

Auxin as a positional signal in pattern formation in plants

(cambium/indole-3-acetic acid/*Pinus sylvestris*/tracheid/wood)

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ABSTRACT By using a novel, extremely sensitive and specific gas chromatography-mass spectrometry technique we demonstrate in *Pinus sylvestris* (L.) trees the existence of a steep radial concentration gradient of the endogenous auxin, indole-3-acetic acid, over the lateral meristem responsible for the bulk of plant secondary growth, the vascular cambium. This is the first evidence that plant morphogens, such as indole-3-acetic acid, occur in concentration gradients over developing tissues. This finding gives evidence for a regulatory system in plants based on positional signaling, similar to animal systems.

Formation of patterns is one of the most intriguing phenomena in biology. Pattern development requires that gene activity must be strictly controlled in time and space. Every cell must receive information about its position and express the appropriate genes. The existence of morphogenetic fields has been suggested as a possible source of such information in both animal and plant systems (1–4). This field is thought to consist of one or more diffusible and physiologically active substances (morphogens) which originate from organizing centers. The concentration gradients created would then influence on tissue and organ differentiation. Current examples of morphogens of this sort in animals are retinoic acid in vertebrate limb development and activin in early amphibian development (5, 6). In plants, positional signaling has been discussed mainly on the basis of the orderly induction of leaf and root primordia and the organization of vascular tissues (7), but neither the mechanisms nor the morphogens behind these patterns have been elucidated. Auxins and cytokinins are plant hormones that have been characterized as important signals in plant development, being involved in the induction and maintenance of meristems as well as in plant polarity (8). Not surprisingly, these substances have been proposed to function as positional signals in pattern specification (9, 10). To date, this proposition has not found much support due to the lack of data proving that such endogenous plant hormone gradients are found in plants (11, 12).

The formation of secondary vascular tissues is a well described phenomenon of patterned growth in plants. This pattern has both radial and longitudinal components (13). Phloem and xylem differentiate radially on each side of the lateral meristem (the vascular cambium). The cambial derivatives which form xylem first pass through a zone of cell expansion and then a zone of secondary wall formation and, finally, a zone of programmed cell death. Phloem derivatives expand and differentiate forming a living tissue (Fig. 1). A longitudinal component also exists in temporal and spatial patterns of cambial cell division, as well as in the morphology and composition of different types of xylem elements. In trees, the amount and characteristics of the final wood product is an outcome of the different components of this patterned growth (14).

A key organizer of cambial growth and vascular development is an auxin, indole-3-acetic acid (IAA) (15–18). It is a very important morphogen as it has the potential to induce differentiation of vascular strands in callus and explants. In intact plants, the polar flow of IAA is essential for the initiation of spatially organized patterns of vascular tissues as well as for maintaining of the vascular cambium. Moreover, in both conifers and angiosperm trees, IAA has been demonstrated to affect most aspects of secondary cambial growth, including cell division, secondary wall thickness and final size of xylem cells, notably vessel size in angiosperms and tracheid size in conifers (19).

Failure to develop a unifying concept for the role of IAA in the regulation of patterns of vascular tissue in both primary and secondary plant bodies is due to a limited knowledge not only of IAA perception mechanisms, but of its metabolism, transport and final distribution. However, in this report we demonstrate the existence of a steep concentration gradient of IAA over the vascular cambium and its derivatives in mature *Pinus sylvestris* trees, by using a novel gas chromatography-mass spectrometry technique coupled to cryosectioning. The occurrence of this distribution indicates a role for IAA in positional signaling.

MATERIALS AND METHODS

Blocks (2 × 5 cm) consisting of extraxillary tissues and a few annual rings were chiseled out at breast height during active (late June) and dormant (mid-January) periods from *Pinus sylvestris* (L.) trees (≈120 years old, 19 m tall, 34 cm in diameter at breast height, location 64°14'N, 19°46'E) and immediately frozen in liquid N₂. After trimming the blocks, specific developmental zones from the cambial region were isolated for IAA analysis by tangential centripetal cryosectioning at –20°C with a HM 505 E microtome (Microm Laborgeräte, Walldorf, Germany) equipped with a steel knife (Fig. 2). Each section was 30 μm thick, ≈3 × 12 mm, and had a fresh weight of around 1 mg. For initial orientation of the block to obtain sections parallel to the cambium, transverse sections were cut with a razor blade from both ends of the specimen. The two unstained transverse sections were mounted in glycerol and inspected under a Zeiss Axioplan microscope, using Nomarski optics. By measuring the distance from specimen surface to cambium in each corner of these transverse sections, it was possible to detect deviations from the parallel. The specimen was then reoriented in relation to the knife and the procedure repeated until the sample was properly lined up. The radial position of each section was similarly determined by obtaining transverse sections with a razor blade from both ends after every second to fourth section. The two sections were inspected under the microscope. Cells in three files, one in the middle and the others at either end, were counted and the developmental stage of the outermost cell determined by its anatomical appearance. As the surface area of the specimen

