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Relationship between formation of earlywood vessels and leaf phenology in two ring-porous hardwoods, *Quercus serrata* and *Robinia pseudoacacia,* in early spring

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Abstract In deciduous ring-porous hardwoods, three-dimensional networks of the current year's earlywood vessels play an important role in the movement of water. In the present study, we analyzed the formation of earlywood vessels along the main stem and leaf phenology in two deciduous ring-porous hardwoods, *Quercus serrata* and *Robinia pseudoacacia*. We introduced a dye in an effort to monitor the timing of initial water conduction through the network of the current year's earlywood vessels. The formation of earlywood vessels began before bud break. At bud break, perforation of the first earlywood vessels was evident at the top of the stem but not at other positions within the stem. Perforations were observed along the entire stem when small leaves became visible and then leaves continued to develop. These results suggest that conduction of water via the network of the first earlywood vessels of the current year might not contribute to the initial growth of buds but might play an important role in the development of the current year's leaves and shoots.

Keywords Deciduous ring-porous hardwoods · Dye introduction · Earlywood vessels · Leaf phenology · Water conduction

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Introduction

In hardwoods, the three-dimensional network of vessel elements in the stem plays an important role in the movement of water from roots to leaves [1]. In deciduous ring-porous hardwoods, the large-diameter earlywood vessels that are formed in early spring are the main conductors of water [2–5]. However, in winter, earlywood vessels loose water as a result of cavitation during freezing and thawing [1, 3, 6]. Thus, the water conduction that results from the continuous formation of networks of the current year's earlywood vessels is very important for the growth of ring-porous hardwoods. The main driving force for conduction of water through vessel networks is the negative pressure that is due to transpiration from leaves. Therefore, it is important to determine the relationship between the timing of the formation of networks of the current year's earlywood vessels and the development and growth of the current year's leaves and shoots.

The differentiation of earlywood vessels in deciduous ring-porous hardwoods is initiated prior to bud break [7–14]. Moreover, formation of vessel elements is induced in disbudded seedlings of ring-porous hardwoods [14, 15]. These observations show that bud growth is not a prerequisite for the induction of formation of earlywood vessels in ring-porous hardwoods.

Zasada and Zhaner [7] proposed that the network of first earlywood vessels in the current year's annual ring should be functional before the expansion of leaves. Suzuki et al. [10] reported that the formation of secondary walls of vessel elements in the stem at breast height was completed from 1 week before to 3 weeks after leaf expansion in several ring-porous hardwoods. González-González et al. [12] and Takahashi et al. [13, 16] observed the simultaneous onset of lignification of cell walls of first earlywood vessels in the

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main stem at breast height and onset of emergence of leaves. Imagawa and Ishida [8] observed that perforation of vessel elements (disappearance of end walls of vessel elements) in the main stem at breast height and bud break occurred simultaneously in *Kalopanax pictus*. These results suggest that water conduction through the network of the current year's earlywood vessels and initiation of growth of leaves might start almost simultaneously. However, Atkinsin and Denne [9] reported that the first mature vessels were present in the upper stem of *Fraxinus excelsior* just before bud break while no mature earlywood vessels were evident at the base of the stem, when leaves were expanding. Therefore, the functional network of the current year's earlywood vessels might not be formed along the entire stem when growth of leaves begins. In addition, the timing of lignification of first earlywood vessels is different among vertical positions of the trees [9, 16]. Thus, the temporal relationship between the formation of networks of vessel elements in the current year's earlywood and the initiation of water conduction remains to be clarified in deciduous ring-porous hardwoods.

In this study, we compared the timing of formation of networks of earlywood vessels with the current year's leaf phenology in two deciduous ring-porous hardwoods, *Quercus serrata* and *R. pseudoacacia*, in early spring. We examined the timing of the differentiation of the current year's earlywood vessels at different heights on the main stem since the maturation of earlywood vessels progresses basipetally down the stem in ring-porous hardwoods [9]. We also introduced a dye to determine the time at which water conduction through the network of the current year's earlywood vessels began.

Materials and methods

Plant materials

Experiments were performed with deciduous ring-porous hardwood trees, *Q. serrata* Murray (5–8 years old; average height, 6.5 ± 0.6 m; average diameter at breast height, 6.3 ± 0.8 cm; $n = 11$) and *R. pseudoacacia* L. (3–5 years old; average height, 6.8 ± 0.5 m; average diameter at breast height, 5.3 ± 0.9 cm; $n = 11$) growing on the campus of the Faculty of Agriculture of Shinshu University in Minami-Minowa, Nagano (30°52′N, 137°56′E, 783 m

