degraded by enzymes or any other means. Furthermore, the number of secondary xylem fragments captured by these IP strands was limited, and not as many as one might imagine. This was supported by the following observations. The captured fragments of the secondary xylem remained as part of the eIP or cIP strands with the same angular shape as when they were captured (referring to long arrows in Figure 4.6d; black asterisks in Figure 4.7c; asterisk in Figure 4.8c). In addition, most of the other secondary xylem tissues escaped from capturing and were pushed outside the cIP and bIP strands.

The conspicuous proliferation of parenchyma cells, like the case in *Aquilaria*, was reported in lianas (Cabanillas *et al.* 2017). Parenchyma destroyed or cleaved lignified tissues into several isolated fragments of different sizes and was therefore named disruptive parenchyma. These ruptures made empty spaces formed within the secondary xylem. Eventually, the disruptive parenchyma invaded these spaces and continued to proliferate. This event was very similar with the proliferation of both enlarged and confluent IP strands that were seen in *Aquilaria*.

The disruptive parenchyma started to proliferate in all directions in the lianas stem. However, a series of proliferations of eIP and cIP strands were performed just in one to three IP layers inside from the girdled edge, corresponding to a 400~1000 μm deep zone. This depth seemed to be programmed to allow the IP strands to destroy the secondary xylem less than 1 mm distant from the girdled surface and proliferate further until the regeneration of secondary tissues in case the bark was lost.

Although IP strands were sensitive to mechanical stress, such as peeling off the

bark, no deformation of IP strands was observed except those in a narrow zone. IP deformation could be regulated by chemical signals, such as IAA, which was implicated in many signal transduction pathways. Most of the IAA synthesized in mature leaves was transported throughout the plant via the phloem. In woody plants, auxin transport also occurred in the vascular cambium (Uggla *et al.* 1996). There was a risk that the flow of auxin might be obstructed after peeling the bark.

However, it has been demonstrated that radial sieve tubes commonly presenting in ray tissues of *A. sinensis*, together with axial sieve tubes presenting in IP strands of the species formed a three-dimensional network that appeared to be useful for the transport of auxin as well as photosynthates (Luo *et al.* 2020). This potential capacity of IP strands to make a banded IP circumferentially by successive tangential connections and proliferation of parenchyma cells in such a narrow zone right under the branch surface was a principal event throughout the complete regeneration of vascular cambium. This unexpected and efficient ability of IP strands was revealed here for the first time.

4.4.3. Regeneration of vascular cambium and phellogen

It was a prerequisite for any tree to regenerate vascular cambium and phellogen to maintain sustainable growth and protection against other living organisms. After peeling off the bark, regeneration of vascular cambium and phellogen have different chronological sequences in different species. According to some reports, the formation of a ligno-suberized layer in the bark was a precondition for the growth of a wound periderm (Novitskaya 1998; Oven *et al.* 1999; Chano *et al.* 2015). The wound periderm differentiated in the callus tissue before the differentiation of vascular cambium. Following the establishment of the wound periderm, a wound cambium was formed in lime trees (*Tilia* sp.) by the differentiation of callus tissue, possibly as the result of the growing radial pressure, due to the increasing thick tissue layer and the wound periderm (Stobbe *et al.* 2002), or the stimulation by endogenous IAA released from its combined form (Mwange *et al.* 2003).

In particularly, it was found in *Broussonetia papyrifera* that the concentration of endogenous IAA, which was mainly distributed in viable tissues or cells and the nascent callus, played an important role in triggering the differentiation of vascular tissue (Mwange *et al.* 2003). On the other hand, in some subtropical tree species in Asia, the wound periderm and the wound cambium were shown to be formed almost simultaneously (Li & Cui 1988). However, the restoration of vascular cambium in *Eucommia ulmoides* Oliv began earlier. At 15 days after girdling, discontinuous cambial cells appeared under newly formed sieve elements and an intact cambium was formed 21 days after girdling (Pang *et al.* 2008).

In *A. sinensis*, meristematic cells with an ordered arrangement were observed in some confluent IP strands until two weeks after girdling, and cork cells with a sparse or layered arrangement were produced in confluent IP strands nearly at the same time. This synchronous situation of two meristems was maintained thereafter. Therefore, it was likely that regeneration of vascular cambium and phellogen in *A. sinensis* proceeded in synchrony.

4.4.4. Differentiation of wound xylem before the regeneration of vascular tissue

Throughout the intensive differentiation of debarked branches one to four weeks after girdling, IP strands increased their bulk by cell division of the component parenchyma cells mainly on the adaxial side and infrequently on the abaxial side, resulting in the production of three types of derivatives before the regeneration of vascular cambium. The derivatives included new parenchyma cells, resinous cells and new tracheary elements. The larger the volume of IP strands, the larger were the strands of tracheary elements. The thick-walled tracheary elements exhibited average length and width almost similar with those of the surrounding parenchyma cells and were organized as wound xylem two weeks after girdling. These sclerotic tracheary elements with simple perforations in the end or lateral walls and occasionally reticulate thickenings in lateral walls, formed a belt of wound xylem at the advanced stage of differentiation. Considering above observations, it was confirmed that the tissue composed of sclerified wound xylem occasionally associated with reticulate tracheary elements was unique and quite different from the other examples described for wound xylem formation after injury (Stobbe *et al.* 2002).

It was worth noting that the component parenchyma cells in IP strands performed not only as the food reserve, but that they also had the meristematic nature of the cambium, which was revealed after wounding by girdling. IP strands in healthy *A. sinensis* were differentiated from vascular cambium during the development of secondary xylem (Luo *et al.* 2018). The regeneration of vascular cambium started and ended exclusively from the redifferentiation of IP strands after girdling.

In previous publications on debarking, the vascular cambium was suggested to regenerate directly from the differentiation of callus tissue, which was formed by different types of cells such as parenchyma cells, immature xylem cells and ray cells. In the present investigation, the component parenchyma cells in IP strands were found to be involved in the formation of wound xylem, and its further differentiation in advance of the regeneration of vascular tissue suggested that parenchyma cells of IP strands played a novel and crucial role during the recovery of cambium and its derivative secondary tissues.

Finally, from a different perspective, this study has investigated the process of wound healing in branches of *Aquilaria* that created IP strands. According to Shigo (1984), compartmentalization in trees was a defense process, by which boundaries were formed to isolate the injured tissues and thus resist the spread of pathogens. In agarwood-producing species, all such boundaries were constructed exclusively in the IP strands from beginning to end. In this sense, agarwood-producing species had a unique barrier protection mechanism.

4.5. Summary

The time course and structural features of stem response within six months following complete girdling in branches of *A. sinensis* were examined to determine the potential role of IP tissues in this mechanically derived response.

The time course study showed that IP strands in the narrow zone of 400 to 1000 μm deep from the girdled surface, connected to each other, merged tangentially and become larger through continuous proliferation of parenchyma cells in them.

It was elucidated that the vascular cambium, as well as its derivative secondary xylem and phloem, regenerated fully through redifferentiation of IP strands. It was confirmed that vascular cambium regenerated within one month after girdling based on observation of new vessels, IP strands, primary and secondary phloem fibers.

The regenerated processes of secondary tissues proved that IP tissues compensated the functions of the external secondary phloem. *A. sinensis*, one of the representative agarwood-producing species evolved efficient mechanisms for injury response and tissue regeneration.

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Chapter 5 Structure, origin and function of radial sieve tube

5.1. Introduction

Commonly in agarwood-producing species such as *Aquilaria* and *Gyrinops*, interxylary phloem (IP) strands are isolated from each other and embedded in the secondary xylem as scattered islands. Even in the vertical direction, the terminations of the IP strands do not connect to the other IP strands. So far, it is not known as to how the sieve tubes in an isolated IP strand could translocate photosynthates to those in other IP strands. One possibility is the presence of ray parenchyma tissues passing through the discrete phloem islands in secondary xylem. Pfautsch *et al.* (2015) suggested that functional links between xylem and phloem transport could be facilitated by radially aligned and interconnected ray parenchyma tissues. Whether this suggestion is applicable to the agarwood-producing species that produce IP for facilitating the translocation of photosynthates has not been explored.

As summarized in earlier reports, radial sieve tube elements have been found in different genera and have been grouped into at least three categories depending on the mechanism by which they are formed. The three categories are: (1) naturally developed radial sieve elements in secondary phloem (Chavan *et al.* 1983; Rajput & Rao 1997; Rajput 2004b; Angyalossy *et al.* 2012); (2) naturally developed radial sieve elements in secondary xylem (den Outer & Veenendaal 1981; Lev-Yadun & Aloni 1991; Angyalossy *et al.* 2012; Gondaliya & Rajput 2016); and (3) artificially or traumatically induced radial sieve elements (Sharma *et al.* 1980; Aloni & Barnett 1996).

Although there is a lack of inherent correlation between the occurrence of radial sieve tubes and the families, genera, and morphologies of investigated samples (herbs, shrubs or trees) (Rajput *et al.* 2022), a considerable number of genera with IP tissues have also differentiated radial sieve tubes (Den Outer & Van Veenendaal 1981; Lev-Yadun & Aloni 1991; Van Veenendaal & Den Outer 1993; Rajput & Marcati 2013; Gondaliya & Rajput 2017). Based on the aforementioned literature, the feasibility of discrete IP strands in agarwood-producing species being connected by sieve tubes presenting in ray tissues to form a continuous transport network remains an unresolved issue, until sieve tubes have been confirmed in ray tissues of the secondary xylem in *Aquilaria sinensis*, *A. malaccensis*, *A. crasna* and *Gyrinops versteeghii* (Thymelaeaceae). The present study describes the occurrence and structure of radial sieve tubes and discusses their function in *Aquilaria* and *Gyrinops*, the two well-known agarwood-producing species.

5.2. Materials and Methods

5.2.1. Materials

The materials studied came from different species, provenances, and stem diameters (Table 5.1). All samples were immediately fixed in 4 % formaldehyde after being collected.

Table 5.1. A list of agarwood specimens used in this study.

Specimen No.	Material	
	Stem diameter	Species, age and provenance
I	1st to 15th internodes	Collected from the main stem of a 10-year old tree of <i>Aquilaria sinensis</i> cultivated in the greenhouse of Southwest Forestry University, Kunming, P.R. China.
II	3 cm diameter branch	Collected from the main stem of a 10-year old tree of <i>A. sinensis</i> cultivated in the plantation of Guangdong, P.R. China.
III	Wood blocks	Used in our previous studies (Luo <i>et al.</i> 2018) and collected from the main stem of a 10-year old tree of <i>A. sinensis</i> cultivated in the same plantation as II.
IV	1st to 10th internodes	Collected from a branch and stem of 6-year old trees of <i>G. versteeghii</i> , growing in the plantation of Lombok island, Indonesia.
V	1.2 cm diameter branch	Collected from the same tree described in IV.
VI	Wood blocks	Collected at breast height from the main stem of <i>A. crasna</i> , <i>A. microcarpa</i> and <i>A. malaccensis</i> planted in an agarwood plantation in Pekanbaru, Indonesia.

5.2.2. Optical, fluorescence and SEM observation

Transverse, radial and tangential sections of 6~8 μm thickness, from different internodes embedded in paraffin, were obtained using a rotary microtome (Leica RM2145). Meanwhile, transverse, radial and tangential sections of 15~25 μm thickness were obtained from small shoots and blocks embedded in polyethylene glycol (PEG) 1500 using a sliding microtome (Yamato-Koki Co Ltd, TU-213).

For fluorescence microscopy observation, sections were stained with 0.005 % aniline blue aqueous solution in 0.15 M K_2HPO_4 at pH 8.2 for 30 min at room temperature (Currier & Strugger 1956). For double staining, sections were stained with 0.5 % safranin for 4 h followed by 1 min 0.5 % fast green staining, or with compound dye including 1.0 % safranin and 0.5 % astra blue for 4 h.

Some of the aniline blue stained sections with marked sieve tubes used for fluorescence microscopy were washed and dehydrated by the normal procedures prior to freeze drying. After freeze drying, the sections were coated with platinum at 30 mA

for 90 s by means of an Ion Spattering Apparatus (JEOL JEC-3000FC) and examined by field emission scanning electron microscope (FE-SEM) (JEOL JSM-7800F) at 2.0 kV accelerating voltage with a 9~10 mm working distance.

5.2.3. Statistical analysis of the number of radial sieve tube strand

For calculation of the number of radial sieve tube strands and IP tissues in the secondary xylem, serial cross sections were made from material (V). For counting the number of sieve tube strands, preliminary testing was carried out, indicating that aniline blue staining coupled with fluorescence microscopy gave the best result. Therefore, twenty-five serial sections were stained with aniline blue and the number of radial sieve tube strands was counted using fluorescence microscopic pictures. After this step, all slides were washed with deionized water and double-stained with a mixture of safranin and astra blue. Then, the cross-sectional images were photographed with a stereoscopic microscope (Wild M3Z) and the whole area of the secondary xylem in individual sections was measured with image J. Furthermore, the number of IP strands was counted directly on the figures of transverse sections.

5.3. Results

5.3.1. Young stem structures of *Aquilaria* and *Gyrinops*

Figure 5.1 shows typical transverse sectional images of the fourteenth internode in *A. sinensis* and the sixth internode in *G. versteeghii*. Taking the more developed structure in Figure 5.1a as an example, approximately, four layers of IP strands were embedded in the secondary xylem (long double-headed arrow) which were surrounded by the secondary phloem (short double-headed arrow), being interrupted by the cambium. Primary xylem (arrow) and intraxylary phloem tissues (circles showing some of them) were located inside, between secondary xylem and pith. The size and distribution of IP strands in the two genera did not show noticeable differences. The presence of IP strands with tangential widths wider than 400 μm and axial lengths longer than 10 mm was very common. It was also common that one to fifteen rays passed through individual IP strands depending on their tangential widths.

The structure and development of IP and intraxylary phloem tissues in *G. versteeghii* were like to the earlier observations on *A. sinensis* (Luo *et al.* 2018; Luo *et al.* 2019). Furthermore, not only the location and developmental sequences of IP strands, but also the occurrence and structural characteristics of radial sieve tubes were also similar in both genera.

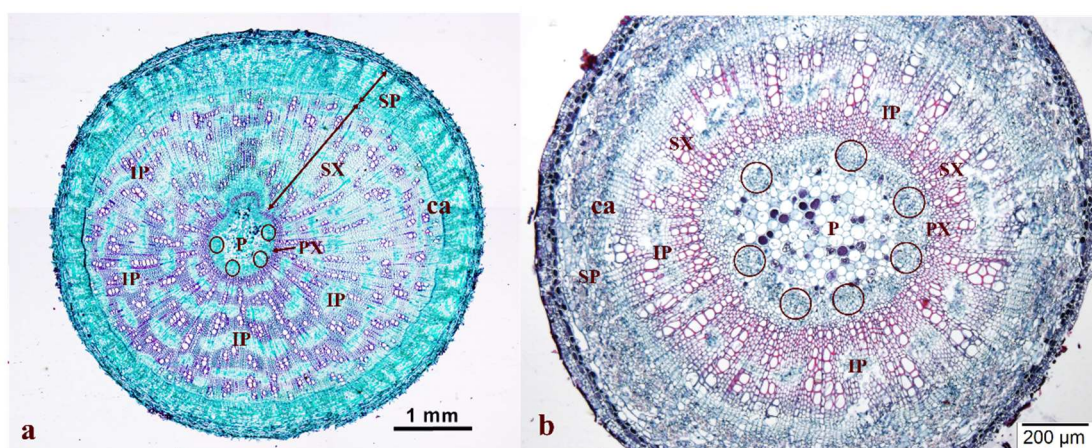


Figure 5.1. Transverse sections of the fourteenth internode in *A. sinensis* (a) and the sixth internode in *G. versteeghii* (b) show the general anatomies of the both genera. ca: cambium; IP: interxylary phloem; P: pith; PX: primary xylem; SX: secondary xylem; SP: secondary phloem; circles: intraxylary phloem.