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Abbreviation: IAA, indole-3-acetic acid.

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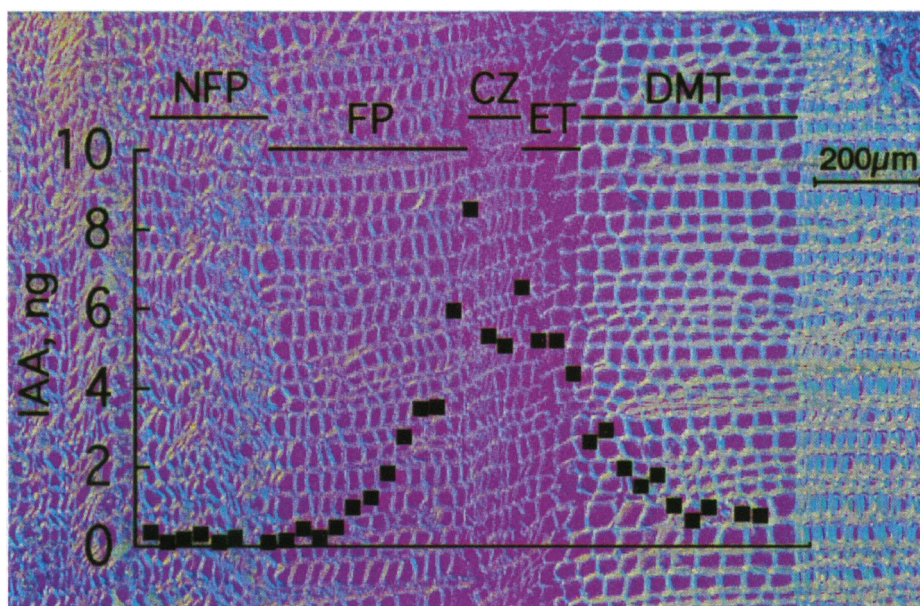


FIG. 1. Interference micrograph of a transverse section from the cambial region of *Pinus sylvestris*. The radial auxin gradient from this particular tree (Fig. 3C) is overlaid on the photograph. NFP, zone of nonfunctional phloem from previous years growth; FP, zone of differentiating and functional phloem; CZ, cambial zone; ET, zone of expanding differentiating tracheids; DMT, zone of differentiating tracheids forming secondary walls as well as mature dead tracheids. The zone of differentiating phloem is much narrower than the zone of differentiating xylem due to the low ratio of phloem to xylem cells formed, and is therefore more difficult to define.

got smaller due to transversal sectioning, the length of each section was measured after each transverse sectioning.

Quantitative measurements of endogenous IAA in each tangential section was done by isotope dilution and extremely sensitive and specific GC-selected reaction monitoring-MS techniques by using a double-focusing magnetic sector tandem instruments (JEOL JMS-SX/SX102A) (20).

Fresh weight and water content were determined in parallel section series obtained from all trees. Each section was weighed before and after drying in 80°C for 24 hr on a Mettler MT5 balance.

RESULTS AND DISCUSSION

The IAA content in 30- μ m tangential sections obtained across the cambial region was measured by gas chromatography-

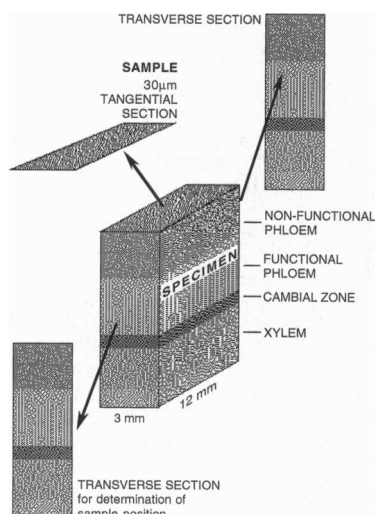


FIG. 2. Schematic drawing of the specimen block from which tangential sections for IAA measurement and transverse sections for determination of sample position were obtained.