Fig. 1 Light micrographs showing the cambial zone on 18th May. Thin cell plates (*arrows*) were observed in some cambial cells along the entire stems of *Quercus serrata* (**a**, **b**) and *Robinia pseudoacacia* (**c**, **d**). **a** The cambial zone at the *top* of a stem of *Q. serrata*. **b** The cambial zone in a stem of *Q. serrata* near the ground. **c** The cambial zone at the *top* of a stem of *R. pseudoacacia*. **d** The cambial zone in a stem of *R. pseudoacacia* near the ground. *Bars* 25 μm (**a**, **c**) and 50 μm (**b**, **d**)
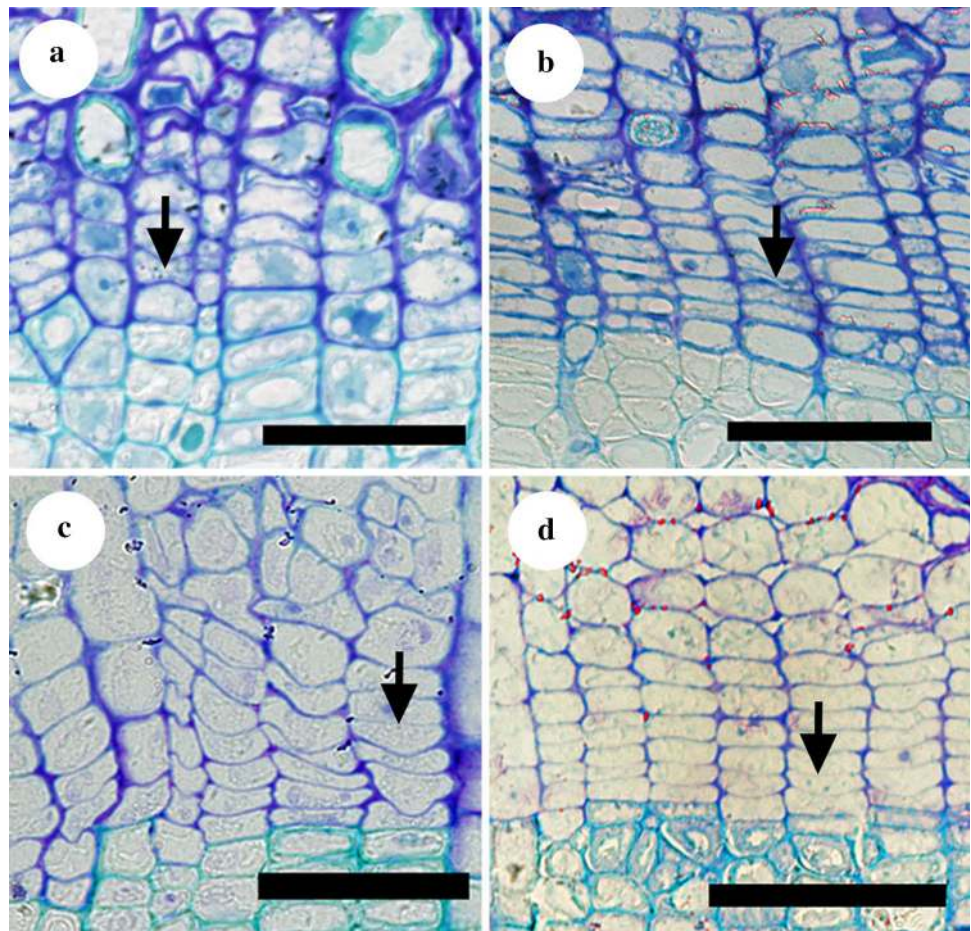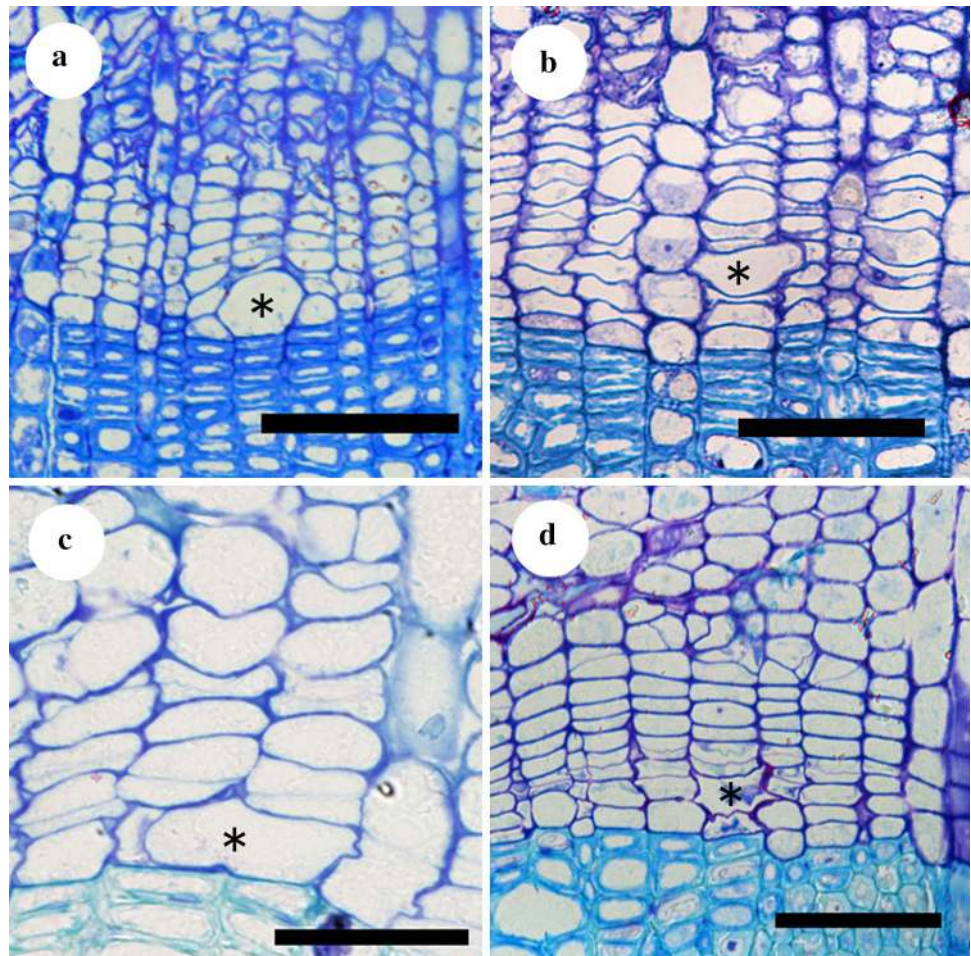
Fig. 2 Light micrographs showing the cambial zone, differentiating secondary xylem cells and enlarging vessel elements (*asterisks*) on 26th March. Enlarging vessel elements were observed at the *top* (**a**) and near the ground (**b**) in a stem of *Q. serrata* and at the *top* (**c**) and near the ground (**d**) in a stem of *R. pseudoacacia*. *Bars* 50 μm (**a**, **b**, **d**) and 25 μm (**c**)

a.s.l.), Japan, were used. We felled one tree of each species at 3- to 14-day intervals from mid-March to May, 2009.