5.3.2. Occurrence of radial sieve tube

The differentiation of secondary xylem and IP strands in *A. sinensis* and *G. versteeghii* initiated in the second and the third internodes respectively. Development of radial sieve tubes was delayed compared to the axial ones located in IP strands in these two species. Radial sieve tubes were rarely observed in serial cross sections obtained from the third, the sixth, and the eleventh internodes of *A. sinensis*; however, one radial sieve tube strand was found in a transverse section of the fifteenth internode of *Aquilaria*.

Occasionally, radial sieve tubes in *G. versteeghii* were observed between the first and the second layers of IP strands adjacent to pith in the secondary xylem (dotted ellipse in Figure 5.2a). It meant that after the development of IP, radial sieve tubes immediately began to be produced. Another radial section of a sixth internode of *G. versteeghii* revealed a radial sieve tube strand consisting of two elements, which initiated differentiation in the cambial zone (Figure 5.2b). The figure shows two sieve plates that exhibited bright fluorescence in the end walls of a radial sieve element (dotted ellipse), indicating that radial sieve tubes, like the IP strands, could be directly produced by the division of vascular cambium.

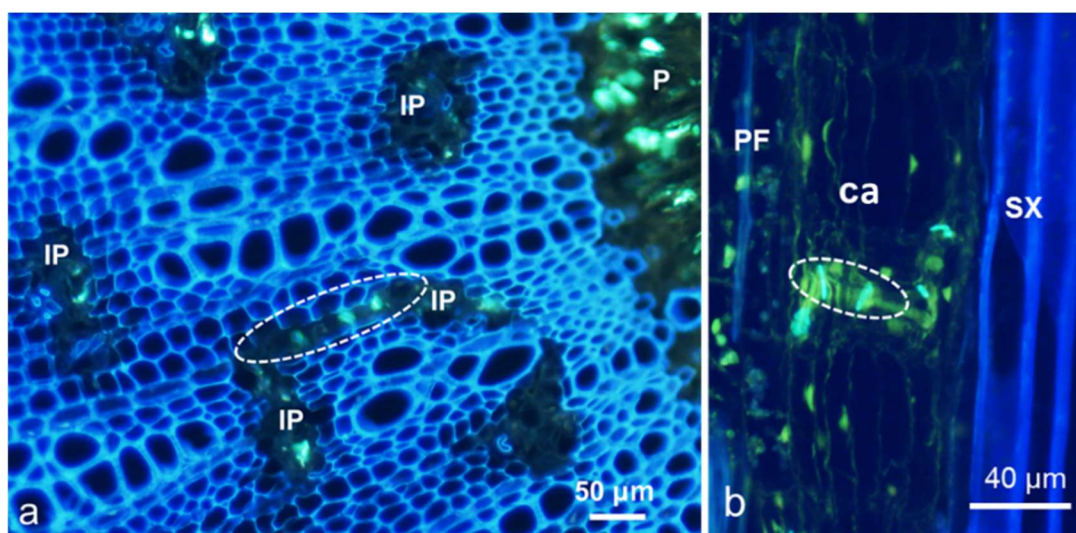


Figure 5.2. Fluorescence microscopic images of *G. versteeghii* taken after aniline blue staining.
a: Cross section of a 1.2 cm-diameter branch shows the occurrence of a radial sieve tube strand (dotted ellipse) bridging two IP strands close to pith. IP: interxylary phloem; P: pith.
b: Radial section of the sixth internode shows a differentiating radial sieve tube strand (dotted ellipse) in the cambium. ca: cambium; SX: secondary xylem; PF: secondary phloem fibre.

Figure 5.3a shows a tangential section of the ray tissue in the secondary xylem at a distance around 8~10 mm from the cambium. At least three ray tissues were observed in the IP, but the cell walls of these tissues were unligified and therefore only faintly visible in outlines. The four bright spots emitting fluorescence were derived from callose accumulated on the sieve plates of the radial sieve tubes. The presence of another two fluorescent spots in the ray tissue adjacent to the IP (arrow) indicated that radial sieve tubes were present in this ray tissue and were surrounded by largely unligified ray parenchyma cells except for eight marginal cells. All ray tissues except those inside the IP and adjacent to the IP were ligified. Several multiseriate rays were observed at the outer margin of IP, all of which had scattered ligified parenchyma cells (arrows in Figure 5.3b).

Observation of the tissues adjacent to the pith of the innermost xylem blocks confirmed that all axial and ray parenchyma cells of the IP strands were largely unligified throughout the growth rings. Figure 5.3c shows a transverse section of the secondary xylem near the pith of a disk (maximum diameter 14 cm, minimum diameter 12.5 cm) collected from a 7-meter-high trunk. As indicated by the arrows, most of the phloem cells of the IP strands had collapsed. Areas stained green indicated the presence of unligified cells, while the areas containing secondary xylem tissues were stained reddish-purple, indicating that the cells were ligified. As shown in Figure 5.3c with the ellipse, the cell walls of the ray tissues that occupied by radial sieve tubes were thin walled unligified.

Tangential sectional images of the unligified ray tissues at a distance around 8~10 mm from the cambium show cell divisions of single ray parenchyma cell in the biseriate ray (arrow in Figure 5.3d) and of five ray parenchyma cells in the three-seriate ray (arrows in Figure 5.3e). As seen in these examples, cell divisions of the ray parenchyma cells in immature ray tissues were often observed throughout the present study.

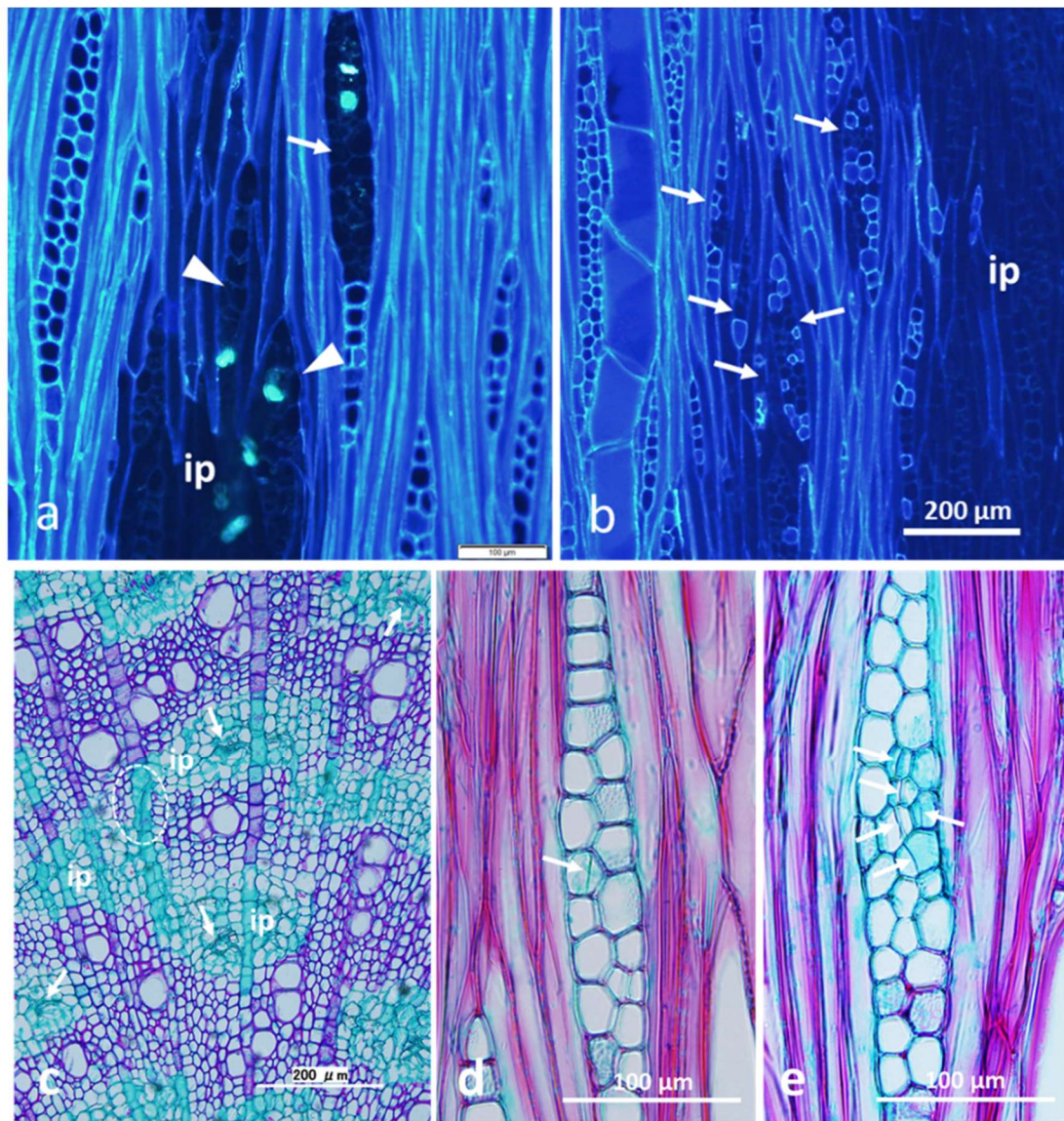


Figure 5.3. Fluorescence and double stained microscopic images show that unignified ray parenchyma cells present in both IP strands and secondary xylem and that unignified elements in ray tissues are undergoing division and producing incipient radial sieve tubes.

a: No fluorescence in the ray tissues inside the IP except bright spots from four sieve plates. A ray tissue close to IP (arrow) is unignified except for marginal ray cells.

b: Ray tissues inside IP are completely unignified, but ray tissues in the outer margin of IP (arrows) show mixture of lignified and unignified ray parenchyma cells.

c: Cross sectional image of secondary xylem near the pith shows collapsed phloem cells in IP strands, lignified xylem tissues and unignified ray tissues containing sieve tubes (ellipse).

d: Tangential section shows division of a ray parenchyma cell (arrow) in a biseriate ray.

e: Tangential section shows almost simultaneous divisions of five ray parenchyma cells (arrows) in a multiserial ray.

In Figure 5.4a, three nascent radial sieve tubes (short arrows) with companion cells that appeared to have differentiated were observed. At the same time, a large radial sieve tube with differentiated sieve plates on both end walls (black and white arrowheads) was observed in the same ray of the secondary xylem. The latter radial sieve tube was considered to be more mature than the former, because it was larger in size and had differentiated sieve plates in both end walls. The sieve plate indicated by

the black arrowhead was connected to an axial sieve tube (asterisk). Incidentally, the latter radial sieve tube strand consisted of only single sieve element with sieve plates on both end walls bridged by veil-like band (long arrow).

Furthermore, two types of radial sieve tubes were observed in tangential section of another multiseriate ray at 6~8 mm away from the cambium (Figure 5.4b). One type consisting of two radial sieve tube elements, was larger in size and appeared to have differentiated almost simultaneously (long arrows), while the other was slightly smaller and intermingled between the larger ones and appeared to be more recently differentiated nascent radial sieve tube (short arrow).

As a result of intensive divisions, radial sieve tube clusters composed of four or more elements sometimes could be observed in multiseriate ray tissues. SEM and fluorescence microscopy images of the corresponding positions in Figures 5.4d, 5.4e and 5.4f show that a cluster of radial sieve tubes appeared in the multiseriate ray at 8~10 mm away from the cambium, which consisted of four sieve tube elements. The radial sieve tubes were unusually aggregated in this ray tissue. The blue fluorescence of the cell wall just below the aggregated sieve tubes (asterisk in Figure 5.4f) indicated the presence of a lignified fiber in ray tissue. Subsequent observations revealed that phloem fibres frequently extended horizontally into ray tissues, which were found to be due to the fibres normally extending vertically into the IP strands, but for some unknown reasons, the fibre tips bent horizontally and elongated parallelly to the ray tissues or radial sieve tubes (arrowhead in Figure 5.4c).

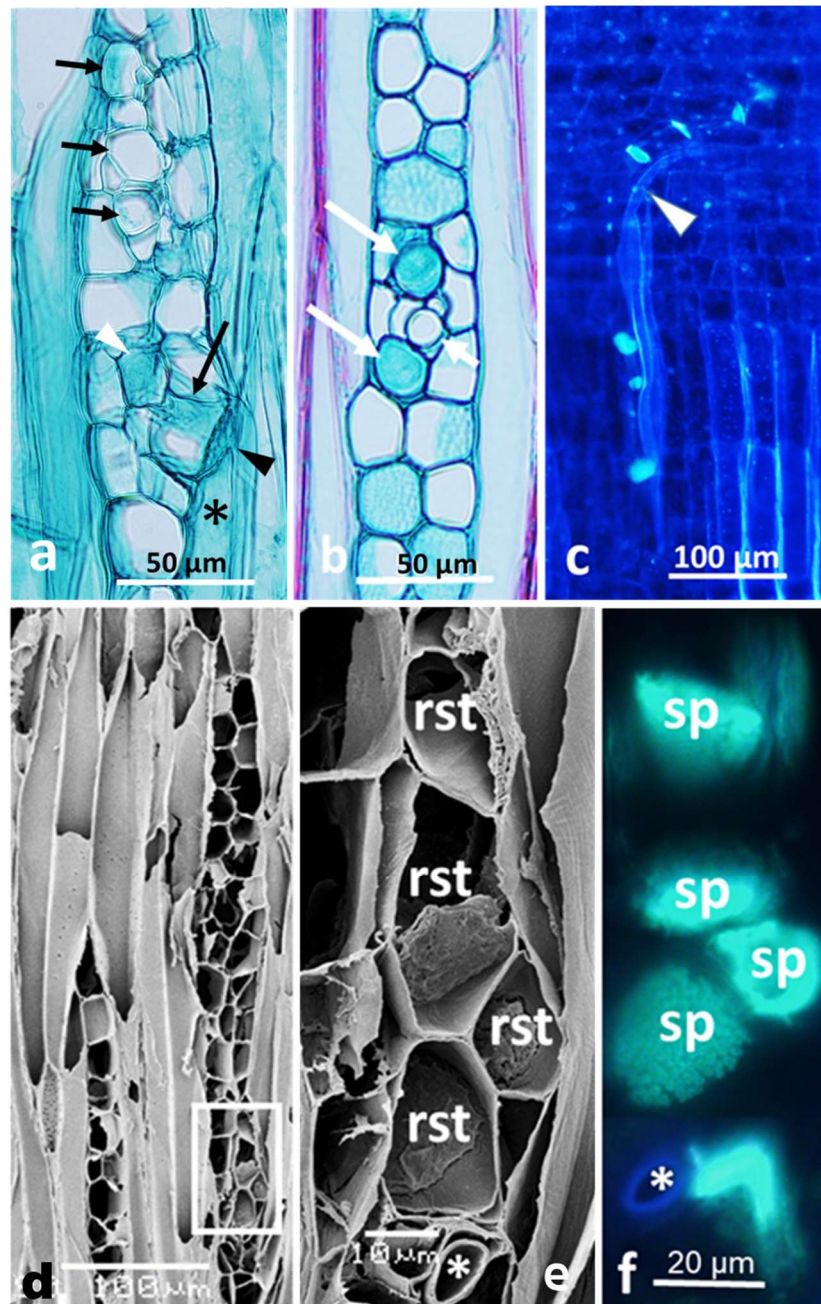


Figure 5.4. Double stained, fluorescence stained and SEM images show the incipient and differentiated radial sieve tube clusters in ray tissues.

a and b: Two types of radial sieve tubes are observed simultaneously in the ray tissues of differentiating secondary xylem, one showing immature radial sieve tubes (short arrows) with companion cells and the other showing more differentiated sieve tubes (long arrows) with sieve plates and veil-like banded slime. Asterisk: axial sieve tube; black and white arrowheads: sieve plates.

c: Fluorescence microscopic image shows that tip of a fibre (arrowhead) intrudes radially parallel to the sieve tube in ray tissue.

d: SEM image of a multiserial ray at 6~8 mm away from cambium shows a cluster of radial sieve tubes (rectangle).

e: Higher magnification SEM image of the rectangle in c, shows four radial sieve tubes with different sizes of sieve pores. rst: radial sieve tube; asterisk: lignified fibre.

f: The four radial sieve tubes corresponding to e, show strongly fluorescing sieve plates and a lignified fiber with blue fluorescence. sp: sieve plate; asterisk: lignified fibre.

