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Vascular Tissue Development and Regeneration in the Model Plant *Arabidopsis*

Ewa Mazur and Jiří Friml

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Abstract

Development of vascular tissue is a remarkable example of intercellular communication and coordinated development involving hormonal signaling and tissue polarity. Thus far, studies on vascular patterning and regeneration have been conducted mainly in trees—woody plants—with a well-developed layer of vascular cambium and secondary tissues. Trees are difficult to use as genetic models, i.e., due to long generation time, unstable environmental conditions, and lack of available mutants and transgenic lines. Therefore, the use of the main genetic model plant *Arabidopsis thaliana* (L.) Heynh., with a wealth of available marker and transgenic lines, provides a unique opportunity to address molecular mechanism of vascular tissue formation and regeneration. With specific treatments, the tiny weed *Arabidopsis* can serve as a model to understand the growth of mighty trees and interconnect a tree physiology with molecular genetics and cell biology of *Arabidopsis*.

Keywords: *Arabidopsis*, vascular tissue, vascular cambium, secondary xylem, auxin, auxin transporters, cellular polarity, PIN proteins

1. Introduction

Various species and systems were used for the analysis of vascular tissue [1–5]; however, *Arabidopsis* has been demonstrated to be the most suited plant for studies of molecular biology and developmental genetics due to its model status [6]. This in combination with rapid and effective induction system for vascularization [7, 8] with features, such as functioning vascular cambium and secondary vascular tissues found in woody plants, established in *Arabidopsis* will provide decisive advantage over previous approaches.

In this review, we summarize information concerning secondary vascular tissue development in *Arabidopsis* including cambium ontogenesis and xylogenesis, with accompanying changes in auxin distribution, directionality of its flow, and cellular polarity defined by auxin transporters (PIN family proteins), which have been indicated to be involved in regulation of vascular tissue patterning and regeneration [7–9].

2. Secondary vascular tissues in woody plants

Vascular tissue is a well function conducting system typical for all woody plants, among them in trees. In young plants, characteristic primary tissues such as procambium, primary xylem, and primary phloem, develop. During the secondary growth, vascular tissue undergoes the transition from primary into the secondary vascular patterning. Vascular cambium, secondary xylem, and secondary phloem form a closed ring on the stem circumference. They are arranged in the radial rows as a consequence of periclinal divisions of cambial cells [10–12]. The secondary growth is mostly characteristic for all woody plants, and production of the secondary vascular tissues is an important developmental feature of the plants [12, 13].

2.1. Vascular cambium

Vascular cambium plays a crucial role in the secondary growth and vascular tissue patterning in woody plants [14, 15]. Activity and functioning of vascular cambium decide about the amount of the secondary phloem and xylem, which are produced outward and inward the vascular cambium, respectively [12, 13]. This meristematic tissue is built from two types of cells: ray cambial cells producing secondary rays—transverse conducting system in tissues—and fusiform cambial cells, producing elements of the longitudinal conducting systems in woody plants. Characteristic feature of the vascular cambium is intrusive growth of the fusiform cambial cells and their periclinal divisions [13, 16, 17]. The intrusive growth is restricted to the ends of growing cells, when two neighboring cells grow in opposite directions. Periclinal divisions of cambial cells decide about production of cambial derivatives and secondary tissue element differentiation.

Vascular cambium is the tissue very much sensitive to mechanical injuries, such as wounding or grafting. However, it can easily regenerate under suitable conditions. It has been experimentally shown that cambium regeneration is mostly dependent on the tensile stress and pressure. Results obtained by Brown [18] indicate that cambium activity, cell divisions, and xylem formation can be easily affected by the pressure externally implied to the cambial strips. It is also documented in *in vivo* experiments with the wounding stems of *Larix europea* that regeneration of this meristematic tissue can dynamically progress under the tensile stress and pressure implication. In such cases, even low pressure (25 kPa) implied to cambium in wounded areas decides about its very rapid regeneration. Lack of the mechanical factors leads to abundant callus tissue production [19, 20].

It has been postulated that appropriate functioning of vascular cambium and its cyclic activity, i.e., periclinal divisions during the seasons, is strictly correlated with auxin signaling and auxin responses [21–23]. From the studies on *Populus tremula* L. × *Populus tremuloides*, it appears that the highest auxin concentration is found in the layer of cambium. Auxin plays here a key role in the regulation of cambial cell divisions and elongation [23]. According to the results obtained with the vascular cambium in *Pinus sylvestris*, thickness of the cambial layer is directly dependent on the auxin concentration in the tissue, which stimulates frequency of divisions [21, 22]. The highest activity of vascular cambium is found at the beginning of the vegetative seasons, in early spring, which is correlated with the first periclinal divisions of the cambial cells, production of new cambial derivatives, and their differentiation into new secondary vascular tissues. Otherwise, the lowest cambial activity in winter, during the dormant period, is strictly correlated with decreasing both temperature and hormonal levels in cambium layer [24]. Periclinal divisions are limited and almost completely stop; thus, the vasculature is not produced during this time. However, it was experimentally shown that such situation could be easily reversed after exogenous auxin application [25]. As a consequence, activity of cambium and periclinal divisions of cambial cells can be resumed by auxin. Thus, from all the studies on the woody plants, it appears that elevated auxin response in cambial cells as well as fluctuations of auxin (maxima/minima) in cambium plays decisive role in seasonal nature of the trees, for example, switching on/off dormant periods [21, 22]. Changes of the cell wall components [26–28] and gene expression during the cyclic activity of vascular cambium [29, 30], correlated with the rapid cytoskeleton rearrangement in differentiating cells [31], indicate that this meristematic tissue plays a crucial role in the secondary growth of woody plants and decides about their adaptation to variable environmental conditions.

2.2. Secondary xylem

In the most typical form, secondary xylem, also called a wood, is found in stems and roots of the woody plants. The secondary xylem, a longitudinal conducting system in trees, develops from the cambial derivatives, which during the maturation process is differentiated into elements of the wood-like vessels, fibers, and tracheids [13, 15].

Vessels of the secondary xylem form strands parallel to the longitudinal axis of the organs—stems or roots. Every vessel strand is consisted of single vessel elements, the so-called vessel members, connected with each other by open perforation plates localized on their apical-basal ends [12]. It is postulated that direction of vessel differentiation is dependent on direction of auxin flow. Thus, in nondisturbed stems, vessels developed according to the polar auxin transport (PAT) in the apical-basal direction whereas in incised organs—according to newly established direction of auxin flow—circumventing the wounded regions. Correlations between auxin flow and vasculature patterning were experimentally documented in woody plants after wounding [32] as well as nonwoody models [4, 5, 8]. Characteristic feature for all types of vessels (primary protoxylem, metaxylem, and secondary xylem vessels) is the secondary cell wall. Different patterning of the secondary cell wall is realized during vessel maturation process and depends on the type of vessel [14]. During the maturation process, protoplasts of differentiating vessels disappeared. Frequently, the

lumen of the vessel members is enlarged in comparison to other tracheary elements, mainly in a wood of such species as *Fraxinus excelsior*, *Quercus borealis*, or *Ulmus americana* [13]. In many cases, length of the vessel members is different than the length of the fusiform cambial cells from which they developed. Interestingly, longitudinal vessel strands change the orientation to the longitudinal axis of stems. Such fluctuations are observed as the wavy grain patterning of wood in many trees [33].

Fibers of the secondary xylem are recognized as one of the longest tracheary elements, characterized by the tapered cell ends and reduced lumen. As a consequence of their intensive intrusive growth, fibers could be even few times longer than the fusiform cambial cells and their derivatives. Particular type of the woody fibers is the so-called gelatin fibers, developed as a layer of the reaction wood in many deciduous as well as coniferous trees, i.e., *Populus* sp. or *Picea* sp. Inner layer of secondary cell wall of these fibers is built mainly from cellulose. The presence of the callose and callose-like cell wall components plays here an important role in mechanical properties of the wood [34].

Tracheids, other tracheary elements of secondary xylem, are nonperforated, long cells with the bordered pits. Dependently on the type of a wood, tracheids are classified as (1) vessel-like tracheids arranged in longitudinal, similar to vessels conducting strands, commonly found in *Carpinus* sp., *Ulmus* sp., *Acer* sp., or *Tilia* sp.; (2) tracheids differentiated around the vessels with enlarge lumen, adjacent to them, and strictly surrounding; and (3) fiber-like tracheids [35]. The last of them develop as a conducting and storage water system but also play mechanical functions and in some species could be the main component of the softwood of gymnosperms [36]; they are found also in some angiosperms, i.e., in *Populus* sp.