selected reaction monitoring-mass spectrometry (Figs. 1 and 3). This technique involves detection of daughter ions originating from specific, metastable parent ions, which results in extremely high specificity. Thus, some of the problems with interfering substances usually encountered with traditional techniques for plant hormone measurement are avoided. Together with the high sensitivity of the double focusing magnetic sector instrument, accurate measurements in small amounts of tissue can be performed without much sample purification (20). The radial distribution pattern of endogenous IAA across the cambial region exhibited a peak level in the cambial zone where cell division takes place, steeply decreasing toward the mature xylem and phloem. This pattern reflects the content of IAA in each 30- μ m tangential section, expressed on a cm^2 basis. Fresh weight per cm^2 section did not vary significantly over the differentiating cells, and the average for the three trees was 2.5 ($\text{SE} \pm 0.14$) mg/cm^2 section. In the nonfunctional phloem, density decreased by $\approx 20\%$. Water content was highest in the cambial zone and radially expanding tracheids, ranging from 90% to 95%, and gradually decreased to between 55% and 85% in the nonfunctional phloem and mature xylem. By using the average fresh weight per cm^2 section, concentrations can be calculated. In the meristematic cambial zone concentration ranged between 3 and 6 $\mu\text{g}/\text{g}$ fresh weight in three different trees. With a water content of 90%, the molar concentration in the cambial zone cells is estimated to be between 19 and 38 μM . From the peak level in the cambial zone, the concentration was calculated to decrease to about 80 ng/g fresh weight in the maturing xylem cells and nonfunctional phloem. Thus, the difference in IAA concentration was about 50-fold over a 250- μm radial distance. As neither weight nor water content in comparable sections over the same region varied much, it can therefore be concluded that the radial distribution pattern of IAA reflects a true concentration gradient, and not differences in dry matter in the different sections. This finding demonstrates for the first time that plant hormones can have a distribution pattern as required for positional signaling. The visualization of the radial IAA gradient will help us to understand its role in the regulation of cambial growth and wood formation.