Experiments with acid fuchsin dye

Experiments with acid fuchsin dye were conducted from 20th April to 9th May 2009 to evaluate whether the network of the current year's earlywood vessels was functional. The experiments were performed as described by Sano et al. [17] and Umebayashi et al. [5, 18]. A plastic funnel was attached, with vinyl adhesive tape, to the stem of each sample tree 0.3–0.8 m above the ground. The funnel was filled with a 0.2 % (w/v) solution of acid fuchsin (Wako Pure Chemical Industries, Osaka, Japan). Notches were cut at the mouth of the funnel, with a chisel, to the depth of the previous year's secondary xylem. The dye flowed from the funnel, through the notches, into the stem. After introduction of dye for 30 min, the tree was felled and disks were cut from the stem at 2-m intervals from the base of the stem to the apex. Then, the bark was peeled from the stem and the distribution of dye in vessel elements at the surface of the stem was examined with the naked eye. The maximum ascent of dye, defined as the distance from the site of introduction of the dye to the top of the colored zone, was recorded.

Observation of formation of vessel elements

Disk samples were taken from the main stem at 2-m intervals from the base to the shoot apex. Blocks containing phloem, cambium and xylem (approx. $2 \times 2 \times 4$ cm^3) were taken from the "south" side of the disks and fixed in 3 % glutaraldehyde at room temperature. Fixed blocks were washed in phosphate buffer and cut into small blocks (approx. $2 \times 2 \times 5$ mm^3). The small blocks were dehydrated in a graded ethanol series and embedded in epoxy resin. Transverse and radial sections were cut at thickness of 1–5 μm on a rotary microtome (TC-65; Leica, Germany and Microm HM340; Thermo Fisher Scientific, U.S.A) with tungsten steel knives. Sections were stained with 0.5 % toluidine blue and examined by light microscopy and polarized light microscopy (Axioskop; Carl Zeiss,

Germany). The progress of differentiation of the first earlywood vessels was divided into four stages as follows: stage 1, division of cambial cells; stage 2, expansion of earlywood vessels; stage 3, deposition of secondary walls in earlywood vessels; and stage 4, perforation of earlywood vessels. Division of cambial cells was identified by the presence of a thin cell plate in cambial zone [19–21]. Expansion of vessel elements was identified by the presence of enlarged cells in the cambial zone [8]. Deposition of secondary walls in vessel elements was detected by monitoring birefringence of cell walls under polarized light. The end walls of vessel elements are degraded during the final stage of maturation of vessel elements [22, 23].

Such perforations were detected by monitoring the disappearance of end walls of earlywood vessels in radial sections [8].

Observation of leaf phenology

Leaf phenology was monitored at the same time as stem disks were sampled. Photographs were taken of shoots in the upper crown of each sample tree. Definitions of leaf phenology were modified according to Fujimoto [24], González-González et al. [12] and Takahashi et al. [13, 16]. Bud break was defined as open of buds, when a part of leaves became visible at least.
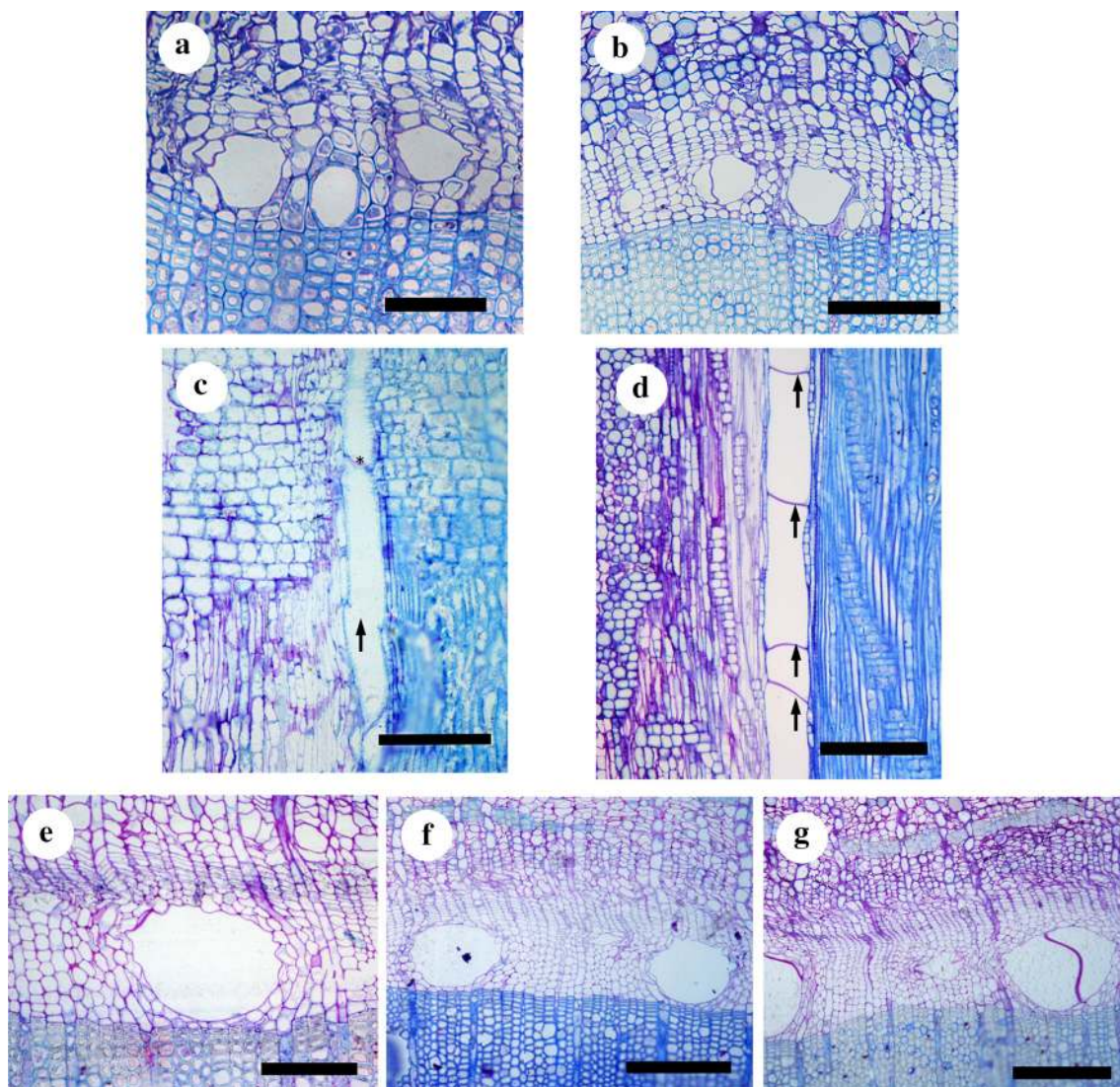