Besides of the dead, water-conducting elements of the secondary xylem mentioned above, in many cases secondary vascular tissue of woody plants is compound with the xylem parenchyma cells, which remain alive for a long time to finally die in the programmed cell death (PCD) process [12].

Thorough knowledge about the genetic and molecular mechanisms involved in vascular tissue functioning, development, and regeneration is eagerly expected. Different molecular components involved in the determination of developmental plasticity of cambial cells have been searched for with special interest focused on the key regulators of vascularization. Genes involved in auxin response, auxin signaling pathways, and tissue and cellular polarity during vascular tissue development induced in vascular cambium should be extensively studied for detailed characterization of this process.

2.3. *Arabidopsis* as a nonwoody plant example for vascular tissue formation

Since many years, *Arabidopsis* is nominated as a good model for studies of vascular tissue formation, because under suitable conditions, *Arabidopsis* can undergo secondary growth in hypocotyls, when enlarged layer of secondary xylem develops during xylogenesis [37]. Xylogenesis in hypocotyls is comparable to xylogenesis in roots. Development of secondary xylem is divided here into two phases: the early phase, xylem is building from vessels and numerous parenchyma cells, and in the second, later phase, also called xylem expansion, enlarged amount of xylem elements

develops mainly vessels and fibers [37, 38]. It is well documented that vascular tissue develops not only in hypocotyls [37] but also in the matured inflorescence stems [39–41], in their basal parts [42–45]. With the use of *Arabidopsis*, the correlation between auxin signaling and tissue polarity has been intensively studied and modeled [46–49]. However, because of lack of some important vasculature features such as a variety of typical phenotype features and functional cambium, these models could not be used for full analysis and description of vascular tissue development and compared to the analogical process in trees. For example, in both *Arabidopsis* models mentioned above, rays were not found. Also intrusive growth typical for fusiform cambial cells was not confirmed. Finally, impressive variety of tracheary elements, among them tracheids, commonly found in trees, were not observed in *Arabidopsis* mature stems and hypocotyls, which underwent secondary growth [50].

In contrast, in the *Arabidopsis* inflorescence stems stimulated mechanically by an artificial weight, the transition from primary to secondary tissue architecture leads to the development of all vasculature features mimicking secondary vascular tissues in woody plants. According to the new approach, immature inflorescence stems (9–10 cm tall) were firstly decapitated with the sharp razor blade (shoot apex and flowers were removed). Next, the artificial weight (2.5 g) was applied to the decapitated apical parts of the stems. Stems were additionally supported by a wood stick to avoid their bending. Importantly, the axillary buds grown above the leaf rosettes were not removed, thus remaining the natural source of endogenous auxin. This experimental approach has been extensively described [7, 8]. It was speculated that the weight carried by the stem serves as a mechanically stimulated signal for wood formation [7, 8, 42, 51]. According to Ko and coauthors [42], mechanical stimulation of immature inflorescence stems of *Arabidopsis* increases polar auxin transport and promotes the secondary growth. It allows designing *Arabidopsis* as a full “tree-like” system. Moreover, the secondary vascular tissues develop in a very short time, namely, in 6 days [7], which is much faster than in hypocotyls [37, 38] or mature inflorescence stems of *Arabidopsis* [44, 45].

In the created *Arabidopsis* “tree-mimicking” model, the development of variety of vascular cambium phenotypes is the most spectacular. According to the obtained results, both types of cambial cells develop: (1) ray cambial cells, very short and almost round cells arranged in single-row rays mimicking transverse conducting system in woody plants, and (2) fusiform cambial cells, long, tapered-end cells, characterized by the intrusive growth and periclinal divisions, play here an important role in secondary tissue element differentiation. The phenomenon of intrusive growth of fusiform cambial cells is described for the first time in the mechanically stimulated *Arabidopsis* stems (**Figure 1A–D**), not found in the previously analyzed models. Neighboring cells start their growth in the opposite directions, but the growth is restricted to the tips of the cells, which slide along the radial cell walls and provide the elongation of the fusiform cambial cells (**Figure 1A, C, and D**).

New approach, based on mechanical stimulation of the immature inflorescence stems of *Arabidopsis* [7, 8, 42, 51], is expected to elucidate the phenomenon of vascular tissue formation and regeneration at cellular and molecular level—processes commonly studied in woody plants, but not fully explained yet, because of some experimental and environmental difficulties in these plants [52]. Thus, *Arabidopsis* comes out as a good model system for vascular tissue patterning.

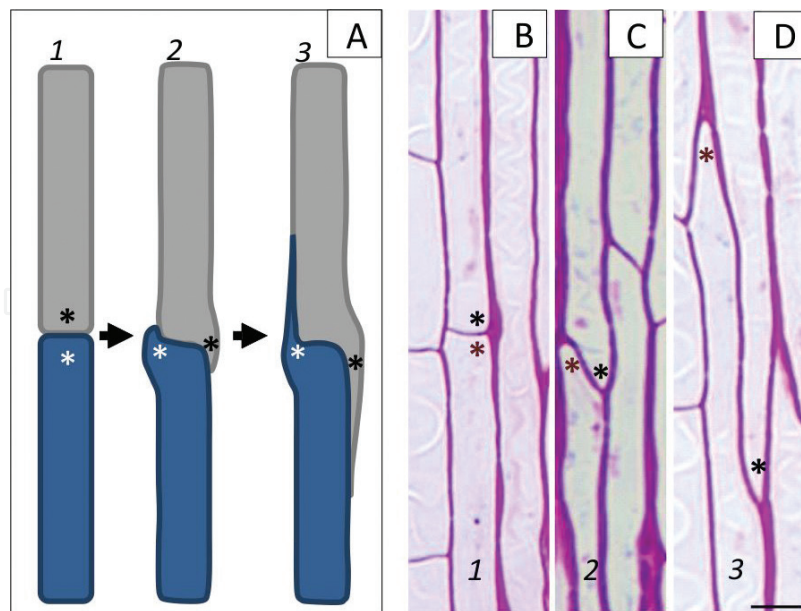


Figure 1. Intrusive growth of the fusiform cambial cells in mechanically stimulated *Arabidopsis* stems. (A) Schematic visualization of two intrusively growing cells ((1) two neighboring cells with still non-intrusively growing ends, (2) beginning of the intrusive growth of the opposite ends, (3) advanced intrusive growth along the radial cell walls of the cells). Asterisks indicate neighbor cells and their intrusively growing ends. (B–D) Intrusively growing fusiform cambial cells in temporal steps corresponding to situation visualized in (A). (B–D = longitudinal tangential sections through *Arabidopsis* stems; Poly/Bed 812 resin sections stained with the Periodic Acid–Schiff's (PAS reaction); bar, 10 μm).

3. Vascular tissue development and regeneration in mechanically stimulated inflorescence stems of *Arabidopsis*

In this paragraph, we will describe in detail the transition from primary to secondary tissue architecture in inflorescence stems of *Arabidopsis* as the important step in obtaining a suitable model for secondary vascular tissue analysis, following the temporal and spatial changes during vascular cambium ontogenesis, xylem formation, and vascular tissue regeneration in weight-induced *Arabidopsis*.

3.1. Ontogenesis of vascular cambium

Ontogenesis of vascular cambium is correlated with temporal and spatial changes on the stem circumference. Usually, formation of a closed ring of cambium is preceded by dedifferentiation of parenchyma cells into cambial cells and the so-called interfascicular cambium development. This process is commonly observed in young woody plants during their secondary growth [12]. It has been confirmed by histological analyses that the first dedifferentiated parenchyma cells are localized next to the vascular bundles in the early stages of the interfascicular cambium development [12]. With the time, the regions of dedifferentiating parenchyma cells are extended and finally enclosed as continuous ring on the stem circumference. The mechanism of these changes is still not clarified. The basic question is which of the cellular events trigger the parenchyma cell dedifferentiation?