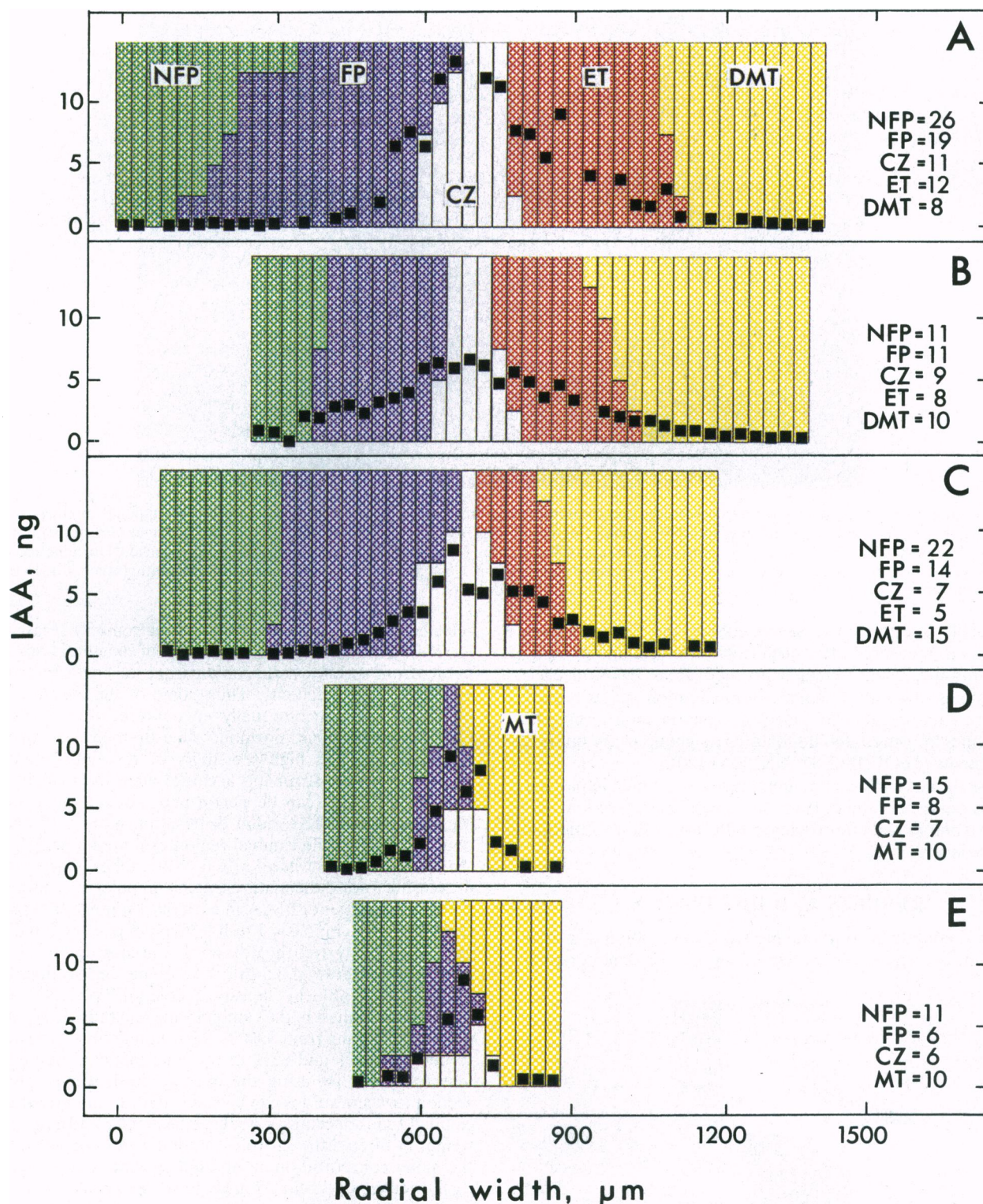


FIG. 3. Radial distribution of IAA in three different *Pinus sylvestris* trees during activity in late June (A–C) and in two different trees during dormancy in mid-January (D and E). Each column represents the 30- μ m tangential section, and its composition of cell types from different developmental zones. MT, mature tracheids; other abbreviations are as in Fig. 1. Endogenous IAA content per cm^2 section is indicated with black dots. The average numbers of radial file cells in each developmental zone are given to the right.

The high concentration of IAA in the cambial zone and its immediate derivatives suggests that this region is either a site of IAA biosynthesis, a site of IAA transport, or both. As the plant genes and enzymes involved in IAA biosynthesis have not been characterized, direct evidence for the cellular, tissue or organ site of synthesis in intact plants is still wanting. However, a generally accepted view is that buds and developing leaves

are the major sources of IAA in intact plants. This view is based on the observations, in many experimental systems, that physiological events dependent on IAA, such as apical dominance, cambial growth and vascular induction, are inhibited by debudding or defoliation, but are restored by replacing the leaf or bud with exogenous IAA. Moreover, defoliation of developing leaves on intact plants results in a dramatic decrease of

endogenous IAA in subadjacent internodes (21, 22). Apically produced IAA is actively transported in a basipetal polar transport system which has been localized to the cambial zone, its differentiating derivatives and the phloem region excluding sieve tubes (23). Blocking the polar transport system in intact Scots pine shoots with specific inhibitors of polar IAA transport (*N*-1-naphthylphthalamic acid and morphactin), decreased endogenous IAA to trace levels below the block, inhibiting cambial growth (24). This observation clearly demonstrates that the basipetal supply of IAA, needed for cambial growth, is maintained by the polar transport system. The finding of a peak concentration of endogenous IAA in the cambial zone would therefore be a reflection of these cells being the pathway of polar IAA transport rather than a site of IAA biosynthesis.

The IAA gradient would be created by radial diffusion of polarly transported IAA. This conclusion is supported by the finding of Nix and Wodzicki (25), that radiolabeled IAA, which had been apically fed to decapitated *Pinus echinata* internodes, and which had been transported in a polar manner, was distributed in a gradient across the cambial region identical to that of endogenous IAA in this study. It has been suggested that the polar IAA transport pathway involves both symplast and apoplast (23). A radial diffusion of IAA would therefore occur in both of these compartments, whereas the symplastic movement is likely to take place preferentially in the rays (26). To maintain the radial concentration gradient a removal of radially transported IAA is required, either by degradation or by IAA entering mass flow in the vascular tissues.