5.3.3. Structural features of radial sieve tube

Radial sieve tubes were detected as several bright dots by callose staining on sieve plates in *Aquilaria* and *Gyrinops*. These radial sieve tubes were found in uniseriate as well as in multiseriate rays embedded in secondary xylem and they served as bridges between two or several independent IP strands. Uniseriate rays that included sieve tube elements were slightly wider than the others (Figure 5.5a). Multiseriate rays in *Aquilaria* and *Gyrinops* sometimes showed a group of two to four or more radial sieve tube strands that occurred side-by-side in parallel rows (Figure 5.5b). Most of these sieve tubes had slender cytoplasmic bodies and showed slightly twisted or bent shapes. Every radial sieve tube element was commonly associated with 1~2 companion cells and formed a functional unit similar with the axial sieve tube elements in IP strands.

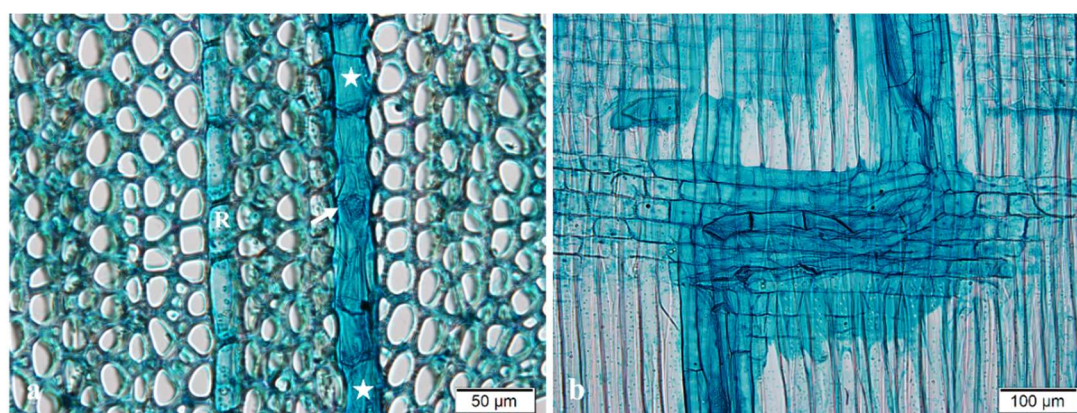


Figure 5.5. Cross and radial sections show radial sieve tube strands in ray tissues of *A. malaccensis*. a: Cross section shows a radial sieve tube strand (stars) which is wider than the normal uniseriate ray (R). Arrow: sieve plate. b: Radial section shows multiple and parallel radial sieve tube strands within the same ray tissue.

Generally, radial sieve tube elements were shorter than axial sieve tube elements, and lengths of most radial sieve tubes were about 40~60 μm . However, some of them were only 20 μm in length. Diameters of most radial sieve tubes were about 15~25 μm and some of them exceeded 30 μm . Measured from tangential sections, the average area of 50 radial sieve tubes was $412 \pm 152 \mu\text{m}^2$. Usually, radial sieve tubes were connected to each other through the simple sieve plates on their end walls, forming radial transport channels of varying lengths.

A SEM image in Figure 5.6a and the corresponding fluorescence microscopic image in Figure 5.6b show a long radial sieve tube strand bridging two IP strands in the early stage of secondary xylem differentiation, which was at a distance around 300~600 μm from the cambium (inset in Figure 5.6a). Fluorescence emitted from the sieve pores was weak compared to those of more matured sieve plates (Figures 5.3a and 5.4f). It should be noted that Figure 5.6b was taken with a longer exposure time to clearly display the positions of the sieve plates. The fluorescence microscopic image combined with SEM show that most of the sieve pores of the four sieve plates could be identified and were filled with small amount of callose (inset in Figure 5.6b, a higher magnification of the sieve plate, no.4), but nearly no open holes were observed in the center of the sieve pores. However, circular outlines of the sieve pores were faintly

visible in the sieve plate (arrows). In addition, some parts of the sieve plate and the cytoplasmic slime bodies detached from the sieve plate retained the round outlines of the sieve pores (arrowheads).

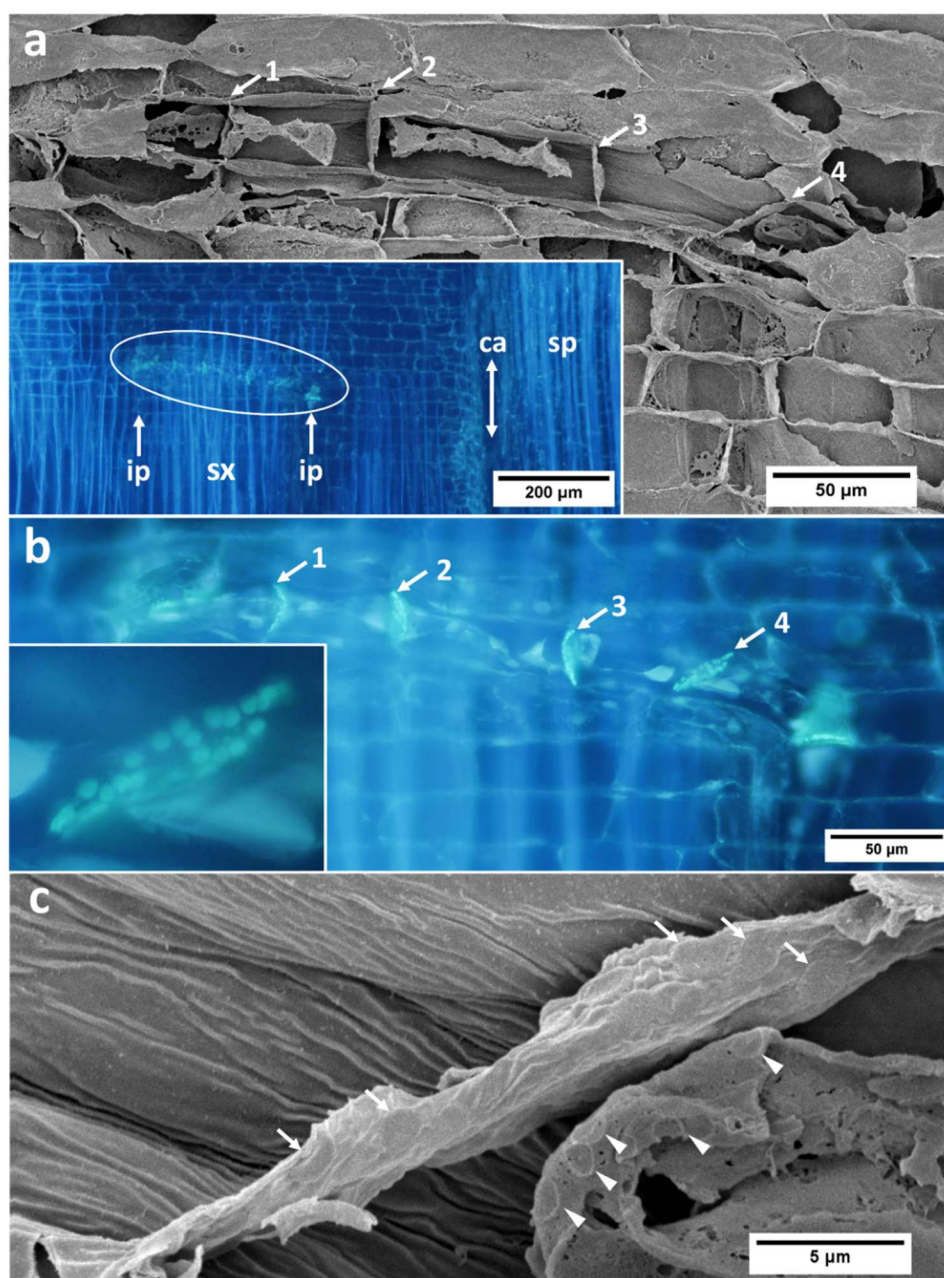


Figure 5.6. Fluorescence microscopic and SEM images of nascent radial sieve tube strand close to cambium.

a: SEM image shows 4 nascent sieve plates (arrows 1 to 4) in the early stage of differentiation observed on radial section. Inset: fluorescence microscopic image of the same sieve tube strand as a, taken at a distance around 300~600 μm from cambium. ca with double-headed arrow: cambium; ip: interxylary phloem; sp: secondary phloem; ellipse: differentiating radial sieve tube strand.

b: Fluorescence microscopic image taken at the same position as a. Inset shows enlarged image of the No.4 sieve plate with unopened sieve pores.

c: Higher magnification image of the No.4 sieve plate in a and b, shows circular outlines (arrows) and impressions (arrowheads) of sieve pores on the cytoplasmic slime bodies detached from the sieve plate.

The shape of sieve pores and the size of filamentous or tubular slimes penetrating the pores seemed to be considered as practical indicators of the maturity of radial sieve tubes. Therefore, the sieve plates of three radial sieve tubes in mature secondary xylem at distances of 6~8 mm from cambium were characterized.

The sieve plate in Figure 5.7a was covered with a dense cytoplasmic slime body, and a branched filamentous slime (arrow) extending from the slime body penetrated the sieve pores. The sizes of most filamentous slimes passing through the pores were 0.2~0.4 μm . The distribution of the sieve pores was uneven, with sparse and even no sieve pores in some areas (Figure 5.7b). The sizes of the sieve pores ranged from 0.2 to 0.6 μm , and the outlines of the callose collars ranged from 0.7 to 1.0 μm .

In Figure 5.7c, the sieve plate was covered with dense cytoplasmic slime body and thick tubular slimes penetrated the sieve pores. The sieve pores were 1~2 μm in size and the outlines of their callose collars were 1.5~2.5 μm . The larger sieve pores and thicker tubular slimes in Figure 5.7c indicated that the radial sieve tube was in a more mature state. At nearly the same distance from the cambium, appearance of different developmental sieve tubes indirectly proved that radial sieve tubes were not only derived from the vascular cambium, but also could be redifferentiated in the secondary tissues.

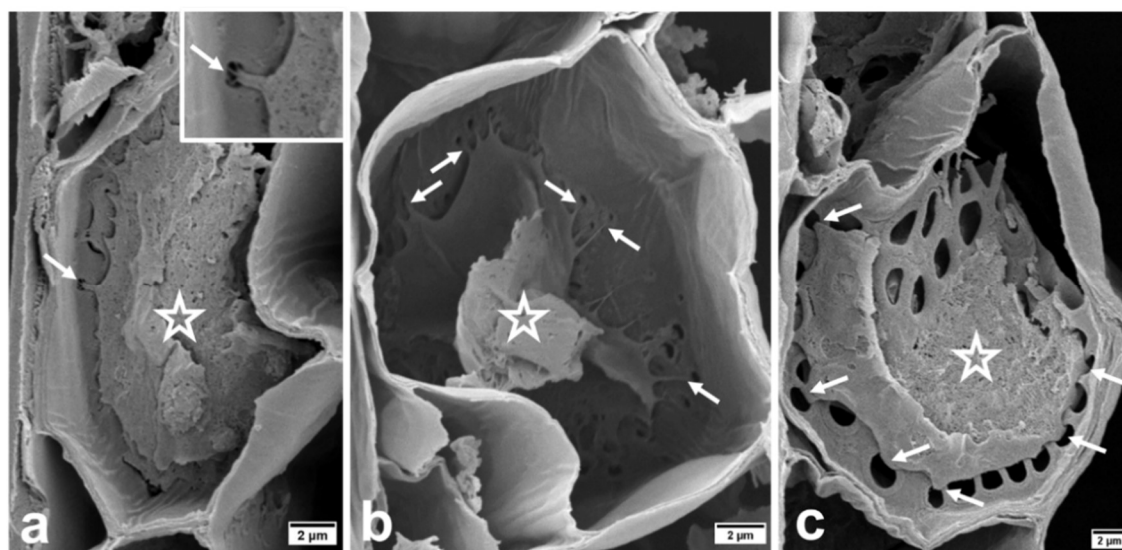


Figure 5.7. SEM images of sieve plates of radial sieve tubes in the secondary xylem at 6~8 mm away from the cambium.

- a: Dense cytoplasmic slime body (star) almost covers the entire sieve plate. Branched tubular slime is shown by an arrow (inset).
- b: Numerous branched filamentous slimes penetrate the sieve plate.
- c: Thick tubular slimes penetrate large sieve pores. Star: cytoplasmic slime body; arrows: tubular slimes of different sizes.

Two different types of connecting areas were observed on the lateral wall of radial sieve tube, depending on the size and shape of the sieve pores. One type, as shown in Figure 5.7, was more common on the simple sieve plate of the radial sieve tube, with wide callose collars and highly differentiated large pores (asterisk in Figure 5.8), while the other type consisted of minute sieve pores scattered or slightly clustered on the lateral wall of the radial sieve tube (dotted square in Figure 5.8). Inset on the upper right

of Figure 5.8 is a magnified image of the scattered minute sieve pores. These sieve pores were approximately the same size as those found on the lateral wall of axial sieve tube and were generally distributed along the axis of radial sieve tube, rather than being evenly scattered throughout the cell wall. Due to the limited deposition of callose around the tiny pores, the fluorescence emitting from them was relatively weak after the same staining procedure.

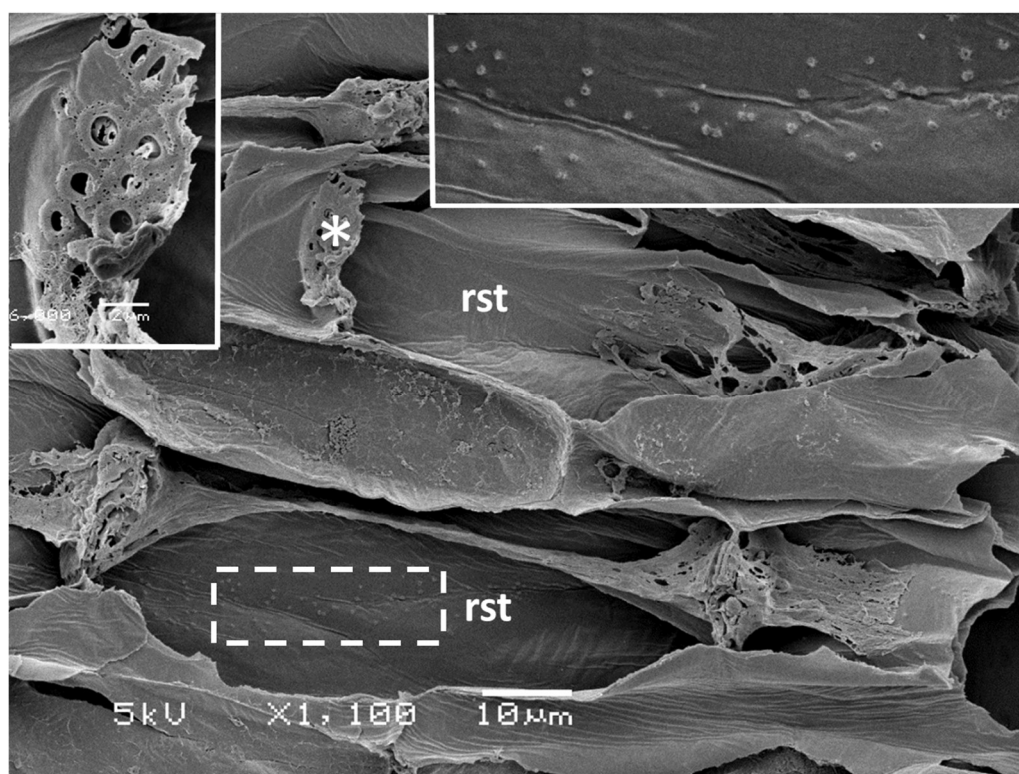


Figure 5.8. Two radial sieve tube strands parallelly cross the ray parenchyma strands showing two types of connecting areas on their cell walls. Radial sieve tube in the lower side shows scattered minute sieve pores in the lateral wall. rst: radial sieve tube; asterisk: sieve plate; inset on the left shows enlarged image of sieve plate in the upper radial sieve tube strand, while inset on the right shows enlarged image of the dotted square filled with scattered minute sieve pores.