In mechanically stimulated *Arabidopsis* stems, vascular cambium develops from fascicular cambium and interfascicular cambium bands (**Figure 2**). Fascicular cambium develops as a primary meristematic tissue in vascular bundles, localized in the inner parts of immature stems characterized by primary tissue architecture. The vascular bundles are separated by interfascicular parenchyma bands with nonpericlinally dividing parenchyma cells (**Figure 2A and B**). Outside these regions, few layers of cortex and single layer of the epidermis are situated. Middle parts of the stems consisted of the enlarged, thin-cell wall pith parenchyma cells. One- or few-layer supporting tissue with characteristic thick-cell wall interfascicular fibers plays mechanical function in immature stems (**Figure 2B**). In immature stems of *Arabidopsis*, 6 days after weight application, the architecture of the basal parts of such stems diametrically changes. At the beginning of the secondary growth, interfascicular cambium develops in the interfascicular regions of stems, as a consequence of parenchyma cell dedifferentiation (**Figure 2C and D**). Interestingly, the most inner layer of interfascicular parenchyma cells dedifferentiates into the interfascicular cambium. Typically, it is a single layer of parenchyma

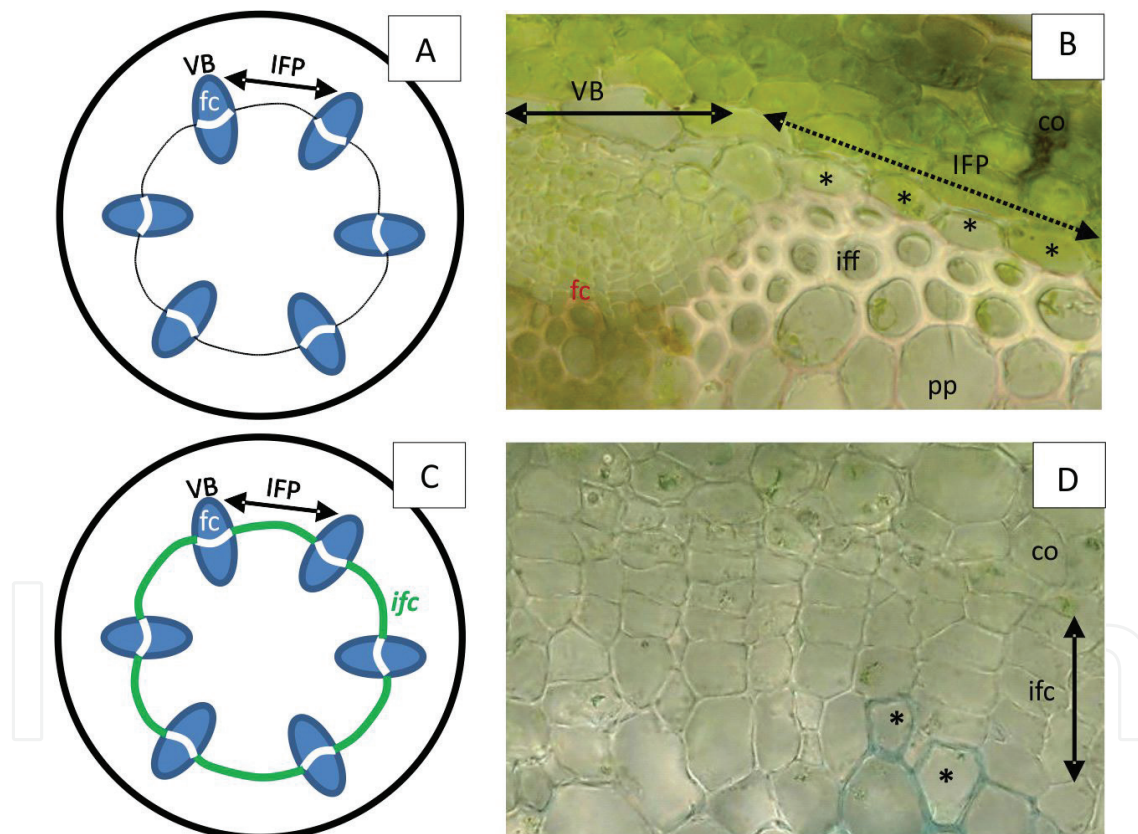


Figure 2. Transition from primary to secondary tissue architecture in weight applied inflorescence stems of *Arabidopsis*. (A) Schematic visualization of the tissue arrangement in immature inflorescence stems of *Arabidopsis*. (B) Cross section through the basal parts of stem with the primary tissues—vascular bundles with fascicular cambium are separated by the interfascicular parenchyma. The most inner layer of interfascicular parenchyma cells will dedifferentiate into cambial cells (asterisks). (C) Schematic visualization of the secondary vascular tissues with closed ring of vascular cambium on stems circumference. (D) Layer of periclinally dividing interfascicular cambial cells as a part of the ring of vascular cambium. The first developed vessels are indicated by asterisks (lignin in the secondary cell walls stained with 0.05% Toluidine Blue O aquatic solution). B and D, handily made cross sections through basal part of stems (VB, vascular bundle; IFP, interfascicular parenchyma; iff, interfascicular fibers; co, cortex; pp, pith parenchyma; fc, fascicular cambium; ifc, interfascicular cambium); bars, 20 μ m.

cells localized between vascular bundles. Finally, during the transition from the primary to the secondary tissue architecture of *Arabidopsis* stems, fascicular and interfascicular cambium forms fully enclosed ring of vascular cambium on stem circumference (**Figure 2D**).

The whole process of cambium ontogenesis is strictly correlated with such cellular events as elevated auxin response in interfascicular parenchyma, polarity of parenchyma cells dedifferentiating into the cambium, their periclinal divisions, and changes of their cell wall components [7]. The most spectacular seems to be correlations between auxin response and tissue polarity during cambium ontogenesis in analyzed *Arabidopsis* stems. Already in the first few days, auxin concentration distinctly arises in the dedifferentiating parenchyma cells. At the early stages of the interfascicular cambium development, maximum auxin concentration is detected in parenchyma cells localized in the nearest neighborhood of vascular bundles, whereas in the later stages of this process, the zone of the cells with elevated auxin response is gradually extended toward the middle parts of the interfascicular regions, in the next few days after weight application [7]. Polarity of the interfascicular parenchyma was monitored by the PIN-FORMED1 (PIN1) protein localization in differentiating cells. The PINs are well-known auxin transport proteins involved in the cellular efflux of auxin and polar auxin transport in plant tissues [53]. In many developmental processes, the establishment of local PIN-dependent auxin gradient in cells is strictly correlated with cellular divisions and developmental reprogramming [54, 55].

During analyzed process of cambium ontogenesis, tissue polarity is rapidly established in *Arabidopsis* stems. Amazingly, polarity of interfascicular parenchyma is indicated by polar localization of PIN1 auxin transport protein, which localizes at the basal plasma membranes of differentiating cells [56]. It has been documented that the protein appears in the basal plasma membranes of dedifferentiating parenchyma cells, not previously found in parenchymatic cells of immature mechanically noninduced *Arabidopsis* stems [7]. Moreover, both of the events—elevated auxin response and tissue polarization—are accompanied by periclinal divisions of the parenchyma

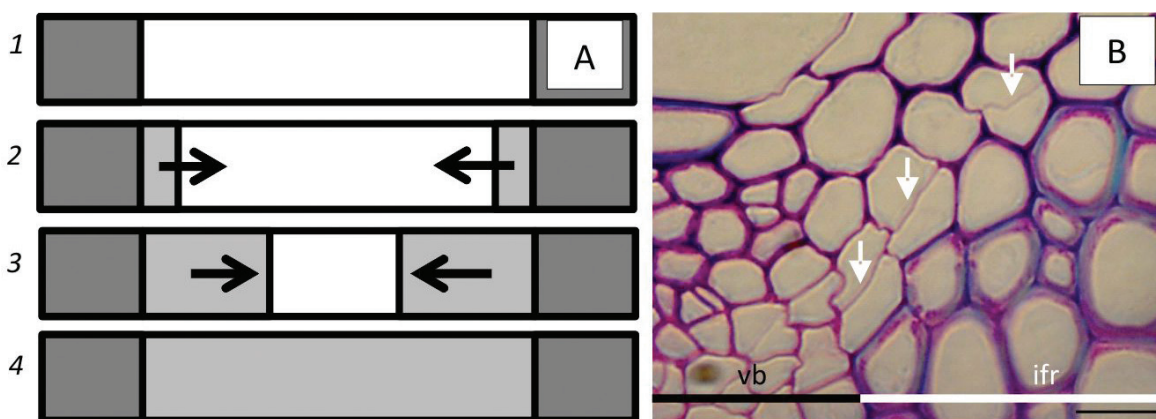


Figure 3. Periclinal divisions of interfascicular parenchyma cells and interfascicular cambium development in weight-induced *Arabidopsis* stems. (A) Schematic visualization of the temporal changes in interfascicular parenchyma regions with gradually extended zone of periclinally dividing cells (1–4 = four steps of the changes from vascular bundles, dark grey; zone of dividing parenchyma cells, grey; nondivided parenchyma cells, white; arrows indicate the direction of changes). (B) Periclinal divisions of the interfascicular parenchyma cells in the neighborhood of the vascular bundle (arrows); Poly/Bed 812 resin section stained with the periodic acid-Schiff's (PAS reaction); *vb*, vascular bundle; *ifr*, interfascicular region; bar, 20 μm .

cells (**Figure 3**). Divisions are temporarily correlated with the cellular events mentioned above and maintained in space. Namely, the first periclinally divided cells appear in the neighborhood of vascular bundles (**Figure 3A and B**), but later the zone of dividing cells slowly extends toward the middle part of interfascicular regions. In consequence, parenchyma cells dedifferentiate into cambial cells, which definitely changes architecture of the interfascicular regions and decides about development of the interfascicular cambium (**Figure 3A**). According to the obtained results, it is tempting to conclude that auxin plays the most important role during cambium ontogenesis in *Arabidopsis* stems. Auxin seems to be a primary signal for cellular fate reprogramming and a crucial clue for stimulation of the dedifferentiation process in the interfascicular parenchyma zones.