Fig. 3 Light micrographs showing transverse and radial images of differentiating vessel elements in *Q. serrata* on 16 April. Vessel elements with deposition of secondary walls were observed at the *top* (**a**) of the stem and 6 m above the ground (**b**). Radial images show partial perforation (*arrow* no end wall) and end walls (*asterisk*) in vessel elements at the *top* (**c**) of a stem. End walls of vessel elements (*arrows*) were found in the stem 6 m above the ground (**d**). Enlarging vessel elements were observed 4 m (**e**) and 2 m (**f**) above the ground and near the ground (**g**). *Bars* 50 μm (**a**), 100 μm (**b**, **c**) and 200 μm (**d**–**g**)
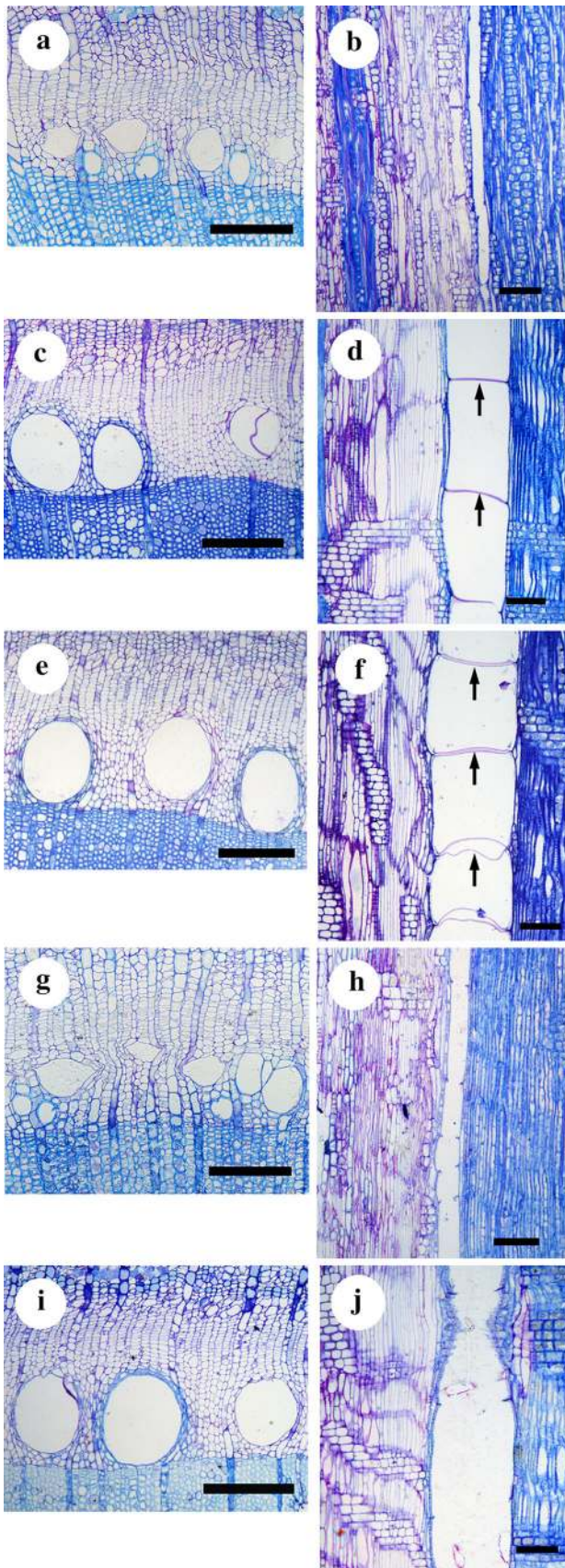
Fig. 4 Light micrographs showing transverse and radial images of differentiating vessel elements in *Q. serrata* on 20th and 24th April. On 20th April, deposition of secondary walls in first earlywood vessels was evident at the *top* of the stem (**a**), 4 m above the ground (**c**) and near the ground (**e**). Radial images revealed the perforations (no end walls) at the *top* of the stem (**b**), while end walls of earlywood vessels (*arrows*) were observed 4 m above the ground (**d**) and near the ground (**f**). On 24th April, earlywood vessels with deposition of secondary walls were observed at the *top* of the stem (**g**) and near the ground (**i**). Radial images revealed that perforations at the *top* of the stem (**h**) and near the ground (**j**). *Bars* 100 μm (**a**, **b**, **d**, **f**–**h**, **j**) and 200 μm (**c**, **e**, **i**)

Results

Formation of earlywood vessels

On 18th March, we observed thin cell plates in some cambial cells along the stems of *Q. serrata* and *R. pseudoacacia* (Fig. 1). Thus, cambial reactivation had started within entire stems before 18th March. On 26th March, we observed many dividing cambial cells and enlarging vessel elements throughout the entire stems of *Q. serrata* and *R. pseudoacacia* (Fig. 2).