5.3.4. Frequency of radial sieve tube strand in the secondary xylem

The radial sieve tube strands in cross sections were observed at a relatively low frequency compared to that of IP strands in all the samples examined in this study. The frequency of radial sieve tube strands and IP strands was investigated in a branch wood with 1.2 cm diameter of *G. versteeghii*, which already produced a large amount of IP strands. The number of radial sieve tube strands were counted from 25 serial cross sections stained by aniline blue and the number of IP strands was counted from 9 cross sections. Further, the area occupied by secondary xylem was measured in transverse sections after staining with compound dye of safranin and astra blue.

Table 5.2 shows the results of measurements. Usually, radial sieve tubes were connected to the axial sieve tubes embedded in IP tissues. They frequently bent or deviated from the ray tissue direction, making it difficult to accurately count the number of radial sieve tube elements. Instead, the frequency mentioned here was characterized

by the number of continuous radial sieve tube strands. The total surface area measured in 25 transverse sections was 2811.9 mm². The total number of radial sieve tube strands was found to be 97, irrespective of their length. The average number of radial sieve tube strands per mm² was 0.034. Fifty of 20 µm-thick sections could be cut in 1 mm-thick secondary xylem. Therefore, 1.7 radial sieve tube strands were calculated per mm³. On the other hand, total number of IP strands was 11050 in total area of 1214.7 mm². The average number of IP was calculated as 9.1 per mm².

Table 5.2. The number of radial sieve tube strands and IP strands in the secondary xylem of *G. versteeghii* counted on sections.

Serial section number	Area of secondary xylem (mm ²)	Number of radial sieve tube strand	Number of IP strand
1	121.4	4	–
2*	121.3	3	1113
3*	121.3	1	1105
4*	117.5	0	1105
5*	122.9	2	–
6*	120.1	5	1095
7*	125.1	3	1102
8*	119.2	6	–
9*	124.2	6	1102
10*	120.2	6	–
11*	121.3	3	–
12*	121.3	7	1109
13*	121.3	5	1098
14*	121.3	5	1114
15*	121.3	6	1107
16	108.7	4	–
17	91.7	0	–
18	83.5	4	–
19	108.4	1	–
20	102.1	3	–
21	84.9	6	–
22	90.9	4	–
23	111.7	6	–
24	108.4	2	–
25	101.9	5	–
Total	2811.9	97	11050

– No measurement of IP strands number.

*Complete section without defects in the secondary xylem.

It was worth mentioning that connection between radial and axial sieve tubes was a common feature in all the species of *Aquilaria* and *Gyrinops* that were investigated. The frequency of radial sieve tube strands was reasonably related to the morphology,

dimension and distribution of IP in the secondary xylem. The fine structural characteristics, the phloem anastomoses composed by the radial and axial sieve tubes and their influence on the frequency of different types of connections would be further discussed in detail in Chapter 6.

5.4. Discussion

The study sheds light on the origin, developmental process, cellular structure, distribution and frequency of radial sieve tubes in the ray tissues of five common agarwood-producing species in *Aquilaria* and *Gyrinops* (Thymelaeaceae), in order to comprehensively understand the structural characteristics and compositions of the phloem network in these species developing diffuse-typed IP tissue and the ability related to agarwood production.

5.4.1. General features of radial sieve tube

Ray tissue generally consisted of parenchyma cells, but other elements such as tracheids, vessels, resin ducts and sieve elements also could be found in rays of various species (Lev-Yadun & Aloni 1995; Angyalossy *et al.* 2016). Particularly, occurrence of sieve tube elements in ray tissues was not a rare feature in the agarwood-producing species of *Aquilaria* and *Gyrinops*. The combined observations confirmed the existence of sieve tubes in rays in three species of *Aquilaria* and one species of *Gyrinops*. These two genera are commonly characterized by the development of dispersed IP strands in secondary xylem. When radial sieve tubes were observed, most of them had connections with the IP strands.

As early as 1981, den Outer and Van Veenendaal reported the existence of radial sieve tubes and companion cells in very wide aggregate rays of *Azima tetraacantha* (Salvadoraceae). However, the study did not confirm the direct connection between axial and radial sieve tubes. Additionally, unlike *Azima*, *Aquilaria* and *Gyrinops* generally produce uniseriate as well as multiseriate rays including solitary and/or multiple radial sieve tube strands, but it was more common to find 1~3 sieve tubes in one multiseriate ray.

Lev-Yadun and Aloni (1991) suggested a new type of xylem ray with many “ray centers” in *Suaeda monoica* (Amaranthaceae). In some huge rays, radial strands of phloem were observed, and the number of phloem strands increased with the enlargement of the xylem ray in the centrifugal direction. Compared with the multicentric distribution of sieve tubes in huge aggregated ray of *Suaeda monoica*, tangential sections of all the studied samples of *Aquilaria* and *Gyrinops* showed radial sieve tubes mostly as a solitary cell/strand or in small groups like those found in the five tropical species reported by Rajput (2004). Radial sieve tubes generally developed from the margin as well as the center of the ray tissues, and they were observed in contact with axial sieve tubes. The present observations of radial sieve tubes in secondary xylem were consistent with those in Rajput’s (2004) study on secondary phloem.

5.4.2. Occurrence of radial sieve tube

IP tissues in *Aquilaria* and *Gyrinops* usually started to develop in the lower part of the third internode in young shoots and grew to about four to five layers in the fourteenth internode. The earliest radial sieve tube strand was observed in the secondary xylem adjacent to the pith, immediately following the differentiation of the first layer of IP strands. In the previous study, it has been confirmed that the first-formed IP strand appeared in the third or the fourth internode (Luo *et al.* 2019). It meant that the initiation of radial sieve tube differentiation should not be later than the development of the fourth internode. Considering the discontinuous distribution of IP strands in the secondary xylem, once the island-like phloem strands began to develop, they needed to be involved into a phloem network for performing the transportation function. Therefore, it was very reasonable for the radial sieve tube to be formed immediately following the production of IP.

Regarding the origin of radial sieve tubes, the sieve tube strand observed in the sixth internode of *G. versteeghii* differentiated in the vascular cambium and emitted weak green fluorescence (Figure 5.2b). The strand was composed of only two sieve elements, and each end of the strand seemed to connect with an axial sieve tube strand through common sieve plate, which emitted dim fluorescence. Due to the fact, that the incipient connection strand was found in the cambium zone, it was reasonable to believe that radial sieve tubes could be directly generated from the vascular cambium and the radial sieve tubes were already connected with the axial ones in the early stage of development.

In general, IP tissues included multiple layers of axial parenchyma cells and several lines of ray parenchyma cells crossing the strands, in addition to axial sieve tubes, companion cells and fibres. The cell walls of all the parenchyma cells in the IP strands remained unlignified for several years after the surrounding secondary xylem differentiated and matured from the cambium and even all the phloem fibres in IP strands have become lignified. This feature allowed the ray parenchyma cells contained in IP strands, in other words the ray cells that passed through or were adjacent to IP, to keep the possibility of redifferentiation.

According to the literature, radial sieve tubes have been documented to differentiate from ray cells in phloem tissue of various species of angiosperms (Gondaliya & Rajput 2017; Rajput & Rao 1997; Rajput 2004b; Rajput *et al.* 2022). In the stems of *Coccinia indica* L., axial parenchyma cells dedifferentiated into meristem, which later redifferentiated into IP tissues (Patil *et al.* 2011). Some parenchyma cells in the secondary xylem of liana and shrubs that formed earlier during vegetative growth redifferentiated into sieve elements (Rajput 2002; Rajput *et al.* 2013). Furthermore, parenchyma cells could directly develop into xylem elements (Aloni 1987; Lev-Yadun & Aloni 1995) and sieve elements (Galatis 1991; Eleftheriou 1993; Lev-Yadun & Aloni 1995). It was also proposed by den Outer and Van Veenendaal (1981) that IP strands differentiated from parenchyma cells in *Azima tetracantha*. In *A. sinensis*, intraxylary phloem elements have been reported to differentiate from the parenchyma cells that constituted the pith (Luo *et al.* 2019). All the researches convincingly demonstrated that radial sieve tubes in agarwood-producing species could originate not only from the

vascular cambium but also from the unlignified ray parenchyma cells.

5.4.3. Structure of radial sieve tube

There were many structural similarities between radial and axial sieve tubes in *Aquilaria* and *Gyrinops*. First of all, both axial and radial sieve tubes were always surrounded by layers of unlignified parenchyma cells throughout the secondary xylem. Secondly, both were associated with one to several, most commonly 1~3 companion cells, forming sieve tube-companion cells (SE-CCs) functional groups. Additionally, they were connected to each other through the sieve plates at end walls, forming axial and horizontal transport channels of varying lengths, but compound sieve plate with a very sharp angle to the cell axis has not been found in radial sieve tubes, which was a common feature of axial ones. On sieve plates, the sieve pore sizes and the collars composed by callose deposition were also very similar in the two types of sieve tubes.

Regardless of the types of sieve plates, the end walls of axial and radial sieve tubes both had highly differentiated and wide sieve pores, which exceed the pores in the lateral walls, some of them larger than 1 μm in diameter. The single or branched slime bodies passing the pores as revealed in Figure 5.7 might play a role in increasing the intercellular connectivity (Amsbury *et al.* 2018).

Independent from the sieve pores constituting sieve plates, the occurrence of minute sieve pores in the lateral walls of radial sieve tube strands in both *Aquilaria* and *Gyrinops* showed the following features: scattered or clustered sieve pores were surrounded by callose deposits and lined up in a direction parallel to the cytoplasmic flow in the radial sieve tube strand. The distribution of minute sieve pores in nearly horizontal direction rather than all over the lateral walls of radial sieve tubes, suggested a more effective transport pathway between the sieve tube and the surrounding ray parenchyma cells, especially in the case of *Aquilaria* and *Gyrinops* that produced uniseriate or biseriate rays. Metabolite transport might occur through the minute sieve pores as an alternative to pore-plasmodesmata units described by Cayla *et al.* (2015).

5.4.4. Frequency of radial sieve tube

After measuring the area of secondary xylem in a small branch of *G. versteeghii* and counting the number of radial sieve tube strands, it was found that a complete cross section included only four radial sieve tube strands on average (Table 5.2). Compared to the occurrence of a great number of IP strands that included more than one axial sieve tube strands per IP, the number of radial sieve tube strands presenting in a cross section seemed almost negligible for serving the translocation of photosynthates.

It was suggested that radial sieve tubes might be temporarily involved in the short-distance rapid transport of food nutrients under stress derived from injuries or other environmental changes, as have been discussed in the cases of radial sieve tubes in the secondary phloem of five tropical tree species (Rajput & Rao 1997; Rajput 2004b). However, it was meaningful to recall the very long axial length of IP strands. For instance, the average length of IP strands in *A. sinensis* was 14 ± 4 mm according to the former results (Luo *et al.* 2018) and the IP strands longer than 10 mm were very

common in *G. versteeghii* (Karmilawati *et al.* 2021).

The significant number of radial sieve tube strands and IP strands, that was 1.7 per mm³ and 9.1 per mm² respectively, presenting enough to connect each other in the secondary xylem. Further, the number of radial sieve tube strands would potentially increase with the increase in thickness of the secondary xylem, which suggested a considerable number of connections between axial and radial sieve tubes. Considering the fact, that IP strands were completely isolated from each other in the secondary xylem and none of the axial sieve tube strands in independent IP had direct contact, the presence of diversified connections between radial sieve tubes in the ray tissues and axial sieve tubes in IP strands could play a significant role in the translocation of metabolites.

5.5. Summary

The radial sieve tubes were newly observed in the secondary xylem of two genera and four species of agarwood, *A. sinensis*, *A. crasna*, *A. macrocarpa*, *A. malaccensis* and *G. versteeghii*. The earliest radial sieve tube was formed in the secondary xylem immediately following the differentiation of the first-formed IP. The radial sieve tube could be directly differentiated from vascular cambium and produced by redifferentiation of unlignified parenchyma cells in ray tissues. Radial sieve tube developed in both uniseriate and multiseriate ray tissues.

In addition to the highly differentiated sieve plates in lateral and end walls, solitary and clustered sieve pores localized in the lateral walls of radial sieve tubes were found to be new channels of photosynthate and other nutrients. The most striking evidence in this study was the direct connections between radial sieve tubes in ray tissues and axial sieve tubes in IP strands.

The average number of radial sieve tube strands per a cross section with an average area of 121.3 square millimeter was 4. Despite its low frequency of occurrence, the presence of direct connections between radial and axial sieve tubes could be effective to serve for the translocation of reserve food materials in the secondary xylem of *Aquilaria* and *Gyrinops*.

Chapter 6. Phloem network created by axial and radial sieve tubes

6.1. Introduction

It was widely recognized that agarwood-producing species could synthesis useful aromatic substances and it has been reported that a whole-tree inducing technique for agarwood production has been developed (Liu *et al.* 2013) and interxylary phloem (IP) is involved in the production of these substances (Liu *et al.* 2019). However, it was a mystery how discrete IP could contribute to the efficient production of useful substances. The principal function of IP depends on the presence of axial sieve tubes, while the formation of a continuous phloem network relies on their connections with radial sieve tubes. Therefore, it is prerequisite to clarify the fine structures and connections of sieve tubes for fully understanding the functions of IP and the composition of continuous phloem network of agarwood-producing species.

To begin with, observation of the normal tissues of agarwood-producing species showed that IP strands were formed from vascular cambium (Luo *et al.* 2018) and scattered as islands in the secondary xylem tissue. Meanwhile, radial sieve tubes were found in *Aquilaria sinensis*, *A. crasna*, *A. macrocarpa*, *A. malaccensis* and *Gyrinops versteeghii* (Thymelaeaceae) (Luo *et al.* 2020). According to the literature, radial sieve tubes have been reported in the secondary phloem and xylem of several species (Den Outer & Van Veenendaal 1981; Lev-Yadun & Aloni 1991; Rajput 2004b). However, the presence of radial sieve tubes in secondary tissues is considered as a rare feature, possibly due to their involvement in the transient and quick transport of photosynthates from the phloem to the xylem (Rajput & Rao 1997; Rajput 2004a).

Subsequent experiments with agarwood-producing species revealed that the active action of IP strands immediately after bark peeling, resulted in spectacularly vigorous and rapid repair of the cambium and vascular tissues (Luo *et al.* 2021). The quick repair and regeneration of tissues is an energy consuming process that relies on abundant and efficient nutrient supply, which depends on the well-developed phloem network in agarwood producing species.

Based on these experiences, it was planned to combine the following two experiments to investigate the details of the phloem transport channels in agarwood-producing species, taking *Aquilaria* as an example. The first is to investigate the relationship of radial and axial sieve tubes with the slime bodies, whose structure and function are not fully understood (Azizpor *et al.* 2022). The second is to clarify the types of connections between radial and axial sieve tubes in the IP strands for understanding the highly sophisticated phloem network in the secondary xylem of *A. sinensis*.

6.2. Materials and Methods

6.2.1. Materials

Stem disks measuring about 3~13cm in diameter and 1~3 cm in thickness were collected at different heights of 2, 3, 4, 5, 6 and 7 m in July 2021, from a 12-year-old tree of *A. sinensis* cultivated in the plantation in Zhongshan, Guangdong, P.R. China. After collection, the stem disks were immediately fixed in 4 % formaldehyde in PBS buffer at pH7.4. Wood blocks of *A. malaccensis* were the same ones used in Chapter 5.