In the described model, vascular cambium could be classified as “functioning” meristematic tissue, which actively produces cambial derivatives. Differentiation of cambial derivatives is a consequence of numerous periclinal divisions of fusiform cambial cells. Finally, the maturation of the cambial derivatives into secondary vascular tissue elements supported functionality of this meristematic tissue in the present model. The sequence of the changes could be useful for all comparative analysis of the cambium ontogenesis and xylogenesis both in *Arabidopsis* model system and the analogical mechanisms studied in woody plants. Thus, the mechanically stimulated *Arabidopsis* model with fully functional cambial meristem could help us in addressing the elusive vascularization mechanisms observed in the woody plants.

3.2. Secondary xylem formation in *Arabidopsis* stems

Reprogramming of the gene expression that accompanies xylogenesis and transdifferentiation of mesophyll cells into tracheary elements was extensively studied in *in vitro* cultures of zinnia (*Zinnia elegans*) [57, 58]. However, the lack of the cambium stage in this experimental system prevents us from deciphering the role of cambium in wood formation. Temporal gene expression pattern accompanies dedifferentiation of cambial cells into cambial derivatives, but their maturation into different types of tracheary elements is poorly characterized. Thus, numerous efforts have been focused on the identification of master regulatory genes required for this transition and revealing the key components of the vascular-differentiation-involved genetic network [48, 59].

In *Arabidopsis* “cambial” model, vascular cambium reveals basic features of functioning cambium important in the following stages of xylogenesis. Periclinal divisions of the fusiform cambial cells lead to the development of secondary xylem derivatives in the early stage of xylogenesis. Changes in later stages of xylogenesis are correlated with maturation of cambial derivatives into tracheary elements and secondary vascular xylem development (**Figure 4**). During this process, such recognizable tracheary elements as vessels, fibers, or tracheids develop and create the layer of secondary xylem. Vessels are easily recognized, because of some diagnostic features such as secondary cell wall and open perforation plates on the opposite ends of the vessel members (**Figure 4C**). Vessels are arranged in threads of longitudinal strands in the vascular tissue. Amazingly, in the present *Arabidopsis* model, impressive variety of tracheary elements is detected, not previously documented in analyzed hypocotyls [37, 38] or adult stems of *Arabidopsis* [39–41].

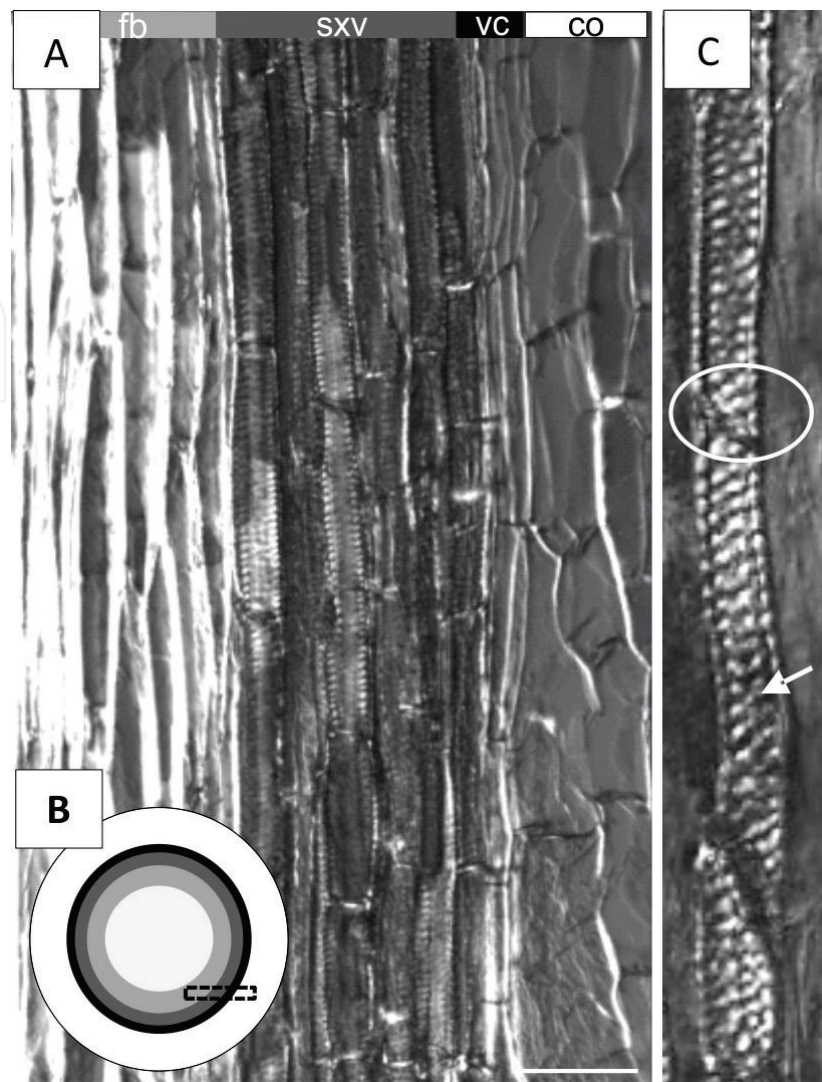


Figure 4. Secondary xylem in the weight stimulated stems of *Arabidopsis*. (A) Secondary xylem elements, like vessels and fibers, are produced from cambial derivatives after numerous periclinal divisions of fusiform cambial cells. Cortex parenchyma is visible outside the secondary vascular tissues. (B) Schematic visualization of the tissue arrangement in stem and localization of the tissues showed in (A) is indicated by the square. (C) Vessel strand developed parallel to longitudinal axis of stem. Characteristic patterning of the secondary cell wall (arrow) and perforation plates developed on the opposite apical-basal ends of neighboring vessel members (circle) determines the most diagnostic features for this type of tracheary elements (A and C, bright-field images in a confocal laser-scanning microscope; *fb*, fibers; *sxv*, secondary xylem vessels; *vc*, vascular cambium; *co*, cortex); bars, 50 μm (A); 20 μm (C).

Patterning of vascular tissue and variety of tracheary elements developed as a dynamically operating water-conducting system and was extensively studied in the woody plants [13, 14]. However, mechanism regulating xylogenesis at cellular and molecular levels remains unclear, and many questions are unanswered. For example, differentiation of tracheids as a type of tracheary elements commonly found in trees, but for the first time detected in mechanically stimulated *Arabidopsis*, led to important conclusions about the involvement of the artificial weight in wood formation. Following stages of xylogenesis involving formation of the variety of tracheary elements, such as recognized tracheids, will be helpful in future analysis.

3.3. Regeneration of vascular tissue in wounded *Arabidopsis* stems

In 1981, Sachs postulated canalization hypothesis according to which vasculature patterning is based on the positive feedback loop between auxin flow and cellular polarity. Consequently, in the primary uniform tissue, cellular auxin transporters emerge as the so-called auxin channels that transport the hormone through the tissue in the polar direction. Emergence of auxin channels is correlated with establishment of cellular polarity inside these specific auxin transport routes. Finally, new vessels develop directly along the auxin channels. Canalization hypothesis is strongly supported by many classical experiments with the incised plants, i.e., by wounding or grafting, which shows that emergence of auxin channels is correlated with increased auxin response and tissue repolarization [1, 2, 4, 5]. It is well documented that initially broadly elevated auxin response in wounded tissues is gradually restricted to narrow auxin channels, in which auxin level is still very high [4]. The obtained results showed that patterning of vascular tissue, explicitly visible during regeneration and new vasculature development, is dependent on new ways of canalized auxin flow.