On 16th April, we noted the deposition of secondary walls in earlywood vessels at the top and 6 m above the ground in the stem of *Q. serrata* (Fig. 3a, b). At the top of the stem, a few vessel elements had already lost their end walls (Fig. 3c). End walls of vessel elements were found in the stem 6 m above the ground (Fig. 3d). Moreover, enlarging vessel elements were apparent 4 and 2 m above the ground and close to the ground in the stem of the same tree (Fig. 3e–g). By 20th April, deposition of secondary walls in earlywood vessels had occurred along the stem (Fig. 4a, c, e). Perforations were observed at the top of the stem (Fig. 4b) but end walls of earlywood vessels were still visible 6, 4 m (Fig. 4d) and 2 m above the ground and, also, close to the ground (Fig. 4f). By 24th April, deposition of secondary walls in earlywood vessels and perforations of earlywood vessels were evident along the entire stem (Fig. 4g–j).

In *R. pseudoacacia*, deposition of secondary walls in earlywood vessels was apparent along the stem on 16th April (Fig. 5a, c). Perforations were observed near the top of the stem (Fig. 5b), but earlywood vessels retained their end walls 6 m (Fig. 5d), 4 and 2 m above the ground and close to the ground. On 20th April, deposition of secondary walls in earlywood vessels was apparent along the entire stem (Fig. 5e, g). Perforations were observed at the top of the stem and 6 m (Fig. 5f), 4 and 2 m above the ground. Partial perforation of earlywood vessels was evident in the stem close to the ground (Fig. 5h). By 24th April,
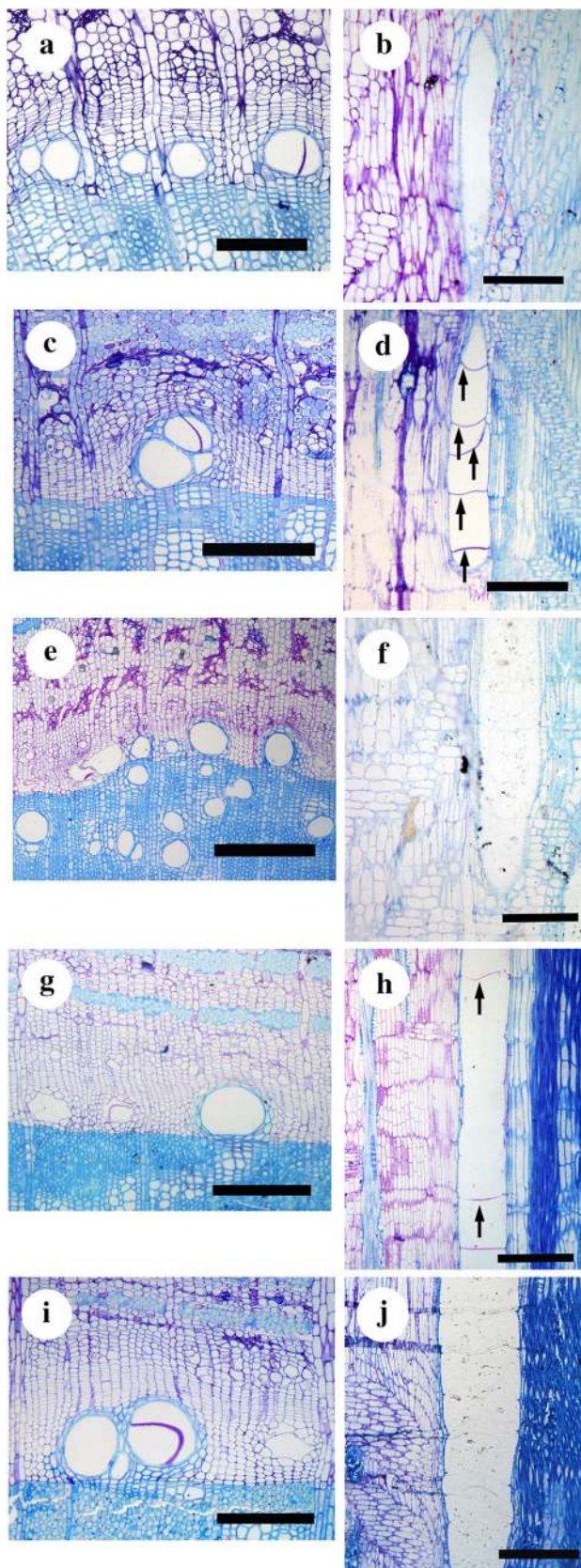
◀ **Fig. 5** Light micrographs showing transverse and radial images of differentiating vessel elements in *R. pseudoacacia*. On 16th April, deposition of secondary walls in the first earlywood vessels was observed at the *top* of the stem (**a**) and 6 m above the ground (**c**). Radial images revealed perforations (no end walls) at the *top* of the stem (**b**), but end walls of earlywood vessels (*arrows*) were still visible 6 m above the ground (**d**). On 20th April, deposition of secondary walls in the first earlywood vessels was observed 6 m above the ground (**e**) and near the ground (**g**). Radial images revealed perforations 6 m above the ground (**f**) and partial perforation of vessels near the ground (**h**). Some end walls of earlywood vessels (*arrows*) were visible near the ground (**h**). On 24 April, deposition of secondary walls in earlywood vessels was observed (**i**) and perforations were observed near the ground (**j**). *Bars* 100 μm (**a**, **b**, **d**, **f**, **g**) and 200 μm (**c**, **e**, **g–j**)

perforations were observed along the entire the stem of *R. pseudoacacia* (Fig. 5i, j).

On 1st and 9th May, we observed mature and differentiating earlywood vessels along the entire stems of *Q. serrata* and *R. pseudoacacia* (Fig. 6a–d).