6.2.2. Optical, fluorescence microscopic and SEM observation

From all disks, wood blocks were cut into cubes approximately 1 cm per side, including bark. After they were embedded in PEG2000, transverse, radial and tangential sections of 20~30 μm thickness were prepared with a sliding microtome.

Firstly, half of the sections were stained with 1.0 % safranin and 0.5 % astra Blue for 1 hour or 1 % safranin in 50 % ethanol overnight, followed by 0.5 % fast green in 95 % ethanol for 6 min. The sections were then washed with deionized water, and microscope slides prepared by conventional methods (Luo *et al.* 2018) were observed under an optical microscope. Secondly, another half of the sections were stained with 0.005 % aniline blue aqueous solution for 30 min to detect the exact locations of the sieve tubes. The sections were mounted and observed under a fluorescence microscope with a filter set (Olympus U-MWU2).

To observe the submicroscopic structures of sieve plates or sieve pores and the slime bodies penetrating sieve pores, a part of the sections was dehydrated through serial passages in different concentrations of *t*-butyl alcohol and air-dried. Dried sections were sputter-coated with gold using an ion sputtering apparatus (JEOL JFC-1100E) and observed using a scanning electron microscope (JEOL JSM-6060) at an accelerating voltage of 5 kV.

6.3. Results

6.3.1. Structures of axial and radial sieve tubes related to phloem anastomoses

The developmental process of IP strands from vascular cambium has been reported in previous works (Luo *et al.* 2018; Luo *et al.* 2020), but the characteristics of axial sieve tubes during differentiation have not been addressed. At this part, the observation focused on the structural changes of axial sieve tubes during cambium original IP production. As a key hub, the structural development of axial sieve tubes had a crucial significance, which endowed IP tissues combined into the continuous transport channel.

Figure 6.1a shows an immature IP and a nascent axial sieve tube strand that has just been differentiated from the cambium. Arrowheads indicate immature phloem fibres located just outside the cambium. The weak blue fluorescence was emitted from the cell walls of differentiated and lignified secondary xylem and phloem, and the weak green fluorescence (arrows in Figure 6.1a) was emitted from the sieve plates of the axial and radial sieve tubes in the immature IP strand (double-headed arrow), which was mostly consisted of the cells with unligified walls. Almost no fluorescence was detected from these cells and the IP strand appeared dark.

Unopened and/or slightly opened sieve pores with weak green fluorescence were clustered tightly in some of the sieve plates observed in the immature IP and the diameter of individual pore with collar, was around 1.0~1.5 μm (inset in Figure. 6.1a). Callose stained with aniline blue was deposited in the periphery of the sieve pores. Occasionally, two or more sieve pores were surrounded by the same callose collar.

Fluorescence microscopy image of another differentiating sieve tube strand revealed that very often a veil-like band of slime containing a P-protein-like structure bridged the sieve plates at both ends of the nascent sieve tube element (Figure 6.1b). When axial or radial sieve tubes were observed under a fluorescence microscope, a veil-like band of cytoplasmic slime body frequently appeared to bridge the sieve plates at both ends of sieve tubes. As shown in Figure 6.1c, the cytoplasmic slime body bridging two end walls of the shorter radial sieve tube strand was distinct, while the slime body bridging the end walls of the longer radial sieve tube strand in Figure 6.1d was dim or translucent.

No such veil-like band was detected in either axial or ray parenchyma cells. In addition, the cytoplasmic slime bodies have never been found in the senescent sieve tubes with mesh like sieve plates, which already lost their regulatory function for nutrients transport. In other words, cytoplasmic slime bodies were essential and only appeared in the active staged sieve tubes for performing the physiological functions of conductive phloem.

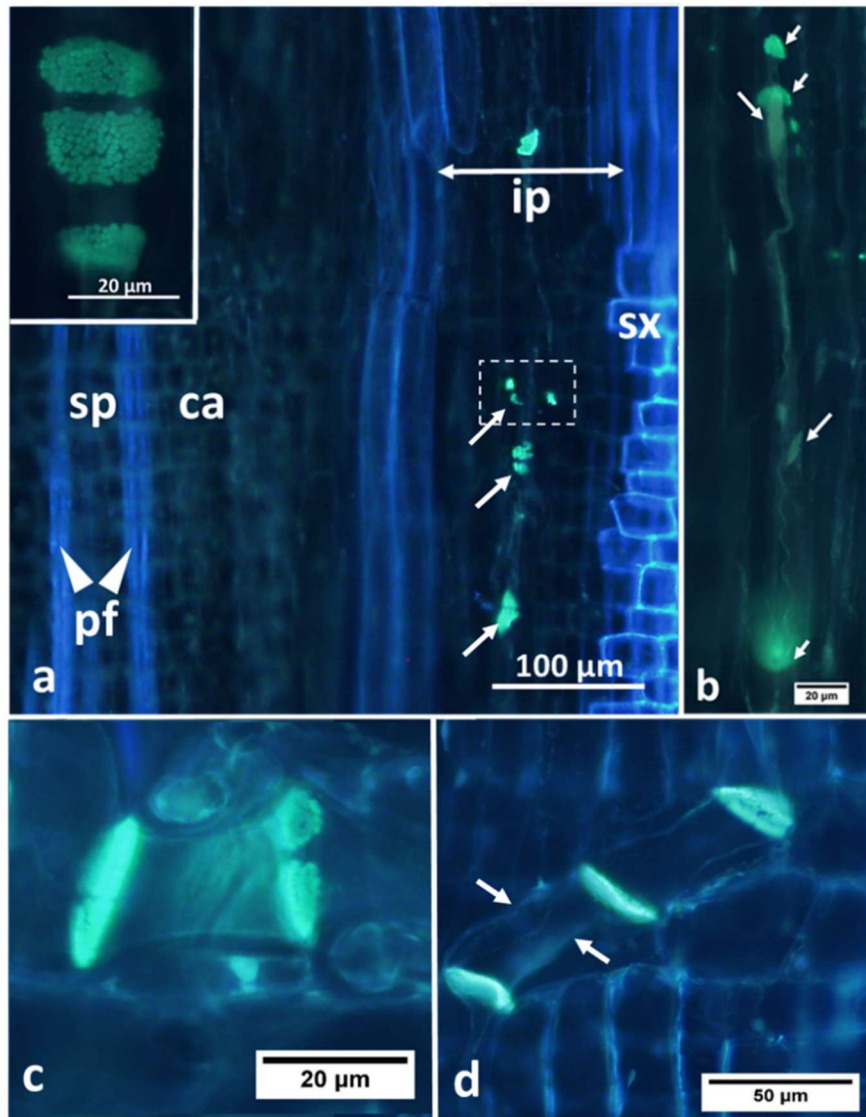


Figure 6.1. Fluorescence microscopic images of newly formed IP strand, sieve plates and cytoplasmic slime bodies in axial and radial sieve tubes.

a: Newly formed IP strand in differentiating secondary xylem. Arrows: sieve plates; arrowheads: secondary phloem fibres; ca: cambium; ip and double-headed arrow: interxylary phloem; sp: secondary phloem; sx: secondary xylem; dotted square: radial sieve tubes; inset: enlarged image of immature compound sieve plate.

b: An immature axial sieve tube with bridging of elongated slime body between two sieve plates. Short arrows: sieve plates; long arrows: P-protein.

c and d: Veil-like slimes bridge sieve plates of radial sieve tube strands. Veil-like slime in c is distinct, while that in d is nearly translucent. Arrows: veil-like slime.

The distance between the axial sieve tube and cambium shown in Figure 6.2a was around 1 mm, but the figure still shows the characteristics of early developmental sieve tubes. The axial sieve tube possessed a very thin sieve plate with a thickness of 0.5 μm and several tiny pores through which slender filamentous slimes passed. Arrows point to the filamentous slimes. Some of them were broken, one end still connected to the membrane and the other end connected to the sieve plate (Figure 6.2a). Inside the inner surface of the sieve tubes marked with stars again showed SEM image of cytoplasmic

slime bodies

In Figure 6.2b, taken at about the same distance away from the cambium as Figure 6.2a, it was noted that filamentous slimes protruding from the sieve pores connected to cytoplasmic membranes (arrows). Sizes of sieve pores in the sieve plate were quite small, with diameters of 0.3~0.5 μm (Figure 6.2b). The ring-like bulge collars formed by callose deposit around sieve pores were not very thick but flat and wide. Sizes of these sieve pores containing callose collars were approximately 1.5~2 μm in diameter. Due to the very wide callose collars around sieve pores, some of them were interconnected to each other. Filamentous slimes protruded from these sieve pores and connected to the membrane that covered the cytoplasmic slime body occupying the inner part of the sieve plate.

While as another sieve tube which was located at 8~10 mm away from the cambium exhibited more mature characteristics (Figure 6.2c). The thickness of its sieve plate was more than 1 μm or close to 1.5 μm . In particular, the filamentous slimes passing through the sieve plate appeared much thicker than those shown in Figure 6.2a and 6.2b. The slimes with this shape were henceforth referred to as tubular slimes.

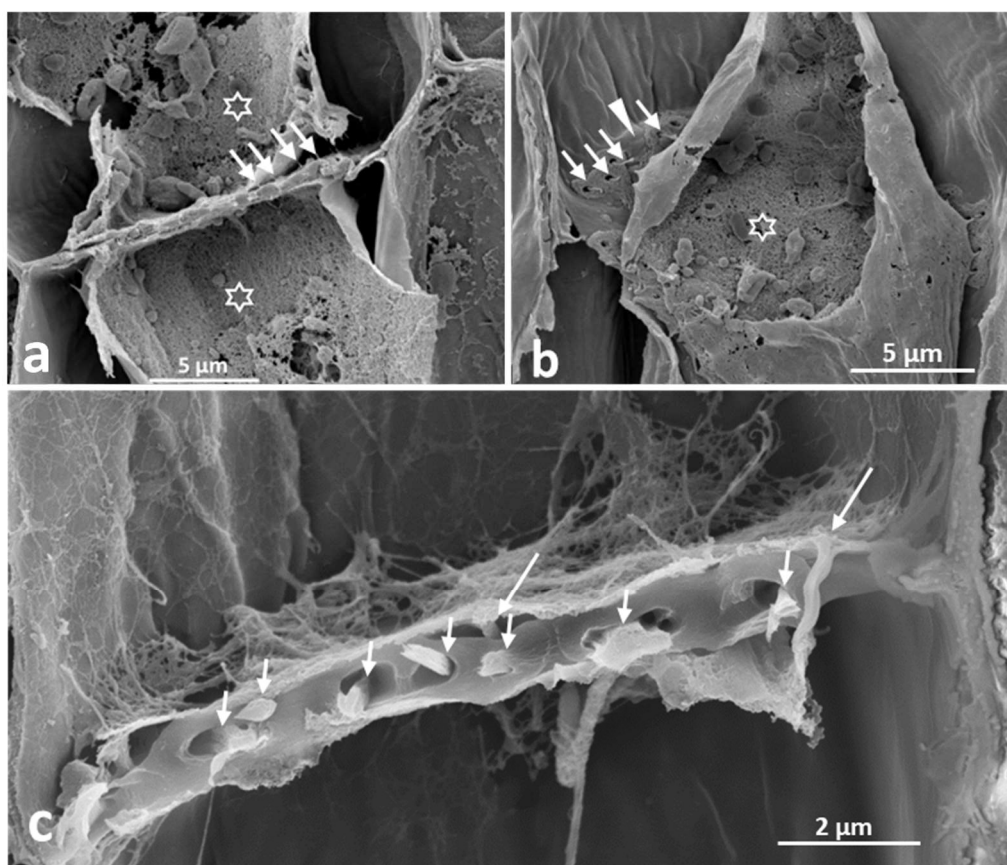


Figure 6.2. SEM images of sieve pores, sieve plates and filamentous slimes in different developmental stages of axial sieve tubes.

- a: Filamentous slimes protrude from nascent sieve pores (arrows). Stars: cytoplasmic slime body.
- b: Sieve plate (arrowhead) and filamentous slimes protrude from immature sieve pores (arrows). Star: cytoplasmic slime body.
- c: Tubular slimes penetrate across sieve pores (long arrow) and inside the sieve plate (short arrows).

Two types of connecting areas occurred in the cell walls of axial sieve tubes due to differences in the size and shape of the sieve pores. One type was composed of highly differentiated large pores, mainly located in the simple and compound sieve plates of sieve tubes as described above. Due to the strongly inclined end walls of some axial sieve tube elements, a few of these sieve areas looked like appearing in lateral walls.

Figure 6.3a shows two axial sieve tubes side by side. At least five connecting areas (arrows) were observed in the strongly inclined end wall of the sieve tube on the left, and three in the end wall of another sieve tube on the right. In all sieve areas, clusters of sieve pores with about 1 μm in diameter were observed. Each pore had a ring-shaped raised collar, which emitted fluorescence when stained with aniline blue (inset in Figure 6.3a), indicating the presence of thick callose deposits. One such kind of area with higher magnification shows distinct and thick tubular cytoplasmic slimes passing through sieve pores, making a highly differentiated sieve area (Figure 6.3b). The size, structure and arrangement of sieve pores in these sieve areas were alike to those of simple sieve plates on the horizontal end walls. Furthermore, the two sieve plates located at the end and lateral walls of the same axial sieve tube (Figures 6.3d and 6.3e) showed no differences in their structures, such as the thickness of the sieve plates and the size of the sieve pores.

Another type of connecting sieve area (Fig. 4a) was observed in the lateral walls of axial sieve tubes, consisting of scattered and/or clustered minute sieve pores with diameters smaller than 0.1 μm (Figure 6.4a). Figure 6.4b shows a higher magnification of one of the sieve areas consisting of clustered minute sieve pores. Each cluster was composed of several to dozens of minute sieve pores. Individual pores were surrounded by slightly raised collars, which emitted weak green fluorescence under UV light (Figure 6.4c), indicating small amount of callose deposits in and around the pores. These sieve pores were mainly concentrated along the cell axis of the sieve tube element. Even after differentiation and maturation of the secondary xylem, the clustered minute sieve pores were often found to be axially interconnected by the veil-like slimes along the lateral walls of sieve tubes, not only leading to the sieve plates on the upper and lower end walls, but also to the other sieve tubes above and below (Figure 6.4d).