Well-functioning vascular cambium plays the most important role for the secondary growth in the woody plants, both secondary xylem formation and stem thickness [14, 21, 22, 60]. Many results revealed an important role for this meristematic tissue during vasculature regeneration process. For decades analysis of vascular patterning and incised vascular cambium regeneration was restricted mainly to trees [61–63] because these woody plants undergo secondary growth with enlarged amount of secondary xylem (wood) and active cylinder of vascular cambium [64]. Studies were based mainly on the histological analysis, thus limited only to the final effects of regeneration. Thus, it was impossible to analyze vasculature regeneration, including vascular cambium, on the cellular and molecular levels. Some experimental studies on trees showed that in the wounded areas, the cambium and vascular tissue regenerate very fast both *in vivo* [19, 20] and *in vitro* [25, 65, 66]. Regeneration is accompanied by numerous anticlinal divisions of cambial cells and their dynamic intrusive growth [19, 20, 64], which finally leads to the reconstruction of vasculature and new vessel patterning in the incised regions [25, 65]. In some instances, when the auxin flow is locally reversed, the so-called circular vessels develop [32, 67, 68]. In the nondisturbed woody plants, circular vessels are often found in branch junctions, above the axillary buds [68], whereas in incised plants, after transversal cuts and exogenous auxin application to stem segments, in wounded regions [32, 67]. Accordingly, circular vessels occur in the form of rings and are presumably induced as a consequence of the circular auxin flow and the establishment of the circular polarity of individual cells that dedifferentiated into this type of vessels [67]. Thus, according to Sachs and Cohen [67], circular vessels develop as a response of individual cells to the auxin flux rather than to the high local auxin concentration. In nonwoody dicotyledonous plants characterized by primary tissue architecture, such as *Phaseolus vulgaris*, *Pisum sativum*, or *Coleus* sp., vasculature is regenerated directly from dedifferentiated parenchyma cells [1–5]. New vessels are arranged either around the wound according to the presumable new auxin flow [69] or form the so-called bypass strands directly through the wound [3] or bridges between the neighboring vascular bundles [70]. Lack of the vascular cambium in the studied nonwoody plants restricted a detailed analysis of regeneration of this meristematic tissue and cellular events accompanying this process. Therefore in the used models, the most intriguing questions are still remained of

answer: (1) what is the role of vascular cambium in vascular tissue regeneration?, (2) which of the cellular events are temporary correlated with the vascular cambium regeneration?, (3) is vascular cambium regeneration mediated by the canalization process? Full verification of the postulated canalization hypothesis and identification of the molecular mechanisms accompanied vascular tissue regeneration are still limited.

Because of the difficulties in using woody plants as a convenient model system [52], mechanisms of cambium regeneration are still poorly understood. With the *Arabidopsis* “cambium” model, it is now possible to monitor vascular tissue regeneration with all cellular events accompanying this process. Thus, in control conditions, i.e., in nonincised stems, polar auxin flow is in the direction from apical to basal part of stems, and according to this flow, new vasculature develops. Otherwise, in incised stems (i.e., wounded stems), polar auxin transport is disturbed; thus, new ways of auxin flow are established. As a consequence, new vessel strand arrangement is changed, because the new vasculature likely developed according to new directions of auxin cell-to-cell transport (**Figure 5**). In wounded *Arabidopsis* stems, threads of new vessel strands develop above or around a wound (**Figure 5A** and **B**, respectively). Interestingly, vessels above a wound regenerated faster, in the first days after wounding (DAW) (2 and 3 days), whereas vessel around a wound differentiated in the next few days, beginning the day 4 and circumventing the incised areas. They developed from cells after their numerous, uneven divisions, and

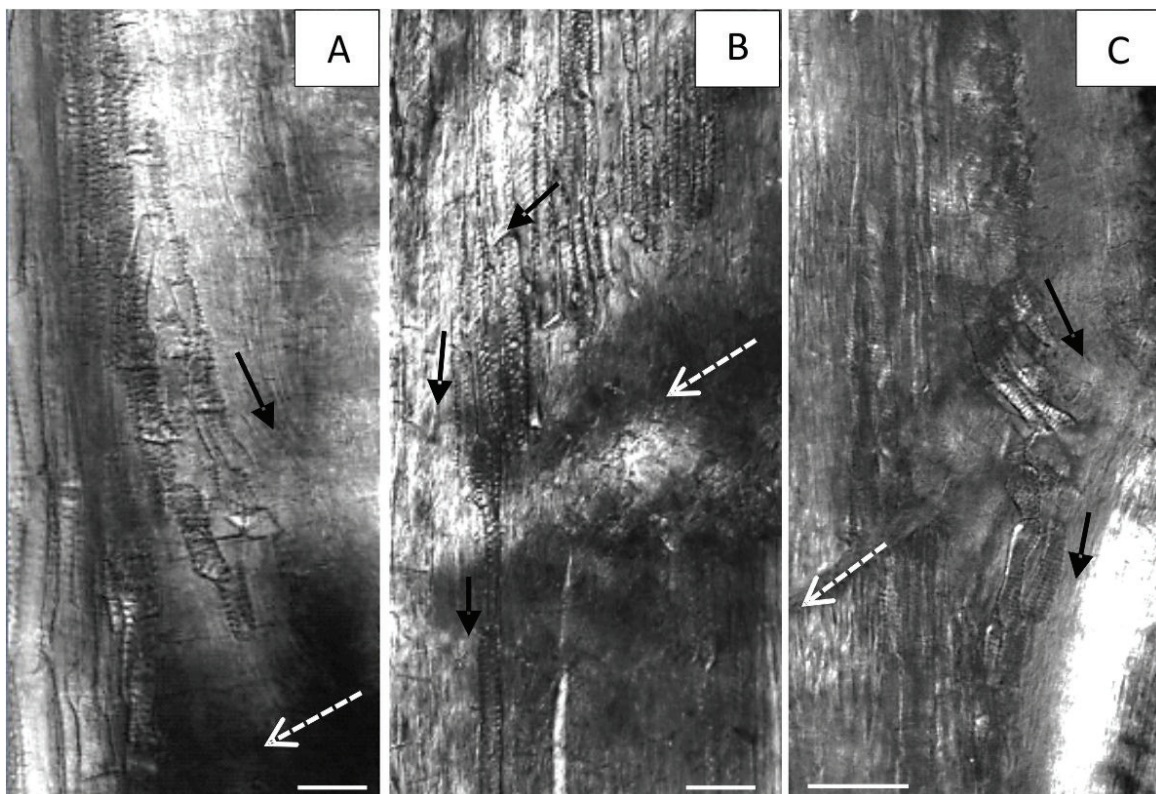


Figure 5. Paths of vessel regeneration in wounded *Arabidopsis* stems. (A) Threads of short vessel members developed above a wound. (B) Vessel strands regenerated around a wound. (C) Vessel “bypass” strands reconstructed partially from the callus tissue developed inside the wound. Arrows indicate regenerated vessel strands. Broken arrows mark places of the wound; bright-field images in a confocal laser-scanning microscope; bars, 50 μm .

what is commonly observed in the wounded tissue. Differentiating vessels were visualized by the activity of the *AtHB8* gene, which belongs to HD-ZIP III family [71, 72]. The *AtHB8* is positively regulated by auxin, and its extensive activity in wounded regions during vascular tissue regeneration suggested that *AtHB8* might play a crucial role in the vasculature development [71, 72]. The last observed way of vasculature regeneration is correlated with callus differentiation (Figure 5C). Namely, in wounded areas vessels develop from previously proliferated callus tissue cells. Such vessels often create the type of “bypass” strands extending above and below the transversal incision.