For the coordination of cambial growth a positional signaling system must exist, from which cambial derivatives interpret their radial position, hence their gene expression. It is well established that IAA stimulates mitotic activity in the cambial zone, induces tracheid element differentiation, and is involved in the control of primary wall expansion and secondary wall thickening (19), although the exact molecular mechanisms behind its action is not known in great detail. Nevertheless, from the results obtained here we suggest that IAA is involved in the positional control system. Structural integrity and cell division in the cambial zone are maintained above a threshold IAA concentration, the center of the gradient. In the event of increased width of the radial IAA gradient, there is an increase in the population of dividing cells in the cambial zone. This notion is supported by the finding of Gregory (27) who observed that the number of xylem cells produced was correlated to the radial number of cambial zone cells and not to their mitotic index. Similarly, auxin-stimulated cell expansion and secondary wall formation would be induced through an increased duration of these events as a result of a wider radial IAA gradient.

That a change in IAA supply to the vascular cambium affects its growth by positional signaling through the width of its radial concentration gradient, rather than by variation in concentrations in specific cell types, is indicated by the finding that IAA concentration in a nondividing dormant cambial zone sampled during mid-winter was similar to the concentration in the corresponding tissue in active trees (Fig. 3). The observation that the dormant cambial zone contains significant amounts of IAA is in line with earlier findings in conifers (19). It is also well established that cessation and reactivation of mitotic activity in the cambial meristem of conifers, in autumn and spring respectively, is due to factors other than IAA availability (19, 28). However, our results suggest that an increased basipetal supply of IAA to the vascular cambium during resumption of shoot growth results in a wider radial IAA distribution rather than a higher IAA concentration in the cell division zone. This observation provides further support for the concept that IAA controls cambial growth by determining the radial number of dividing cells in the cambial zone through

positional signaling. Furthermore, this concept explains the relationship between IAA amount (i.e., the integrated area under the gradient) and cambial growth found in our earlier work (28, 29). Since cells are dividing in areas above a certain threshold, division cannot be considered to be proportional to concentration. Preliminary results support this idea (B.S. and C.U., unpublished data). The radial width of the gradient was positively correlated to cambial growth rate, as well as to the population of dividing cells in the cambial zone.

On the basis of numerous studies on patterns of vascular tissue regeneration in stem wound callus and in grafting experiments, the Warren-Wilsons (10) have argued for the existence of radial concentration gradients of morphogens, including auxin, which determine the organization and differentiation of vascular tissues. Up until now there has been no hard evidence for such gradients. A role for IAA in positional signaling during cambial growth has also been suggested in many studies by Zajackowski *et al.* (30). They reported an oscillating pattern of IAA diffusing out of subsequent stem segments, and suggests that IAA is transported in waves. The frequency, amplitude and vectorial field of these waves, rather than concentration gradients over the meristem, has been suggested to convey positional signaling. However, this hypothesis still suffers from a lack of evidence for the occurrence of auxin waves in intact tissues. In addition, a model for the perception mechanism of the frequency and amplitude of waves has not been presented.

Since IAA is a major organizer for induction and maintenance of vascular tissue it is likely that the observed concentration gradient is the cause of some aspects of pattern development in cambial growth. Experimental manipulation of the gradient shape resulting in an altered growth pattern, as well as perturbation of the gradient resulting in an aberrant pattern, would give further support for this idea. However, it is also clear that the information content of the radial concentration gradient cannot explain all aspects of pattern formation in cambial growth. Determination of fusiform and ray initials, anticlinal and periclinal divisions, xylem and phloem, and the different cell types in these tissues requires additional information. This might consist of gradients of other morphogenetic fields or of cell-cell interactions. Microscale measurement of other growth regulators will give more information about these possibilities. A procedure for accurate measurement of gibberellins in small amounts of tissue is already established (31) and similar techniques for cytokinins and other plant hormones are underway. This development of techniques for hormone measurement in small amounts of plant tissue opens new perspectives for investigations on distribution patterns of plant hormones, and will provide clues about their roles in a range of other growth and developmental responses, such as establishment of embryonic axes, tropism, secondary root formation, apical dominance, and apical growth. From this study it is clear that plant hormones, especially auxins, must be considered in pattern formation.

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