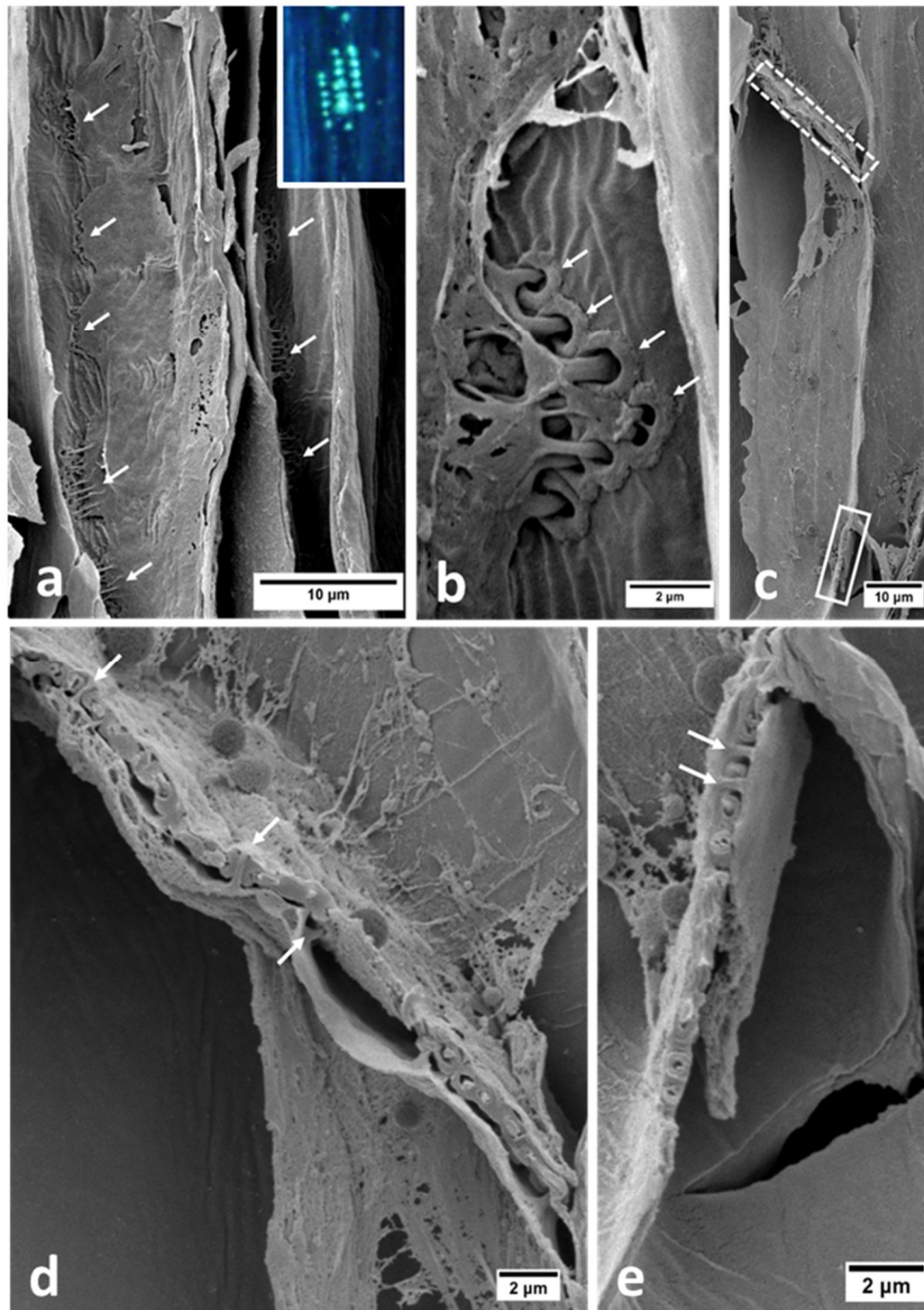


Figure 6.3. SEM images of differentiated sieve areas in axial sieve tubes.

a: Highly differentiated sieve areas (arrows) present in strongly inclined scalariform sieve plates of axial sieve tubes. Inset: fluorescent microscopic image corresponds to strongly inclined scalariform sieve plates.

b: Enlarged image of a sieve area, with thick tubular slimes penetrating through clustered sieve pores. Arrows: sieve pores with thick callose collars.

c: Two sieve plates present in the same axial sieve tube; one at the end wall and the other at the lateral wall.

d and e: Enlarged images of the sieve plates in the end wall (dotted rectangle in c) and the lateral wall (solid rectangle in c).

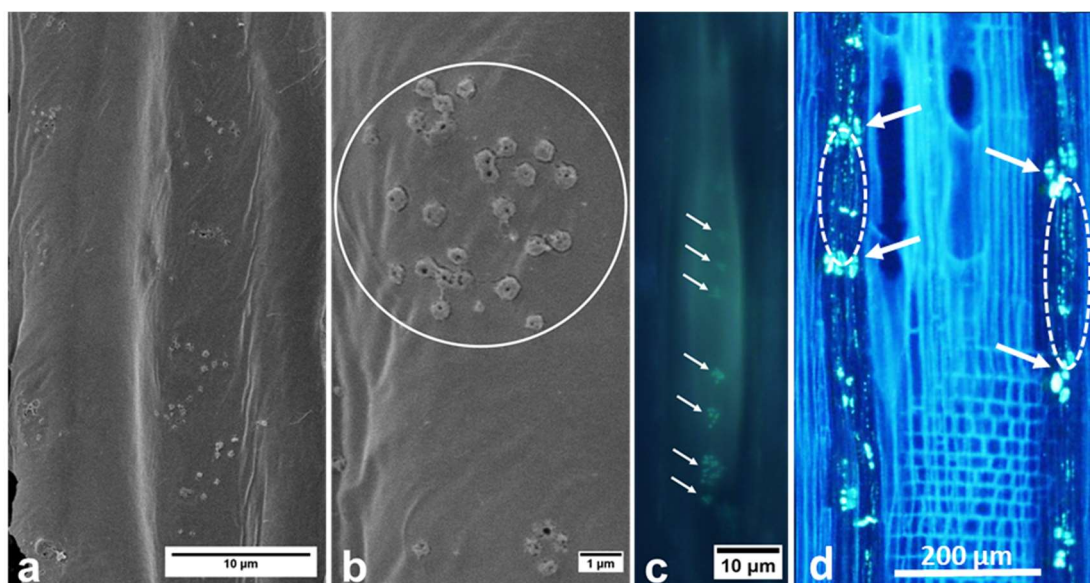


Figure 6.4. SEM and fluorescence microscopic images of clustered minute sieve pores in axial sieve tubes.

- a: Clustered minute sieve pores distribute along the cell axis in lateral wall.
- b: Enlarged image of a clustered minute sieve pores in lateral wall (circle).
- c: Axial linear arrangement of clustered minute sieve pores with weak fluorescence in the lateral wall of a nascent axial sieve tube (arrows).
- d: Clustered minute sieve pores make linear arrays (dotted ellipses) through the axis of two axial sieve tubes in the advanced stage of differentiation. Arrows: sieve plates.

In terms of the connection structures, radial sieve tubes had many similarities with the axial ones. For example, there were also highly differentiated sieve pores on the end walls of radial sieve tubes to facilitate the flow of nutrients within phloem network. During the developmental process, the sizes of sieve pores and callose collars increased many times. Additionally, lateral walls of radial sieve tubes also showed groups of minute sieve pores distributed roughly parallel to the cell axis (Figure 5.7 in Chapter 5), which facilitated the connections between the sieve tubes and adjacent cells, such as the companion cells.

Radial sieve tubes often had tilted cell axes, a feature that allowed them to be connected to axial sieve tubes (solid ellipse in Figure 6.5a). The end wall indicated by “a” was common to both inclined radial and axial sieve tubes, with a large inclination of the cell axis. The inset of Figure 6.5b is a fluorescence microscopic image corresponding to the SEM image of the radial sieve tube strand indicated by the dotted ellipse in Figure 6.5a, clearly showing three sieve plates (arrows a, b, and c), which were strongly fluorescent due to callose deposition in their sieve pores.

Figure 6.5b and 6.5c show enlarged images of the sieve plates, indicated by arrows “a” and “b” in Figure 6.5a. The thicknesses of these two sieve plates were about 1~2 µm. Sieve pores were observed in the cross section of the sieve plates “a”, which were penetrated by a tubular cytoplasmic slime with a thickness of around 0.2 µm (arrow in Figure 6.5b). At the same time, slightly thicker tubular slimes with thicknesses of 0.5~0.6 µm penetrating the interior of the sieve plate “b” were observed by the breakage of the sieve plate (arrowheads in Figure 6.5c). A sheet-like band of cytoplasmic slime body was connected to the sieve plate of the radial sieve tube (star in Figure 6.5c).

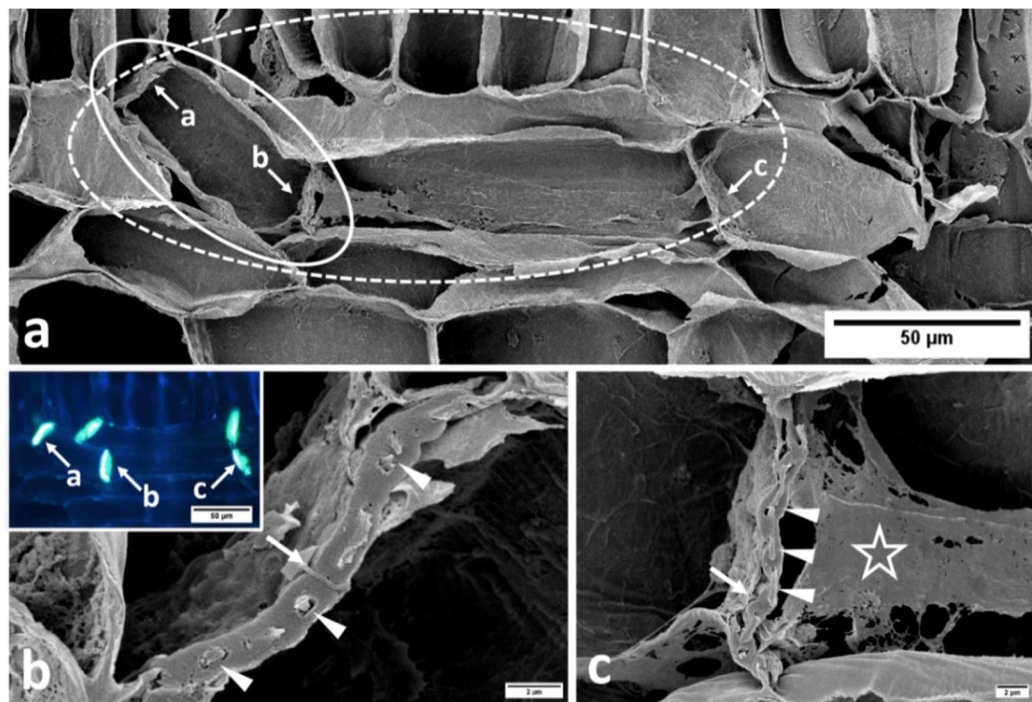


Figure 6.5. SEM images of mature radial sieve tubes.

a: A bent radial sieve tube strand (large ellipse) in mature secondary xylem.

b: Enlarged image of the sieve plate pointed by arrow “a” in a. Arrow: tubular slime crossing the sieve plate; arrowheads: tubular slimes penetrating inside the sieve plate; Inset: a lower magnification fluorescence microscopic image of the area corresponding to a. The arrows a, b, c in a and the arrows a, b, c in the inset point to the same sieve plates.

c: Enlarged image of the sieve plate pointed by arrow “b” in a. Arrow: tubular slime crossing the sieve plate; arrowheads: tubular slimes penetrating inside the sieve plate; star: sheet-like band of cytoplasmic slime body.

6.3.2. Connections between radial and axial sieve tubes

Connection between radial and axial sieve tubes was a common feature in all species of *Aquilaria* and *Gyrinops* that were investigated. An integrated radial sieve tube strand that was not interrupted by other cells or tissues usually consisted of one to several sieve tube elements. Most of the sieve tubes in the middle part of the strand run parallelly with ray parenchyma cells, while the sieve tubes at the opposite ends of the strand frequently exhibited sharp angles. Various types of connections having different angles and directions were observed between radial and axial sieve tubes and summarized as below.

(1) Connection by bending of sieve tube strands

The successive connections between sieve tubes in ray tissues and axial IP strands were observed in Figure 6.6a. Because of their interaction with axial sieve tubes, part of radial sieve tubes obviously deviated from a horizontal direction and exhibited either steep or gentle angles with ray parenchyma cells, especially the elements located at the ends of sieve tube strands. In Figure 6.6a, four radial sieve tubes strands (1 to 4) within a ray tissue were bent towards the vertical direction, giving the impression of curved channels.

(2) Connection of sieve tube strands by an oblique bridge

In some cases, the radial sections displayed very steeply curved sieve tube strands. Each end (arrows) of an oblique sieve tube element was connected to an axial sieve tube, thus forming a S-shaped connection (Figure 6.6b). The sieve tubes oriented in radial or axial direction just like oblique bridges within IP strands (dotted circle in Figure 6.6b) were often observed in radial sections of *A. malaccensis*. Usually, radial sieve tube elements appearing in this type of connection were much shorter than common axial and radial sieve tubes.

(3) Connection of sieve tube strands at a right angle

Figure 6.6c shows two connecting parts at the opposite ends of one radial sieve tube strand that were composed of only two elements in *A. malaccensis*. Sieve plates of radial sieve tubes were attached with cytoplasmic material connecting to axial sieve tubes in both ends separately. The left end of the radial sieve tube strand showed a distinct joining with an axial sieve tube, just like a L-shaped connection (A in Figure 6.6c). The junction on the right end of the radial sieve tube strand was located at the lateral wall or sharply inclined end wall where an axial sieve tube was connected like opposite L shape (B in Figure 6.6c).

Lateral sieve plates constituted another alternative connection between radial and axial sieve tubes. Figure 6.6d shows a T-shaped connection by a common sieve plate (arrow) between an axial sieve tube strand on the left and a radial sieve tube strand on the right in a radial section of *A. malaccensis*.

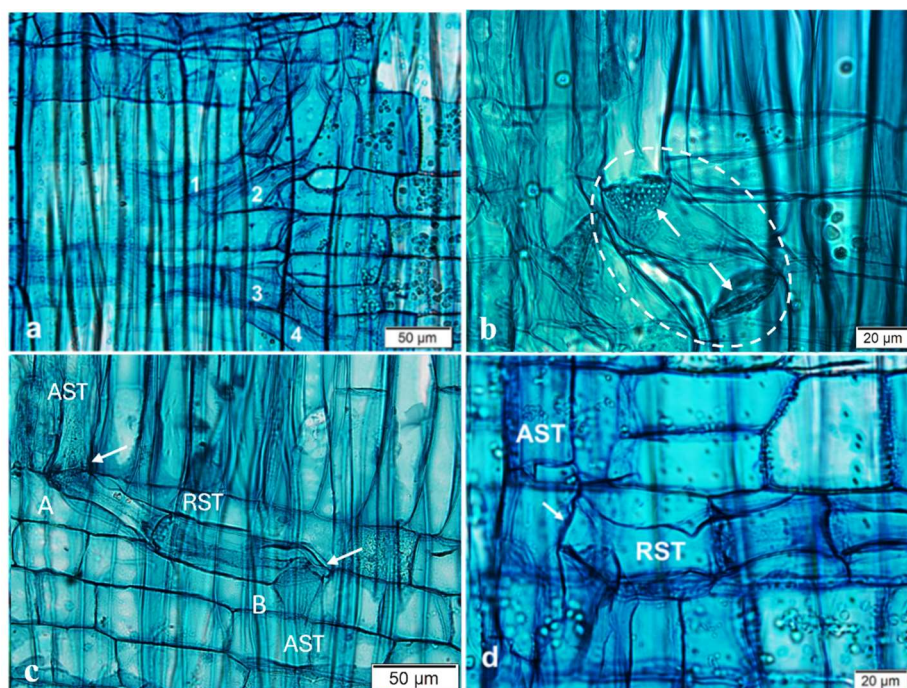


Figure 6.6. Radial sections show different connections between radial and axial sieve tubes.
a: Four radial sieve tube strands (1 to 4) are bending vertically.
b: S-shaped connection (dotted ellipse) of radial sieve tube strand. Arrows: sieve plates.
c: L-shaped connection between radial and axial sieve tubes on the both sides (A and B).
d: T-shaped connection between radial and axial sieve tubes through a common sieve plate (arrow).
AST: axial sieve tube; RST: radial sieve tube.

The number of axial sieve tubes in an IP strand varied from species to species and the sizes of IP strands varied considerably. Radial sectional observations were essential for studying the structure and arrangement of sieve tubes in IP strands and the connections between radial and axial sieve tubes. However, due to the fact, that IP strands in secondary xylem mainly extended along tangential direction, axial sieve tubes in IP strands were mostly clustered or scattered along the same direction. Observations of tangential sectional images yielded unexpected information about the connections within the adjacent axial sieve tube strands. Figure 6.7 provides a very rare situation of the branching of axial sieve tubes.

Tangential sectional image in the secondary xylem, at 8~10 mm away from the cambium, revealed bundles of eight axial sieve tube strands in a 25 μm thick section (Figure 6.7). The dense bundles of sieve tubes were arranged in the axial direction with an intervening ray tissue. Moreover, in the same figure, an axial sieve tube that branched off from the number 2 sieve tube strand and sloped down (arrow) was shown to bind to the other axial strand, independent of bridging by radial sieve tubes in the ray tissue.