Regeneration of vascular tissue in wounded *Arabidopsis* stems is accompanied by temporal and spatial changes following new vessel development. New vessel strands regenerated in the incised regions around a wound develop as a consequence of cambial cell regeneration. Longitudinal continuum of vascular cambium is disturbed after the transversal cut. In such experimental system, rapid auxin response is found as a primary signal of the regeneration. Merely at the first day after incision, elevated auxin concentration is observed above a wound and in the next few days also around a wound [8]. Vasculature regeneration is strictly correlated with tissue repolarization and establishment of new polarity in neighborhood of the wound. Tissue repolarization always preceded emergence of PIN1-positive auxin channels (Figure 6). As a consequence, layer of new vessels develops around a wound, and the regenerated vasculature becomes enlarged

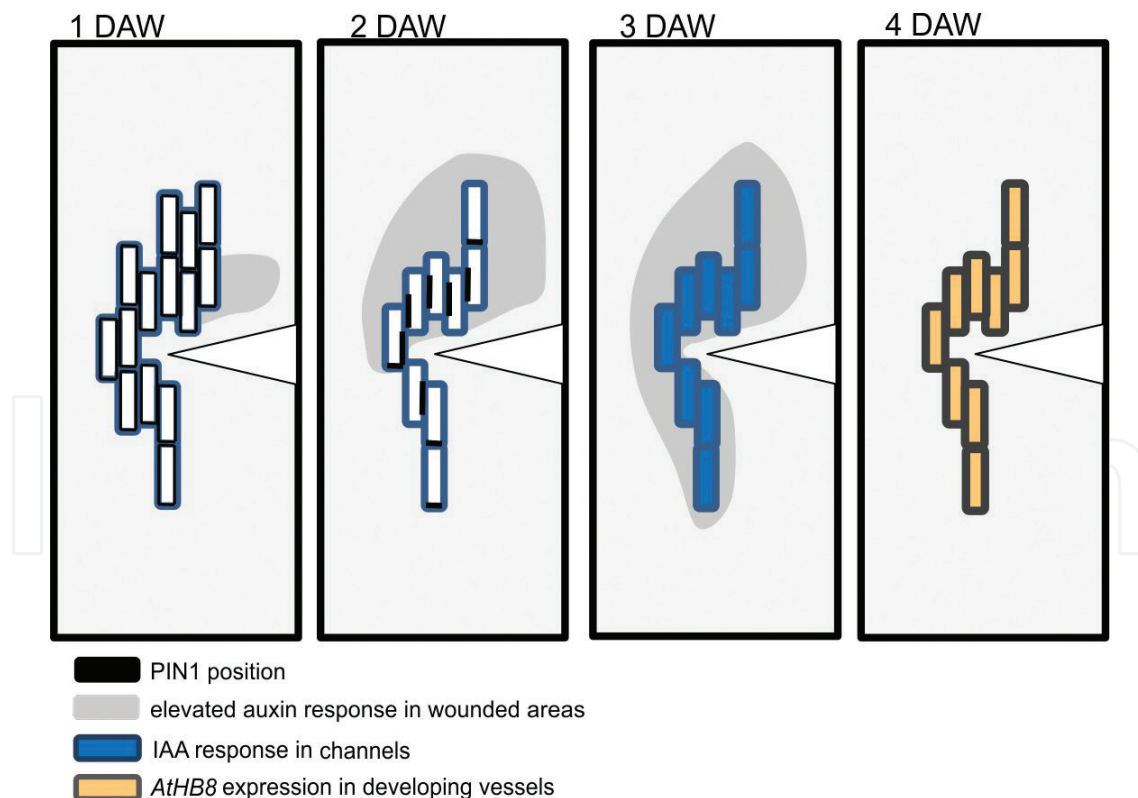


Figure 6. Schematic visualization of the temporal and spatial changes during vascular tissue regeneration in following days after wounding (DAW): re-localization of PIN1 in cells around a wound (1 DAW); establishment of new position of PIN1 at cellular plasma membranes (2 DAW); increased auxin concentration in auxin channels (3 DAW); expression of *AtHB8* gene in differentiating vessels (4 DAW).

in the days following the incision. Analysis of regeneration process in incised *Arabidopsis* stems strongly supported canalization hypothesis. Emergence of new vasculature is correlated here with elevated auxin response and changed polarity in auxin channels, from which new vessel strands develop in the wounded areas.

4. Role of plant hormone auxin and auxin transporters in vascular tissue development

Auxin is regarded as a multifunction plant hormone, which plays a fundamental role in developmental processes during organo- and morphogenesis. Auxin is a primary signal in regulation of many cellular processes, which control oriented divisions, cell elongation, or differentiation. At last, auxin is a key hormonal factor inducing vascularization—vascular tissue development, patterning, and regeneration. Polar auxin transport (PAT) manifested as physiological, basipetal direction of auxin flow represents a unique mechanism specific to plants. The cellular and molecular action of this process, explained in the chemiosmotic model, is based on auxin influx and efflux carriers, namely, AUX and PIN proteins, which actively participate in the cell-to-cell hormone transport [73–75]. The local auxin accumulation, its minima and maxima, or the so-called gradients in tissues are precisely controlled by this process.

4.1. Auxin as a primary signal inducing vascularization

The role of auxin as a primary signaling cue in vascularization has been widely discussed for decades. Experiments with radioactively labeled auxin show its maximum concentration in the meristematic tissues such as cambium [22, 57] and in adjacent cambial derivatives, differentiating into xylem [76]. Periodic fluctuation of auxin concentration in cambium influences the frequency of cambial cell divisions, production of cambial derivatives, and secondary vascular tissues. Disturbance of these correlations leads to many defects in cambium functioning and xylem formation. Using transgenic lines of *Arabidopsis*, elevated auxin response is easily found just in the cambial cells of both types of cambia (**Figure 7**). Auxin concentration is very high in the fascicular cambium bands, primary meristematic tissue in the vascular bundles (**Figure 7**), as well as in the interfascicular vascular cambium on the stem circumference (**Figure 7**).

From the experimental studies on the vascularization *in vitro*, it appears that parenchyma callus tissue is the most convenient for the analysis. Previously uniform callus can form vascular tissue bands or groups of vessels differentiation. However, the process can be realized only in the sufficiently thick callus tissue. It is shown that differentiated xylem is surrounded by cambium-like cells, which additionally are able to produce phloem elements in the inner callus regions. Auxin-dependent vascularization is also shown in the studies with young *Syringa* sp. stems [77]. Combination of auxin and sucrose decides about the induction of vascularization in the axillary buds *in vitro*. Moreover, dependent on the hormone and sucrose concentration, varied vascular tissues develop.

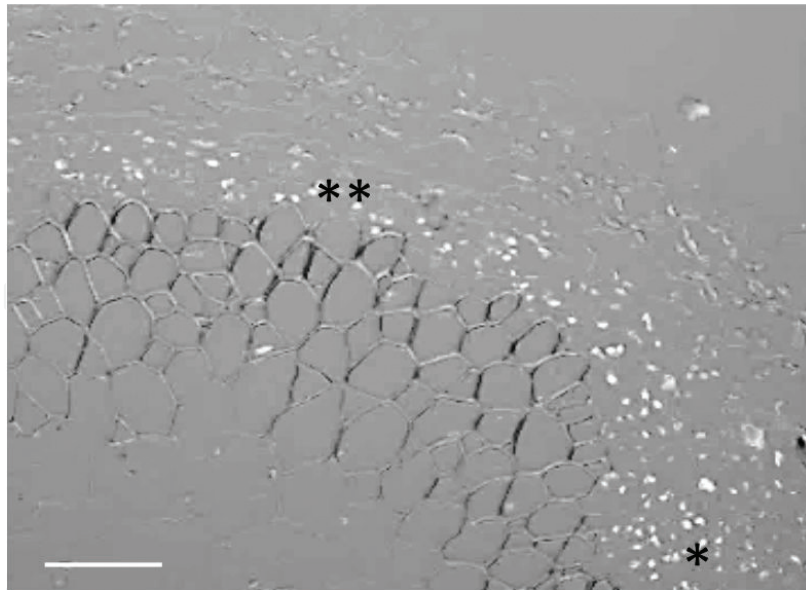


Figure 7. Elevated auxin concentration in cambium of non-incised *Arabidopsis* control stems. Increased auxin level in fascicular cambium (vascular bundle, asterisk) and in the interfascicular cambial cells (double asterisks); cross section through the basal part of Col-0 stem; confocal laser-scanning microscope; IAA immunolocalization-staining with the polyclonal anti-IAA antibody (white color in cambial cells; dilution 1:500); bar, 50 μm .

Several reports discussed auxin as a specific morphogenetic signal triggering cell fates during vascular tissue development and its maturation [78]. Locally created centers characterized by elevated auxin response become more competent for auxin flow through primarily uniform tissues. Auxin waves created in plant organs as a specific system of hormonal information that decide about realization of many developmental programs in plants, among them cambial activity and differential cambial responses [79, 80]. Thus analogically, gradual emergence of auxin channels and gradually narrowing auxin flow finally results in vascular strand differentiation. In other words, canalized auxin flux determined the paths of new vasculature development.