Introduction of dye

In *Q. serrata*, the maximum ascending distance traveled by the dye decreased on 24th April and then increased from 1st May onwards (Fig. 7). On 4th May, the dye reached the top of the stem within 30 min. In *R. pseudoacacia*, the maximum ascending distance traveled by the dye increased rapidly from 24th April onwards. Then, the maximum ascending distance was almost constant from 28th April to 9th May. These results indicate that conduction of water through the network of the current year's earlywood vessels started before 1st May in *Q. serrata* and before 24th April in *R. pseudoacacia*.

Leaf phenology

Winter buds were present on 18th March and were observed until 8th April in *Q. serrata* and *R. pseudoacacia* (Fig. 8a, f). In *Q. serrata*, bud break occurred on 16th April (Fig. 8b). By 24th April, small leaves were visible, as well as many bud scales (Fig. 8c). On 1st May, leaves had expanded and new shoots had increased in length (Fig. 8d). On 9th May, expansion of leaves and elongation of shoots continued subsequently (Fig. 8e).

In *R. pseudoacacia*, we observed bud break on 16th April (Fig. 8g) and small leaves on 20th April. By 24th April, shoots had increased in length and small leaves were visible. From 28th April to 9th May, we observed the continuous expansion of leaves and elongation of new shoots (Fig. 8h, i).

Fig. 6 Light micrographs showing transverse images of the current year's earlywood vessels and differentiating xylem of *Q. serrata* and *R. pseudoacacia* on 9th May. Many mature earlywood vessels and differentiating vessel elements were observed 6 m (**a**) and 2 m (**b**) above the ground in the stem in *Q. serrata*. Mature earlywood vessels and differentiating vessel elements were observed 6 m above the ground (**c**) and formation of earlywood vessels in the first row was observed 2 m above the ground (**d**) in *R. pseudoacacia*. *Bars* 200 μm (**a**, **c**, **d**) and 300 μm (**b**)
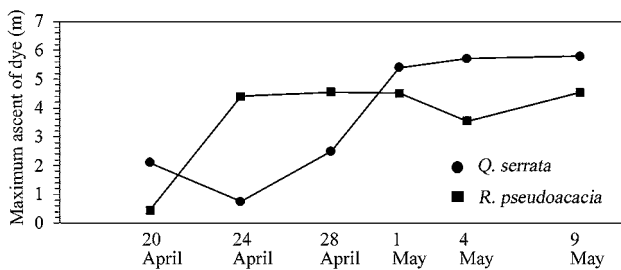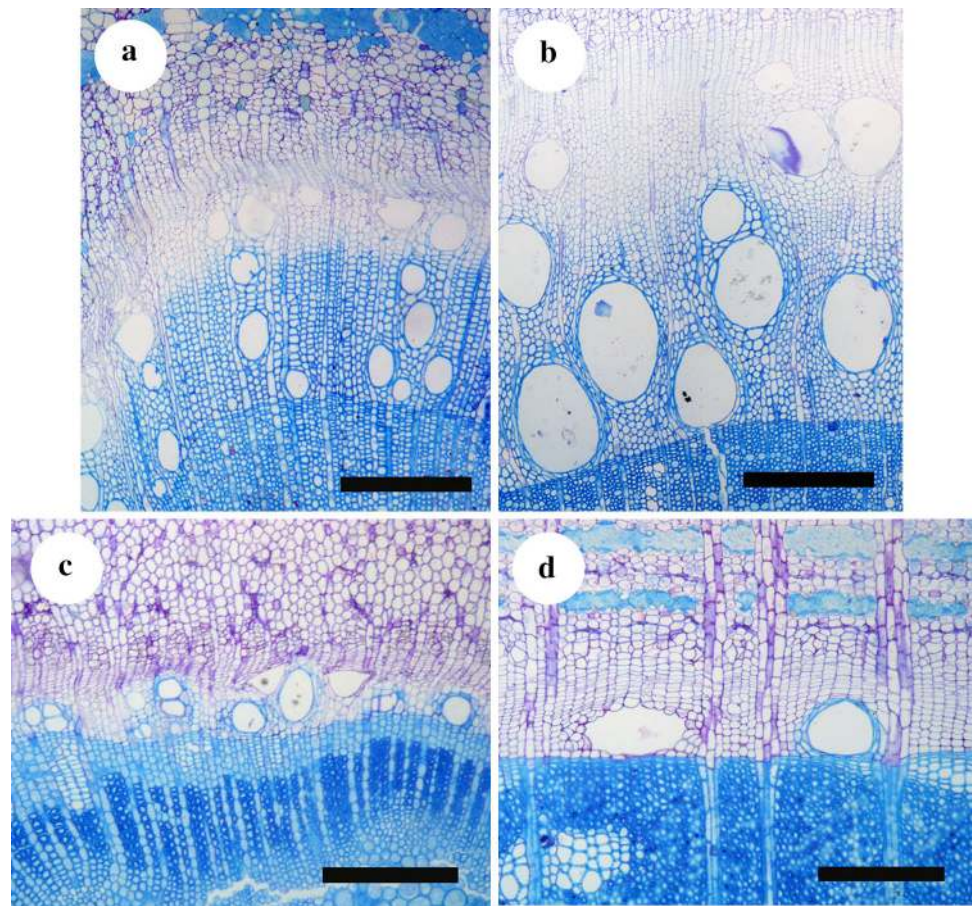

Fig. 7 Maximum ascent of dye in *Q. serrata* and *R. pseudoacacia*. The maximum ascent of dye refers to the distance from the site of introduction of dye to the farthest extent of coloration. In *Q. serrata*, dye reached close to the *top* of the stem on 1st May. In *R. pseudoacacia*, dye reached close to the *top* of the stem on 24th April

Relationship between the formation of earlywood vessel elements and leaf phenology

In both species examined, cambial reactivation and the expansion of vessel elements along the stem were evident prior to bud break (Fig. 9). Deposition of secondary walls in earlywood vessels, the disappearance of end walls of

earlywood vessels at the top of the stems and bud break occurred simultaneously. The formation of perforations was not yet complete along the entire stem at bud break. In *Q, serrata*, completion of perforations along the stem did not occur until small leaves were visible, on 24th April. The movement of dye was maximal on 1st May, indicating that the conduction of water through the network of the current year's earlywood vessels had begun.