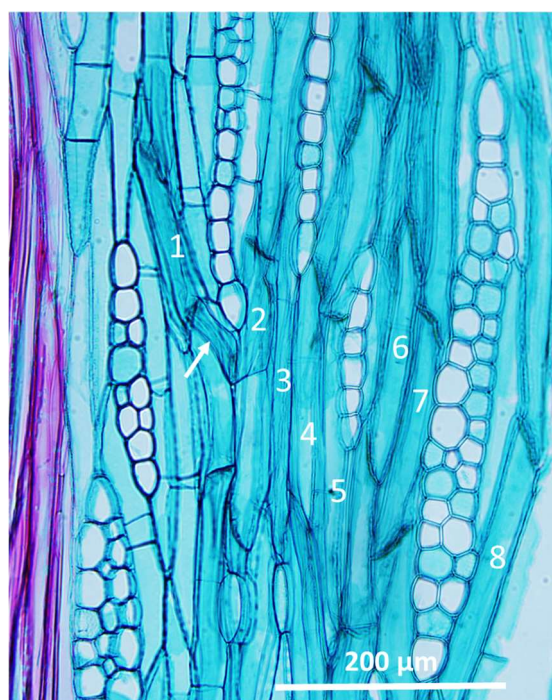


Figure 6.7 Tangential sectional image shows bundled axial sieve tube strands (1-8) in the differentiating stage. The No. 2 axial sieve tube strand branches and connects to the No. 1 axial sieve tube strand.

6.3.3. Phloem network composed of axial and radial sieve tube strands

A. sinensis frequently showed the development of radial sieve tube strands as mentioned earlier, however, substantial data on their locations and how do they bridge the radial and axial sieve tubes was lacking. Therefore, the connections of more than 100 radial and axial sieve tube strands were investigated. In this case, to obtain the quantitative data, the types of connections were classified by examining a great number

of transverse and radial sections prepared from several wood discs of different heights, mainly by fluorescence microscopy. The results were shown in Table 6.1.

The shortest radial sieve tube strand consisted of one sieve tube element, while the longest one consisted of seven sieve tube elements. The radial and axial sieve tubes were connected by common sieve plates, mainly at the end walls. Based on the positions of radial sieve tube strands and IP strands in the secondary xylem, the connections were divided into three types, as shown schematically in Figure 6.8.

(1) Radial sieve tube strand connected to axial strand existing in the same IP. (2) Radial sieve tube strand bridged two independent IP strands. (3) Radial sieve tube strand penetrated IP strand and connected to axial strand in it. The characteristics of the three types of connections were revealed in detail by a combination of fluorescence microscopy and aniline blue staining, as follows.

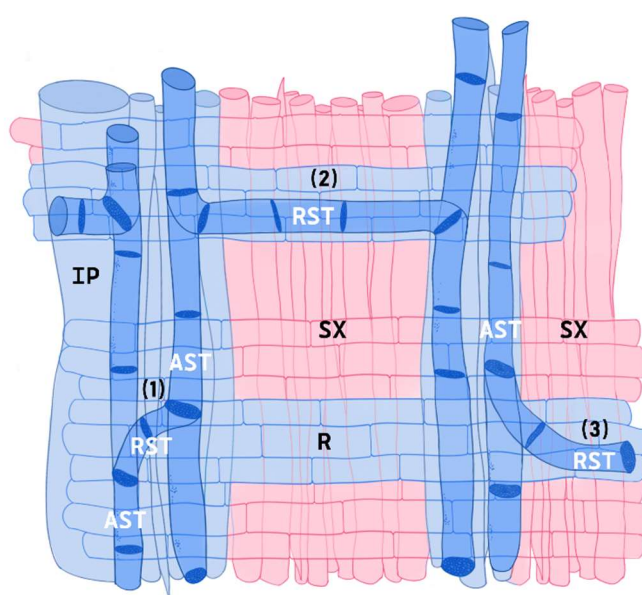


Figure 6.8. Three representative connections between radial and axial sieve tube strands. AST: axial sieve tube strands; RST: radial sieve tube strands; IP: interxylary phloem strands; R: ray tissues; SX: secondary xylem.

Type (1): Radial sieve tube strand connects to axial strand existing in the same IP strand.

Type (2): Radial sieve tube strand bridges two independent IP strands.

Type (3): Radial sieve tube strand penetrates IP strand and connects to axial strand in it.

(1) Radial sieve tube strand connected to axial strand existing in the same IP

The connections between radial and axial sieve tube strands were identified at the three locations within the same IP strand, indicated by the arrows in Figure 6.9a. Two radial sieve tube strands in different ray tissues and two independent axial strands in the same phloem tissue were involved in this type of connection: two radial sieve tube strands could be seen connecting to detached axial sieve tube strands, one in the center and the other in the bottom right-hand corner. Connections within the same IP usually consisted of a smaller number of radial sieve tube elements, with the shortest connection consisting of only one radial sieve element as shown in Figure 6.9e.

(2) Radial sieve tube strand bridged two independent IP strands

The two rows of radial sieve tube strands in Figure 6.9b were connected to IP

strands on both sides. In other words, “bridges” consisted of radial sieve tube strands connected by three independent IP strands. Compared to the connections within the same IP, radial sieve tube strands involved in connections between dispersed phloem strands usually consisted of more sieve tube elements, the number of which depended on the distance between the phloem strands. Moreover, it was often observed that the connections between different IP strands consisted of multiple rows of radial sieve tube strands within the same ray tissue. Rarely, more than six strands of radial sieve tubes were observed bridging two independent phloem tissues, as shown in Figure 6.9c.

(3) Radial sieve tube strand penetrated IP and connected to axial strand

Radial sieve tube strands often extended from the periphery of IP strand and penetrated it, connecting to axial sieve tube strands arranged vertically within the IP as shown in Figure 6.9d. In this case, one end of radial sieve tube strand remained in the ray tissue at the periphery of the IP, while the other end extended towards the IP strand.

The frequency of three types of connections was examined and found that type (1) had the highest frequency (49 %), followed by type (2) (33 %) and type (3) (18 %) (Table 6.1). These results suggested the importance of bridging radial and axial sieve tube strands located within the IP.

Table 6.1 Frequency of three types of connections between radial and axial sieve tube strands.

Serial number	Source of wood disc	Frequency of sieve element/strand	Type of connection		
			Type (1)	Type (2)	Type (3)
1	B	1	1	0	0
2		6	6	0	0
3		2	2	0	0
4	C	1	1	0	0
5		1	1	0	0
6		1	1	0	0
7	D	0	0	0	0
8		7	7	0	0
9		4	4	0	0
10		2	2	0	0
11		1	0	1	0
12		2	0	2	0
13	E	6	3	0	3
14		12	8	1	3
15		9	8	0	1
16		5	4	1	0
17		4	2	2	0
18		2	0	2	0
19	F	0	0	0	0
20		0	0	0	0
21		2	0	2	0
22		6	0	6	0
23		0	0	0	0
24		1	0	1	0
25	H	2	0	2	0
26		1	1	0	0
27		10	1	2	7
28		16	2	10	4
29		4	0	3	1
30		2	0	1	1
		110	54 (49%)	36 (33%)	20 (18%)

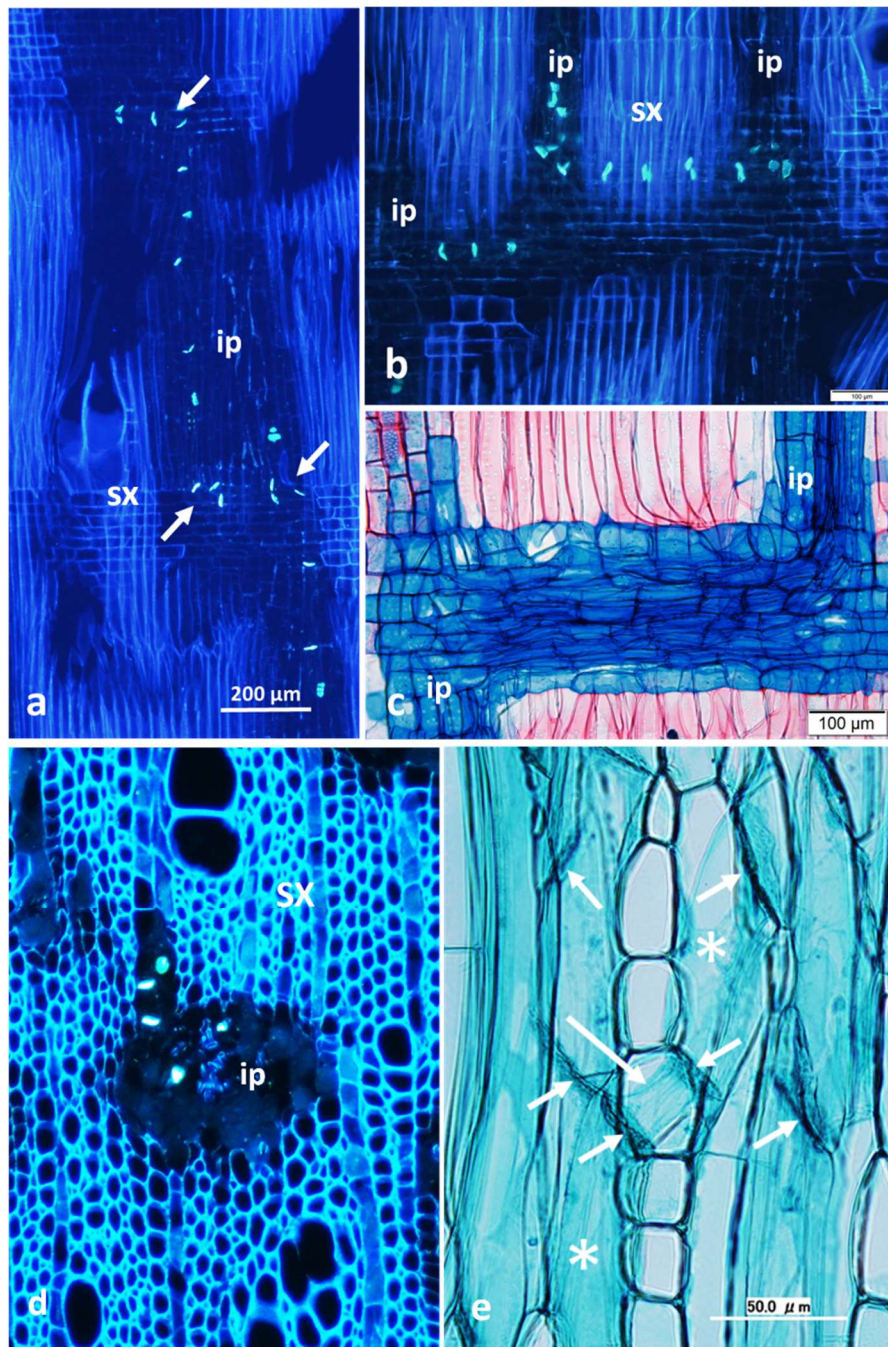


Figure 6.9. Fluorescence and double stained microscopic images of different connections between radial and axial sieve tube strands.

a: Type 1 connection. Two ray tissues cross the IP, with one radial sieve tube strand in the upper ray bridging to an axial strand and two radial sieve tube strands in the lower ray bridging to another axial strand. Arrows: joints between radial and axial sieve tube strands.

b: Type 2 connection. A long and a short radial sieve tube strands bridge three IP strands. Bright spots with green color show sieve plates of both axial and radial sieve tubes.

c: More than six radial sieve tube strands and axial sieve tube strands in two IP tissues form intensive bridging.

d: Type 3 connection. ip: interxylary phloem; sx: secondary xylem.

e: Tangential sectional image shows special type 1 connection, that is single radial sieve tube (long arrow) with two sieve plates connecting to axial sieve tubes (asterisks). Arrows: sieve plates.

6.4. Discussion

6.4.1. Structure related to connections between axial and radial sieve tubes

The previous chapter has already compared and summarize the differences in general features between axial and radial sieve tubes. Here, the study focused on the delicate structure related to "connections" combining two types of sieve tubes and different phloem tissues.

Structural similarities between axial and radial sieve tubes were also found in the sieve areas of the end and lateral walls. The end and lateral walls of axial and radial sieve tubes had simple and/or compound sieve plates. Regardless of the types of sieve plates, the end walls of both showed highly differentiated and wide sieve pores which far exceed the pores in the lateral walls. Filamentous or tubular slimes not only passed through the sieve pores, but also entered the interior of the sieve plates (Figures 6.2c, 6.5b and 6.5c).

Furthermore, sieve pores in the end walls of axial and radial sieve tubes showed similar patterns of development. That was, in the early stage of sieve tubes differentiation, juvenile sieve pores were usually not fully opened and only small clumps formed by callose accumulation were visible in fluorescence microscopic images. As axial and radial sieve tubes become mature, callose partially removal or fusion increased the sizes of sieve pores in their end walls. Similar phenomena have been reported in *Arabidopsis* and *Echium* (Barratt *et al.* 2011; Nedukha 2015; Esau & Thorsch 1984).

Since pore sites were often close together, some callose collars fused during their expansion process and two or more sieve pores were surrounded by the same collar. In fact, comparing the hole diameters of sieve pores, the detectable nascent sieve pores averaged 0.3 ~0.5 μ m, whereas the sieve pores of mature sieve tubes commonly arrived 1.0 μ m or more. Thus, with the growth of sieve tubes, the number of sieve pores in their nascent stages would be slightly higher than the number of sieve pores at maturity, when there was no significant fluctuation in the sizes of sieve areas, indicating that fusion of holes might happen during the differentiation of sieve pores.

Another type of connected area on the lateral walls of axial and radial sieve tubes consisted of minute sieve pores that were either scattered or aggregated into clusters. There were also collars around these pores, but callose deposit was slight. The mass of callose that accumulated at the pore sites corresponded to the size of the lumen of open pores (Esau & Thorsch 1985).

These minute pores found on the lateral walls of axial and radial sieve tubes were very similar in size and arrangement to those observed on the lateral walls of axial sieve tubes in *Cercidiphyllum japonica* and *Castanea dentata* (Angyalossy *et al.* 2016). Furthermore, the linear arrangement of clustered minute sieve pores in the direction of cell axis with the help of veil-like band or lines of slime body suggested that a complex structure consisting of veil-like slimes and clustered minute sieve pores might be involved in the nutrient transport capability of sieve tubes. Considering the total amount

of sieve pores in these “connected structures”, *A. sinensis* would have many alternate transportation channels.

The cytoplasm in sieve tubes was filled with slime bodies, and during the differentiation process of sieve tubes, the cytoplasmic slime bodies were always bound to the sieve plates, which were observed as veil-like bands through the cell axis of sieve tubes by optical microscope and as sheet-like bands by SEM. As the cytoplasmic slimes passed through sieve pores, the slimes thickened from filamentous to tubular shapes as the pore diameters increased with the sieve tube differentiation. In contrast, within individual axial or radial sieve tube, the cytoplasmic slime bodies spread as thin and sometimes thick veil-like band structures, bridging the sieve plates at both ends of the sieve tubes (Figures 6.1b, 6.1c, 6.1d, 6.4c and 6.4d).

In the fluorescence microscopic images, the cytoplasmic slime bodies in axial and radial sieve tubes appeared to be in a state of extension (or flow) in the cell axis direction. Closer examination of individual sieve plate revealed that filamentous or tubular slimes, deriving from the cytoplasmic slime body penetrated the sieve pores (Figures 6.2a, 6.2b, 6.3b and 6.3e), recalling the presence of substantial transport channels through these sieve plates and pores. When cytoplasmic slimes entered the large or minute sieve pores of different sieve plates, they changed into filamentous or tubular forms as described above.