The canalization-predicted vasculature formation is especially observed during regeneration process, in new regenerated vessels after incision [1, 2, 4, 5, 8, 81]. Particularly important contributions to the role of auxin in the vascular tissue differentiation brought studies on *Pisum* sp. [1, 2]. According to all experiments performed by Sachs, vascularization depends on the polar auxin transport, and new vascular band induction depends on the auxin concentration and polarity. Moreover, the early stages of vascular band differentiation are related to the canalization of the polar auxin flow. A key role of auxin in promotion of canalized flow by itself and transport channels formation is commonly accented. However, the feedback mechanism between auxin flow, polarity, and vessel formation as a response to concentration gradients or directional auxin fluxes remains unclear [82, 83].

4.2. Role of auxin transporters in cellular and tissue polarity

The positive feedback loop between polar auxin flow and the polar, subcellular localization of the PIN-FORMED (PIN) auxin transport proteins [56] that, in turn, determine the auxin flow directionality is widely studied [53, 54, 84–86]. Many developmental processes, such as

early embryogenesis or plant organ initiation, are strictly correlated with the establishment of local PIN-dependent auxin gradients that precede cell divisions and differentiation [54, 55, 87]. The expression of auxin efflux carrier genes, like *PIN1*, *PIN2*, *PIN3*, *PIN4*, and *PIN7* was found to peak at the inflorescence stems of *Arabidopsis* during their maturation and secondary vascular tissue development. Changes in PIN localization and tissue polarity in response to auxin that are presumably related to the directional vascular tissue patterning have been observed and modeled [4, 5, 46, 88]. Moreover, in wounded pea or bean epicotyls, the PIN polarity was gradually rearranged marking the position of differentiating vessel strands [4, 5]. Emergence of auxin channels is here visualized by *PIN1* expression of the cellular auxin transporters. In *Arabidopsis* model with mechanically stimulated inflorescence stems, the subcellular *PIN1* position was gradually stabilized and restricted only to cell sides in a first few days after weight application, along the presumable direction of the auxin flow [8]. The auxin-dependent canalization is strongly supported by studies on leaf vein patterning and on the role of the genes encoding the auxin response factor *MONOPTEROS* (*MP*) and *PIN1* [47]. Dynamic expression of both of the genes and gradual establishment of polarized *PIN1* protein localization indicates the direction auxin flow during the vascular tissue patterning in analyzed leaves [47]. Moreover, the other *Arabidopsis* gene *GNOM/EMB30*, which affects apical-basal position of *PIN1*, seems to be required for regulation of the coordinated tissue polarity [6].

The role of auxin transporters in vascular tissue patterning is clearly visible in wounded inflorescence stems of *Arabidopsis*, during vascular cambium regeneration [8]. Rapid tissue repolarization indicated by reposition of *PIN1* at cellular plasma membranes of differentiating cells is emphasized. Dynamic temporal changes in tissue polarity are correlated with varied auxin response and its accumulation above and around a wound. Whereas auxin concentration arises in few hours after wounding, maximum of auxin levels is established at auxin channels and preceded establishment of new polarity in wounded areas of *Arabidopsis* stems. Cellular auxin transporters are characterized with changed position of *PIN1* proteins. Thus, direction of auxin flow through the auxin channels is precisely determined. Both of the events are strictly correlated with each other and play a decisive role in vascular tissue development.

4.3. Auxin signaling pathways in vascular tissue patterning

Two related protein families—Aux/IAA and ARFs—are well-known key regulators of auxin-modulated gene expression and act in the *TIR1*-mediated signaling pathway [89, 90]. Members of ARF family share the characteristic arrangement of a highly conserved DNA-binding domain near the N-terminus, which appear to be capable to auxin response elements (AuxREs)—short conserved sequences (TGTCTC) that have been shown to be essential for auxin regulation of auxin-inducible genes [6]. It is likely that the ARF proteins are strongly involved in the vascularization downstream proteolytic SCF^{TIR1} complex machinery [80]. In support of this, an increased level of ARF transcripts was differentially regulated during the secondary growth, and three of them (ARF2, ARF4, and ARF5) had the most dramatic expression changes, indicating their putative roles in apical-basal signaling and xylogenesis [6, 42]. On the other hand, the *AUXIN-RESISTANT 1* (*AXR1*) gene is required for normal *TIR1* function and, when mutated,

changes the stabilization dynamics of the Aux/IAA proteins [71]. Mutations in the TIR1/AFBs make Aux/IAA proteins insensitive to auxin and can therefore keep ARF transcription factors and auxin signaling repressed. Thus, ARFs together with Aux/IAA proteins constitute a central mechanism in auxin signaling during plant development [91].

Auxin besides regulating a gene expression by the TIR1/AFB pathway can also inhibit internalization of the PIN proteins by a feedback regulation [92]. The underlying perception and signaling mechanism is unclear, but it does not involve transcription regulation and is distinct from the TIR pathway [93]. It may relay on the Auxin Binding Protein 1 (ABP1) since the ABP1 overexpressors increase the PIN internalization and mutations in the auxin binding pocket of ABP1 make the ABP1 effect on PIN internalization auxin-insensitive [88]; however, due to unreliable loss-of-function data [94, 95], this issue requires further clarification.

The identification of spatiotemporal gene expression pattern and the key components of auxin signaling pathway/pathways will greatly contribute to understanding of the molecular mechanisms involved in auxin-induced regeneration switch in cambial cells. In addition, the knowledge on genetic factors, such as ARFs, AFBs involved in the SCF^{TIR1} auxin receptor complex, PIN auxin efflux transporters, or AtHB family of early vascularization markers determining developmental plasticity of cambial cells, can be useful in genetic improvement of woody plants for environment and biotechnology purposes.

5. Genetic control of vascularization processes

Numerous genes differentially regulated during vascularization in woody plants are expected to be identified with the use of obtained *Arabidopsis* model. Many of the genes were indicated to be involved in auxin responses implicating auxin engagement in regulation of vascular tissue development and patterning [42, 43], and the same is expected for vascular tissue regeneration. Experimental data have proven a key role of auxin in variety of developmental processes [80]; however, the molecular, auxin-mediated mechanism involved in vasculature regeneration remains mostly unknown.

As indicated recently, transcription factor genes promoting secondary growth induction [42, 43] can be applied in genetic transformation to improve our knowledge on xylogenesis and regeneration capacity of woody plants. Several regulatory genes, like NAC-domain genes or key regulators of SAM, shoot apical meristem, organization (*WUS*, *STM*, *WOX*, *CLVs*), are closely associated with the vascular tissue formation. It was reported that two NAC-genes, *NAM* and *CUC*, and *ANT*—a cell proliferation marker—play potential roles in vascular tissue differentiation and function [96]. *ANT* regulates organ growth through the maintenance of meristematic tissue activity.

The expression of the homeobox genes (*AtHBs*) is highly increased during xylem production and regarded as a positive regulator of the activity of procambial and cambial cells to differentiate [71, 72]. Baima et al. [72] reported ectopic expression of *AtHB-8* gene during vascular regeneration after wounding in *Arabidopsis*. Extensive activity of this gene was found in the regenerated

tissues, suggesting intensive transcriptional reprogramming during new vessel development. In the model with functioning vascular cambium, expression of *AtHB-8* is observed in differentiating cambial derivatives, in early stages of their maturation into the vessels (**Figure 8**).