In *R. pseudoacacia* on 20th April, perforations were observed everywhere along the stem, except near the ground, when leaves began to expand. On 20th April, the dye hardly moved at all. On 24th April, when perforations were observed along the entire stem, the dye traveled further up the stem, indicating that conduction of water through the network of the current year's earlywood vessels had begun. Completion of perforations along the entire stem and the appearance of small leaves occurred almost simultaneously.

In the two species examined, on 9th May, we observed many mature and differentiating earlywood vessels along the entire stem when leaves expanded and shoots grew longer (Figs. 6, 8).
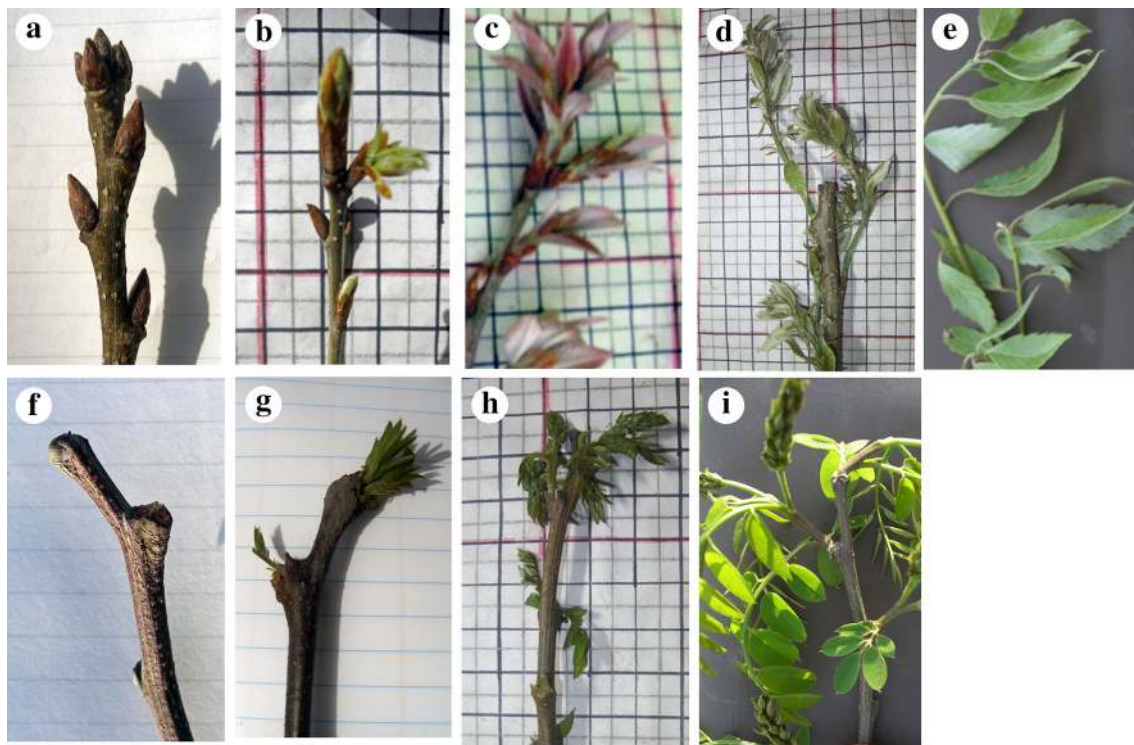
Fig. 8 Leaf phenology of *Q. serratea* (**a–e**) and *R. pseudoacacia* (**f–i**). **a** Winter buds on 18th March. **b** Bud break on 16th April. **c** Bud break and small leaves on 24th April. **d** Expansion of leaves and elongating new shoots on 1st May. **e** The expansion of leaves and elongating shoots on 9 May. **f** Winter buds on 18th March. **g** Bud break on 16th April. **h, i** Elongating shoots and leaves on 1st May and 9th May, respectively

Discussion

We took stem disks from only one tree in each of sampling dates. Therefore, there is a possibility that it is difficult to separate the effects of seasonality and inter-specimen variability of sample trees on our results. We mainly discuss the correlation between formation of earlywood vessels in the main stem at different heights and leaf phenology in the same tree.

It has been reported that earlywood vessels develop in deciduous ring-porous hardwoods before bud break [7–14]. We confirmed that division of cambial cells and expansion of vessel elements along the entire stem began before bud break in *Q. serrata* and *R. pseudoacacia*. We reported recently that the combination of disbudding and localized heating of the dormant stems of seedlings of *Q. serrata* induced the earlier differentiation of vessel elements than that in non-heated stems [14]. Thus, the presence and development of buds are not essential for the start of differentiation of the current year's first earlywood vessels.

We observed bud break on 16th April before the completion of perforations of earlywood vessels along the stems of *Q. serrata* and *R. pseudoacacia* (Fig. 9). Thus, bud growth was initiated before completion of the formation of networks of the current year's earlywood vessels along the entire stem. Therefore, the conduction of water by the network of the current year's earlywood vessels might not contribute to the start of bud growth. By contrast, during dormancy, the previous year's latewood vessels are filled with water in *Fraxinus mandshurica* var. *japonica* [3]. Water that is needed for the initiation of bud growth might be supplied via the network of the previous year's small latewood vessels.