The cytoplasmic slimes contained stretchable materials, probably due to the presence of tubular and bundled actin-like filaments (Evert 2006), which could flexibly change shape depending on the different stages of sieve tubes and adapt to the size of sieve pores to be penetrated. This unique structural and physiological design of cytoplasmic slimes passing through the sieve plates while changing their shapes was more widespread than expected, not only in the axial direction, but also in the horizontal direction. These facts suggest that axial and radial sieve tubes constitute a more functional and diverse phloem network as nutrient transport pathways in the secondary xylem of agarwood-producing species, like *A. sinensis*.

6.4.2. Existence and development of connections in sieve tube strands

The connections between radial and axial sieve tubes shown in the present work were an integral part of phloem anastomoses in *Aquilaria* and *Gyrinops*. In general, phloem anastomoses were very common in higher plants for the aim of fast and alternative communication (Aloni *et al.* 1990; 1995) and were not related with growth habits (Aloni & Sachs 1973). In *Cucurbita* (Cucurbitaceae) and *Dahlia* (Asteraceae), the earliest signs of phloem anastomoses began to be detected from the internode with a length of about 5 mm (Aloni & Barnett 1996). Similar anastomoses of sieve tubes were common features in different internodes of *Coleus* (Lamiaceae) (Aloni *et al.* 1990). In addition to the naturally developing structures, phloem anastomoses composed of a great number of sieve tubes were reported in stems of *Ricinus communis* (Euphorbiaceae) infected by *Agrobacterium tumefaciens* (Aloni *et al.* 1995).

The first sign of radial sieve tubes was observed immediately after the formation of the first layer of IP strands in the secondary xylem at the fifteenth internode of *A.*

sinensis. Distinctive aniline blue staining of radial sieve tubes in *G. versteeghii* appeared in the cambial zone in the sixth internode (Luo *et al.* 2020). Once radial sieve tubes were produced, they could have connections with axial sieve tubes in one or more IP strands. These observations suggested that the network formation of phloem anastomoses depended on the activity of vascular cambium. With the successive differentiation of IP strands, the number of radial sieve tubes increased with increase in the stem diameter. Additionally, the density of phloem anastomoses depended on growth conditions, warmer environment helping to form a high-frequency phloem network (Aloni & Sachs 1973). As a result, all sieve tubes within the plant might gradually establish a collective sieve tube system (Ham & Lucas 2014).

Radial transfer of water from phloem to xylem via ray parenchyma cells was confirmed by fluorescence dye injection (Pfautsch *et al.* 2015). The necessity of sieve tubes differentiation in ray system came from the need for selective transport and intercellular communication via elaborate channels in sieve plates (Amsbury *et al.* 2018). It was suggested that development of radial sieve tubes in the ray system would help shorten the distance for transport of nutrients (Rajput & Rao 1997; Rajput 2004b). Therefore, the fact that four or more strands of radial sieve tubes were often found in the same ray in *Aquilaria*, might suggest that radial sieve tube strands of agarwood-producing species are capable of efficiently shortening the transport of photosynthates.

Finally, what was the source of radial sieve tube strands in the secondary xylem? There were two groups of evidences regarding their origins— one from redifferentiation of ray parenchyma cells, and the other from the division and differentiation of vascular cambium similar with the origins of vessels, ray parenchyma cells and xylem fibres. As described earlier, various types of connections between radial and axial sieve tubes were found in the three species of *Aquilaria* and one species of *Gyrinops*, especially the newly formed connecting strands in cambium zone (Luo *et al.* 2020). It means that the connections of sieve tube strands originate from the cambium and occur almost simultaneously with the differentiation of sieve tubes.

On the other hand, considering the occurrence and development of diversified types of connections between axial and radial sieve tube strands, it is reasonable to assume that radial sieve tubes would additionally be produced through the redifferentiation of un lignified parenchyma cells to fit the fluctuating demand of agarwood-producing species growth during secondary xylem and IP development.

6.4.3. Phloem network between sieve tube strands and its functions

The connections between axial and radial sieve tubes were not uncommon in plants containing IP strands, such as the aforementioned examples. They usually had extremely wide ray tissues, and one to several radial sieve tube strands dispersed within the same ray (Den Outer & Van Veenendaal 1981; Lev-Yadun & Aloni 1991). More recently, it has been pointed out that species with IP tissues frequently showed the development of radial sieve tubes, which helped to develop an anastomosing network for the short distance to connect with adjacent phloem islands (Rajput *et al.* 2022). In all checked samples, width of majority ray tissues in *A. sinensis* was only 1~3 cells.

However, radial sieve tubes could be found in any part of the ray tissues, regardless of uniseriate or multiseriate rays. The high possibility of connections between radial and axial sieve tubes was supported by previous reports on the average numbers of radial sieve tube strands and IP tissues in the unit volume and area in the secondary xylem of *G. versteeghii* (formerly *Aquilaria versteeghii*) branch wood, showing 1.7 per mm³ for the former and 9.1 per mm² for the latter (Luo *et al.* 2020)

Furthermore, present study investigated the geometry of connections between axial and radial sieve tube strands and found that they could be divided into three different types (Luo *et al.* 2020): (i) connection by bending of radial sieve tube strands; (ii) connection by a diagonal bridge between different sieve tube strands; (iii) orthogonal connection of radial and axial strands. Through these observations, different shapes of cell junctions between radial and axial sieve tubes were documented.

To reveal a more substantial network of phloem transport channels, the connections between radial and axial sieve tube strands were examined from different perspectives. In general, the tangential width of IP strands in transverse sections was approximately 200~500 µm or more. Therefore, it was often observed that multiple rays passed through the IP strands, increasing the likelihood of radial sieve tubes connecting to the axial ones inside the IP.

In the cellular configuration of the secondary xylem of *A. sinensis*, the connections between radial and axial sieve tube strands could be classified into three types, depending on the positions of radial sieve tubes and the ways they were connected to the axial ones. (1) Radial strands located in the IP and connected to the axial strands that meant connections between radial and axial strands inside the individual IP. (2) Radial strands bridged the axial ones in two or more IP strands, in other words, radial strands connected IP strands independently distributed in the secondary xylem. (3) Radial strands penetrated IP and connected to the axial strands.

In the first case, it was observed that the bridging radial sieve tube strands tended to be short and obliquely inclined, while in the second case, the radial strands tended to form long, horizontally elongated bridges. The frequencies of the three bridging modes were found to be the highest for type 1 (49 %), followed by type 2 (33 %) and type 3 (18 %). These results indicated the importance of bridging radial and axial sieve tubes within individual IP strand for the establishment of the alternative and efficient phloem network.

Let us consider the significance of type (1) connection. In the previous report, the average length of IP strands was about 14 ± 4 mm, while the average length of axial sieve tubes within them was only 215.7 ± 57.2 µm. By bridging axial sieve tubes in the IP with radial ones at various heights, the phloem network expanded and the IP strand functioned efficiently as a whole. In radial section shown in Figure 6.9a, the connection “bridges” between two axial strands located in the same IP were as short as only one to four radial sieve tube elements, and the axial strands connected by the radial strands were very close. For an IP strand to function as a transportation channel, the radial sieve tubes needed to bridge and connect to multiple axial strands inside the IP. Therefore, the main function of this type of connection was to combine axial sieve tube strands in series through conjunctions with radial strands to form longitudinally extended

transport channels (Figure 6.9a).

Second type of connection was composed of horizontally long strands of radial sieve tubes, forming “bridges” that connected two or more independent IP tissues. As the IP of *A. sinensis* was of diffuse type, type (2) connection allowed the phloem network to be spread over a wider area by linking axial sieve tubes in IP strands that were distributed far apart from each other like islands. In some radial sections, intensive network consisting of multiple rows of radial sieve tube strands coupled with axial strands in two or more different IP tissues was observed (Figure 6.9c). In some case as seen in Figures 5.4c, 5.4e and 5.4f, axially aligned fibres within the IP strands were observed to intrude into the radial tissues and align parallel to radial sieve tubes, which suggested the occurrence of temporarily constructed radial (or ray-like) phloem.

Some researches point out that under normal circumstances, phloem anastomoses in mature internodes rarely functioned as transport channels. According to Aloni and Peterson (1990), translocation was visualized with fluorescein in large area of living phloem tissue after peeling from the xylem. It was suggested that the phloem anastomoses in mature internodes of *Dahlia* served mainly as an emergency system. Further, it was proposed that the dense network of phloem anastomoses developed as a mechanism of adaptation to possible damage in mature internodes by providing flexible alternative pathways for efficient xylem regeneration after wounding in *Cucurbita* and *Dahlia* (Aloni & Barnett 1996).

In agarwood-producing species such as *A. sinensis*, numerous IP strands were distributed throughout the secondary xylem. However, under normal growth conditions, IP strands did not undergo any structural changes and islands of IP were continually to be produced. It has been proven that once the wood was externally injured, as in the process of peeling, the injury would be healed. Because the IP strands in the depth of 400~1000µm from the wounded surface immediately reacted to the injury, and unligified parenchyma cells in IP strands began to divide and multiply. After the bark was ring-peeled along with cambium, the remaining IP strands in the secondary xylem jointed together in a ring-like pattern and new vascular cambium regenerated in almost one month, continuing the formation of new secondary xylem and phloem as before peeling (Luo *et al.* 2021). Thus, in the case of agarwood-producing species such as *A. sinensis*, there was no doubt that the IP strands were primarily responsible for the emergency defense mechanism in the event of a fatal injury to tree body. Contrary to the response of IP strands to injury, no specific changes in number, shape or other aspects of radial sieve tube strands were observed before and after injury.

Given that in the secondary xylem of *A. sinensis* under normal conditions, radial sieve tube strands were (i) always produced somewhere in the secondary xylem by the vascular cambium, albeit partially, (ii) further replenished in the secondary xylem by the divisions of unligified ray parenchyma cells, and (iii) involved in the connections of three types between radial and axial strands that stretched across the secondary xylem, it was unlikely that the radial sieve tube strands were specialized structures formed in preparation for emergency situations. Rather, they might have functions that were yet to be fully elucidated, such as aiding the passage of nutrients by establishing substantial phloem network in the tree body at normal state.

6.5. Summary

The structures of axial and radial sieve tubes were examined by fluorescence microscopy and SEM. Two types of sieve areas were found on the end and lateral walls of axial and radial sieve tubes: one consisted of a cluster of larger, more highly differentiated sieve pores, corresponding to sieve plates, and the other consisted of scattered or clustered minute sieve pores, arranged in a linear array in the lateral wall along cell axis, covered by slime bodies, making a complex structure associated with nutrient pathways. Migration of cytoplasmic slime bodies in axial and radial sieve tubes took place by changing their shapes into filamentous or tubular slimes and passing through the sieve pores, depending on the size of sieve pores at different differentiation stages.

Axial sieve tubes in IP tissues were observed to arrange axially in dense bundles. Ray parenchyma cells often showed cell division to supplement new sieve tube elements during secondary xylem differentiation, which promoted the formation of diverse connections between radial and axial sieve tubes. Based on different shapes, the connections between radial and axial sieve tubes were divided into three categories: radial sieve tube strand bending, S-shaped and orthogonal (L-shaped and T-shaped) connections.

On the other hand, based on the positions of radial and axial sieve tube strands in the secondary xylem, three types of connections were identified. (1) Radial strands connected to axial strands in the same IP; (2) radial strands bridged two or more independent IP tissues; (3) radial strands penetrated IP and connected to axial strands. The highest frequency of type (1) among the three types suggested that it was crucial for the functions of phloem network that radial and axial sieve tubes located inside the IP were connected to establish longitudinal transport channels.

This work was the first account of a structural elucidation of the phloem network in the special agarwood-producing species with IP tissues in the secondary xylem.

Chapter 7 General conclusions

This study took *Aquilaria* and *Gyrinops* as the representatives of agarwood-producing species and combined optical microscopic observation, fluorescence microscopic observation and SEM observation to investigate the anatomical characteristics of the abnormal phloem tissues, intraxylary phloem, interxylary phloem and radial sieve tube in samples of these two genera, including their distributions, morphologies, origins, developmental processes, delicate structures of axial and radial sieve tubes and connections between them to form the continuous phloem network. Based on the observations, the specific functions of the forementioned abnormal phloem tissues were discussed.

The conclusions are as follows:

(1) In addition to the external secondary phloem, agarwood-producing species produced intraxylary phloem, interxylary phloem and radial sieve tube during primary and secondary growth. Observations on the developmental processes of these phloem types revealed that (i) intraxylary phloem differentiated from parenchyma cells in the outer margin of the pith, (ii) interxylary phloem differentiated from the vascular cambium, and (iii) radial sieve tube not only differentiated from the vascular cambium, but also redifferentiated from immature parenchyma cells in ray tissues.

(2) In the early growth of young shoots of *Aquilaria*, the amount of callose staining detected in the intraxylary phloem was more than twice that of the external phloem before differentiation of interxylary phloem, indicating that prior to the initiation of interxylary phloem, intraxylary phloem acted as the principal phloem. After differentiation of interxylary phloem, the amount of callose staining in sieve tubes reversed, which suggested that interxylary phloem took over the role of principal phloem.

(3) When agarwood-producing species suffered exogenous damage, such as girdling, damage healed through the following events: unlignified parenchyma cells in the interxylary phloem strands at 400~1000 μm deepness to the peeling surface began to divide and proliferate, leading to the regeneration of vascular cambium within nearly one month, which reproduced new secondary xylem and phloem, just like before peeling. Drastic structural changes observed in interxylary phloem during abnormal growth provided credible evidence that agarwood-producing species evolved efficient mechanisms for damage defense and tissue regeneration.

(4) The first reported radial sieve tubes in agarwood-producing species and axial sieve tubes both developed highly differentiated sieve plates with larger sieve pores on their end walls. In active sieve tubes, filamentous or tubular slimes were always found passing or entering the sieve plates, connecting to the cytoplasmic membrane bodies of adjacent sieve tubes. In lateral walls of axial and radial sieve tubes, either dispersed or clustered small sieve pores were found along cell axis, together with highly differentiated large sieve pores forming continuous connections between sieve tubes or adjacent cells.

(5) Agarwood-producing species with interxylary phloem frequently showed the development of radial sieve tubes, which helped to develop an anastomosing network for connecting with diffused phloem islands through the following types: (I) radial sieve tube strand connected to axial sieve tube strand in the same IP (49 %), (II) radial sieve tube strand bridged axial sieve tube strand in detached IP strands (33 %), and (III) radial sieve tube strand penetrated IP and connected to axial sieve tube strand (18 %). Considering the occurrence and development of diversified types of connections between radial and axial sieve tube strands, it was reasonable to say that agarwood-producing species acquired highly sophisticated nutrient passage capability during evolution.

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List of Publications

1. **Luo, B.**, Imai, T., Sugiyama, J., Marsoem, S.N., Mulyaningsih, T., Itoh, T., 2020. The occurrence and structure of radial sieve tubes in the secondary xylem of *Aquilaria* and *Gyrinops*. IAWA journal 41(1):109-124. DOI:10.1163/22941932-20170182
2. **Luo, B.**, Imai, T., Sugiyama, J., Qiu, J., 2019. The occurrence and development of intraxylary phloem in young *Aquilaria sinensis* shoots. IAWA journal 40(1):23-42. DOI:10.1163/22941932-40190221
3. **Luo, B.**, Ou, Y.L., Pan, B., Qiu, J., Itoh, T., 2018. Structure and development of interxylary and external phloem in *Aquilaria sinensis*. IAWA journal 39(1):3-17. DOI:10.1163/22941932-20170182
4. **Luo, B.**, Yoshinaga, A., Awano, T., Takabe, K., Itoh, T., 2021. Regeneration of vascular tissue through redifferentiation of interxylary phloem after complete girdling in *Aquilaria sinensis*. IAWA journal 42(4):419-434. DOI:10.1163/22941932-bja10065
5. **Luo, B.**, Yoshinaga, A., Awano, T., Sugiyama, J., 2024. Structure of axial and radial sieve tubes and its functional significance for the phloem network in *Aquilaria sinensis*. IAWA journal IAWA-1733 - in press.