Homeostasis of vascular cambium with its non-disturbed functionality plays an important role in the vascularization [59]. However, genetic control of vascular cambium activity is poorly characterized. The role of the leucine-rich repeat receptor-like kinase (LRR-RLK) families in regulation of this lateral meristem homeostasis, underlying the role of *CLV1* (*CLAVATA1*)—well-known apical meristem marker and *PXY* (phloem intercalated with xylem)—a cambium-specific receptor-like kinase, in this process is suspected [59]. Two receptor-like kinases *MOL1* (more lateral growth1) and *RUL1* (reduced in lateral growth1) identified as opposing regulators of cambium activity were also reported [59]. Recently, the role of the homeobox transcription factor *WOX4* (wuschel-related homeobox 4), as an essential cambium regulator positively regulated by *PXY*, has been revealed [59, 97]. Since many years the correlations between the cytoskeleton dynamic (both actin filaments and microtubules) and activity and functioning of vascular cambium [31, 37, 98] have been widely discussed. It was postulated that changes in

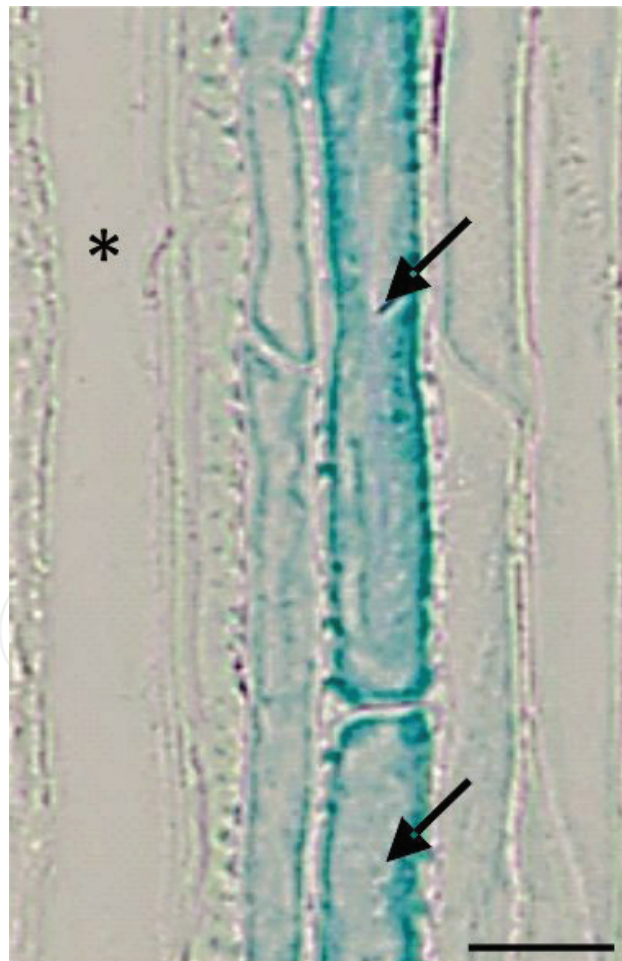


Figure 8. The *AtHB8* gene expression in differentiating vessels. Longitudinal tangential section through the basal part of stem. Expression of *AtHB8* in differentiating vessels (arrows); asterisk indicates matured vessel (*AtHB8:GUS* transgenic line; LR white resin section; bar: 20 μm).

microtubule orientation might be specific cellular marker for cambial cells in the stage of their differentiation into tracheary elements. Analyses of the following steps of this process revealed the dynamic changes in the microtubule orientation in the differentiating cambial cells [98].

The most important role in the whole vascular-formation and regeneration machinery is played by auxin response genes referred to as early/primary auxin response genes. Three major classes of these genes, *Aux/IAA*, *SAURs*, and *GH3*, are characterized by the so-called auxin response elements (AuxREs), the TGTCTC-containing auxin response promoter elements. The genes are specifically induced by auxin, which may rapidly regulate their transcription. Accordingly, when auxin concentration in cells is low, auxin response genes are repressed. Oppositely, when auxin concentration is elevated, transcription of the genes is activated in a few minutes [99], and ARF transcription factors are binding to its AuxRE target sites. It is discussed that dependently on the tissue and developmental stages of the plants, tissue-specific expression of both ARF and Aux/IAA proteins could regulate the transcription of different sets of genes for different developmental processes.

Studies in the *Populus tomentosa* [29] have shown that different groups of genes are activated dependent on the stage of the secondary vascular tissue development. Thus, in the cambial zone, high expression of the *ARK1* gene was observed. It was suggested that *ARK1* regulates cambium activity including cambial cell divisions and differentiation of the cambial derivatives [30]. On the contrary, during the secondary xylem formation, expression of the group of genes responsible for the tracheary element differentiation and maturation, such as *SND1* or *NST1* and *NST3*, was reported [100, 101].

It is widely postulated that important role in the vascularization is played also by gene expression that accompanies this process, like *PLT* (*PLETHORA*) or *TCH* (*TOUCH*) genes encoding calcium-binding proteins. Whereas *PLTs* regulate de novo shoot regeneration in *Arabidopsis* by controlling successive developmental events, *TCH* genes (*TCH2* and *TCH4*) strongly induced by mechanical stimuli like touch and wind [42] may be involved in the signal transduction and secondary xylem formation. Otherwise, two of the *VASCULAR-RELATED NAC-DOMAIN* genes (*VND6* and *VND7*) are reported as positive xylem vessel differentiation regulators in both *Arabidopsis* and poplar [48]. Thus, the elusive mechanism for auxin-regulated vascular tissue patterning might be a part of the extensive genetic network including several hormonal signaling pathways and dynamic spatiotemporal switched on/off gene expression. Deciphering of these yet unknown relationships will help to translate the mechanisms regulated in vascular tissue development and regeneration in woody plants.

6. Emergence of *Arabidopsis* as a good model system to study the vascular tissue formation and regeneration processes

For a long time, the herbaceous plant *Arabidopsis* was postulated as a major model system for developmental plant biology due to its some important features, such as a short generation time and relatively small size of fully sequenced genome [52]. It was used as a modern research tool for different molecular studies, especially thanks to large mutant and transgenic

line collections. Finally, in many studies *Arabidopsis* was also postulated as a perfect plant for secondary growth analysis [37–39, 44, 45, 50, 102, 103].

Although the vascularization and regeneration in *Arabidopsis* have been previously analyzed [3, 81], the role of vascular cambium in these processes has never been addressed. Only just modified and established method to obtain stems with closed vascular cambium rings and some typical features mimicking vascular tissue in trees [7, 51], such as secondary rays and intrusively growing cambial cells or tracheids—unusual example of tracheary elements found in woody plants—gives infinite possibilities to analyze secondary vascular tissue development and provides decisive advantage over previous approaches with the use of *Arabidopsis* stems. This strategy is particularly suited to elucidate different molecular mechanisms and other molecular components involved in auxin-mediated responses, canalization of auxin flow, and cellular polarity, underlying determination of plant developmental plasticity.

The availability of numerous genetic and molecular tools in *Arabidopsis* will provide clarifying picture of the process of vasculature formation as well as reconstruction after wounding and decisively extend the knowledge on molecular control of spatiotemporal vasculature patterning and regeneration both in vivo and in vitro. Resolving the involvement, the mechanism of canalization process will contribute to explain molecular mechanisms involved in vasculature regeneration and provide a useful model for further studies.

7. Conclusions

Specifically manipulated *Arabidopsis* represents a good system for the analysis of vascularization machinery that typically occurs in trees. Obtained results revealed that this process is accompanied with cellular events following cambium ontogenesis, xylem formation, and regeneration: (1) elevated auxin concentration in tissues, (2) new polarity establishment, (3) reposition of PIN1 proteins at plasma membranes of differentiating cells, (4) cellular divisions, and finally (5) cambium and vascular tissue development or regeneration.

Knowledge about the molecular mechanisms regulating vascular tissue development in trees is incomplete, and such studies can take full practical advantages from a recently proposed new approach. Temporal analysis and experiments in trees are hampered mainly because of variability in environmental conditions, their long life cycle, and restricted amount of transgenic lines and mutants available.

Currently, new insights into the vascular tissue formation problematics can be obtained by using *Arabidopsis thaliana* (L.) Heynh. This modest plant commonly used as a genetic model can be also modified to become a perfect experimental “tree-like” model, with a closed ring of functional cambium and secondary vascular tissues with complexity of phenotype features fully comparable to woody plants. This new approach promises to identify additional genetic and molecular components involved in vascular tissue functioning, including the insights into the role of cambial cells in this process in woody plants. Extensive studies with the use of

Arabidopsis model system allow obtaining a more complete data about vasculature development and regeneration under more controlled experimental conditions.

Interdisciplinary scientific approach with the use of *Arabidopsis* as the “tree-like” model promises to pioneer new, original, and valuable information about the mechanisms regulating vascularization and allows defining molecular factors of the auxin-dependent machinery involved in this process. This creates a unique opportunity to compare the processes in *Arabidopsis* with analogical situation found in trees. There is a hope that the obtained knowledge could be applied practically. Plant life conditions are closely related to dynamic environmental and climate changes, which is a global problem of the last decades. Among the most endangered ecosystems are forests, which are shrinking very fast not only because of environmental changes but mostly because of the non-sustainable economic exploitation. Thus obtaining additional options to study typical tree features including their regenerative abilities is important to design efficient strategies for sustainable wood production.

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