In the present study, the completion of perforation of vessel elements along the stem and the appearance of small leaves occurred almost simultaneously in *Q. serrata* and *R. pseudoacacia* (Figs. 7, 9). Transpiration, which contributes to the driving force for the conduction of water through the network of vessels in the stem, might start with the onset of bud break. Then, the amount of transpiration in a leaf and total amount of transpiration in a tree might increase as progress of expansion of leaves. In *Q. serrata*, the maximum upward distance traveled by the dye increased after completion of the perforations of vessel elements along the entire stem. In *R. pseudoacacia*, the maximum ascent of dye and the completion of the perforation of vessel

Fig. 9 The relationships between the formation of earlywood vessels and leaf phenology in *Q. serrata* (**a**) and *R. pseudoacacia* (**b**). Each *symbol* indicates a stage during the differentiation of the first earlywood vessels as follows: *triangles* division of cambial cells, *circles* expansion of vessel elements, *hexagons* deposition of secondary walls in earlywood vessels, and *squares* formation of perforations (disappearance of end walls of vessel elements). *Open symbols* indicate that the respective stage of differentiation of the first earlywood vessels was observed. *Filled symbols* indicate that the respective stage of differentiation of the first earlywood vessels was not observed. Partial perforation of vessels was observed at the *top* of a stem of *Q. serrata* on 16th April and near the ground in a stem of *R. pseudoacacia* on 20th April
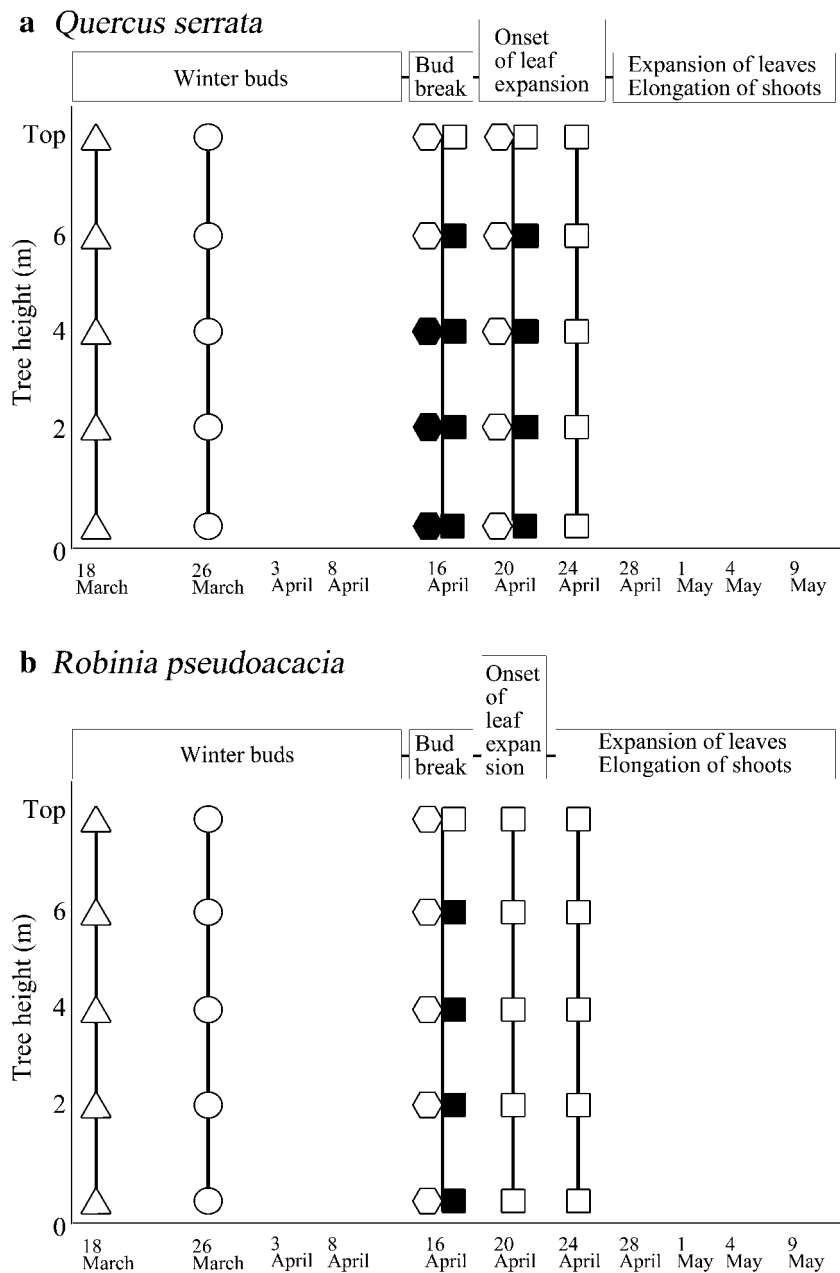
elements along the entire stem were recorded simultaneously. Perforations of all of first earlywood vessels along the entire stem and the onset of growth of leaves might be important for the initiation of water conduction through the network of the current year's earlywood vessels.

The expansion of leaves and the elongation of shoots progressed after completion of the network of the current year's first earlywood vessels in both species examined. This observation suggests that conduction of water through the network of the current year's first earlywood vessels might contribute to the development of the current year's leaves.

In conclusion, we have shown that, in the deciduous ring-porous hardwoods *Q. serrata* and *R. pseudoacacia*, the formation of the current year's earlywood vessel elements starts before bud break and perforations of the first earlywood vessels are completed along the entire stem when small leaves are visible to the naked eye. Then the expansion of leaves and the elongation of shoots continue. Conduction of water through the network of the current year's first earlywood vessels along the entire stem might not contribute to the start of bud expansion but it plays an important role in the development of the current year's leaves. The timing of start of cambial activity and xylem

differentiation is closely related to environmental factors such as temperature [21, 25]. Thus, our findings would contribute to not only the understanding the formation of networks of water transport and the system of water transport in trees but also investigations of climatic effects on growth of trees.

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