

## Responses of plant vascular systems to auxin transport inhibition

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### SUMMARY

To assess the role of auxin flows in plant vascular patterning, the development of vascular systems under conditions of inhibited auxin transport was analyzed. In *Arabidopsis*, nearly identical responses evoked by three auxin transport inhibitor substances revealed an enormous plasticity of the vascular pattern and suggest an involvement of auxin flows in determining the sites of vascular differentiation and in promoting vascular tissue continuity. Organs formed under conditions of reduced auxin transport contained increased numbers of vascular strands and cells within those strands were improperly aligned. In leaves, vascular tissues became progressively confined towards the leaf margin as the concentration of auxin transport inhibitor was increased, suggesting that the leaf vascular system depends on inductive signals from the margin of the leaf. Staged application of auxin transport inhibitor demonstrated that primary, secondary and tertiary veins became unresponsive to further modulations

of auxin transport at successive stages of early leaf development. Correlation of these stages to anatomical features in early leaf primordia indicated that the pattern of primary and secondary strands becomes fixed at the onset of lamina expansion. Similar alterations in the leaf vascular responses of *allysum*, *snapdragon* and *tobacco* plants suggest common functions of auxin flows in vascular patterning in dicots, while two types of vascular pattern alterations in *Arabidopsis* auxin transport mutants suggest that at least two distinct primary defects can result in impaired auxin flow. We discuss these observations with regard to the relative contributions of auxin transport, auxin sensitivity and the cellular organisation of the developing organ on the vascular pattern.

Key words: *Arabidopsis thaliana*, Auxin transport, Provascular tissue, Vascular development, *eir1-1*, *aux1-7*, *pin1*, *mp*

### INTRODUCTION

The vascular tissues of plants form an interconnected system of continuous cell files throughout the plant body (Esau, 1965; Nelson and Dengler, 1997). Two types of conducting tissues, phloem and xylem, are capable of transporting aqueous solutions of photoassimilates and minerals through tubes of aligned cells. All types of vascular tissues differentiate from provascular cells that become recognizable as continuous strands of narrow cells early in organ development (Foster, 1952). In an intact plant, provascular strands differentiate at predictable positions within all major organs, but adaptive responses to wounding or abnormal growth conditions demonstrate considerable flexibility of vascular patterning (Sachs, 1989). Furthermore, if the original connection is interrupted, new vascular strands can be formed even in mature organs (Lyndon, 1990). It therefore seems possible that genetic control of vascular patterning is indirect and acts through partially self-organizing mechanisms.

The molecular mechanisms controlling vascular differentiation during plant organ development are not known, however, the capacity of the plant hormone auxin to induce vascular differentiation is well established (Lyndon, 1990 and

references therein). Auxin-overproducing transgenic plants have increased amounts of vascular tissues (Klee et al., 1987) and application of auxin can induce the formation of new vascular strands from parenchymatic cells of mature organs (Jacobs, 1952). Therefore, developmentally controlled auxin production and distribution could be involved in specifying the sites of vascular differentiation during normal plant organogenesis.

The major auxin in higher plants, indole acetic acid (IAA), is synthesized in shoot apical tissues and actively transported towards the base of the plant (Aloni, 1995; Jacobs, 1952; Sachs, 1981). In roots, the situation is less clear, but it has been reported that a root tip directed auxin flow within the central cylinder is associated with an opposite flow in the root epidermis (Davies and Mitchell, 1972; Tsurumi and Ohwaki, 1978). Auxin transport appears to be mediated by specific cellular transport proteins (reviewed by Lomax et al., 1995) and the directionality of the auxin flow has been attributed to a polar distribution of efflux carriers in the cell membranes (Raven, 1975; Rubery and Shelldrake, 1974). Several polarly localized proteins with possible functions in auxin efflux have been identified (Jacobs and Gilbert, 1983; Gälweiler et al., 1998; Müller et al., 1998). Experimental interference with

auxin transport is possible through a number of chemically heterogeneous compounds, among them 1-N-naphthylphthalamic acid (NPA), 2,3,5-triiodobenzoic acid (TIBA) and 2-chloro-9-hydroxyfluorene-9-carboxylic acid (HFCA), which inhibit polar auxin transport by interfering with the efflux mechanism (Lomax et al., 1995; Bennett et al., 1998).

One peculiarity of vascular tissue induction by IAA is that it occurs in a linear manner: locally applied IAA induces a new vascular strand extending basally from the site of application (Sachs, 1991). The observation is intriguing in three respects: first, the differentiation occurs along a narrow line of cells rather than in a field around the source; second, the signal also mediates oriented differentiation, such that the selected cells eventually form a continuous strand; and third, the response is polar, as local application does not induce vascular strand formation in the apical direction. Experimental evidence suggests that the polar transport of IAA is directly responsible for the directionality of the auxin response (Sachs, 1981 and references therein) and could therefore be of general importance for vascular patterning in organ development. Auxin transport inhibition has been shown to result in increased vessel differentiation (Fosket and Roberts, 1964; Hejnowicz and Tomaszewski, 1969; Sheldrake and Northcote, 1968; Gersani et al., 1986), and therefore a detailed study of vascular responses to modulations of auxin transport could help to define the role of auxin flows in vascular patterning.

In this study we have examined the response of the vascular system of *Arabidopsis thaliana* and three other plants to inhibitor-induced reduction of auxin transport and have related these vascular alterations to those in *Arabidopsis* mutants. Our observations suggest that auxin transport is required for vascular tissue continuity and the restriction of vascular differentiation to narrow strands. Variations in the dosage and stage of auxin transport inhibitor application altered organ shape and vascular patterns in a predictable fashion and revealed that established provascular patterns are unresponsive to further modulations of auxin flows. We show that the *Arabidopsis* leaf provascular pattern emerges as a sequence of continuous arches and that this venation pattern is dramatically altered upon auxin transport inhibition. We relate these alterations to responses in other plant species and to vascular abnormalities observed in *Arabidopsis* mutants.

## MATERIALS AND METHODS

### Plant material and growth conditions

*Arabidopsis thaliana* ecotype Col-0 as well as *Arabidopsis* mutants *eir1-1* and *aux1-7* were obtained from the Arabidopsis Biological Resource Center, (Columbus, USA), *pin1-1* mutants were provided by K. Okada, Kyoto. Seeds of *Arabidopsis*, alyssum (*Aurinia saxatilis* cv Gold dust), snapdragon (*Antirrhinum majus*), and tobacco (*Nicotiana glauca* cv SR1) were surface-sterilized in 70% ethanol for 1 minute, followed by 15% commercial bleach for 15 minutes, washed three times in sterile distilled water and plated in molten 0.4% water agar on top of solid growth medium in 9 cm Petri dishes. Plates were sealed with Parafilm, incubated at 6°C in darkness for 4-5 days and then moved to a growth chamber set at 22°C, 16 hour light (120-150  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ ), 8 hour dark cycle. The time of transfer to growth chamber was considered the starting point of the experiments. Plants were subcultured onto solid medium in magenta boxes (Sigma) after 2

weeks. For staged application of NPA, seedlings were plated and grown as above, then moved from medium without NPA to medium supplemented with 40  $\mu\text{M}$  NPA every 24 hours after transfer to the growth chamber. Seedlings were covered with liquid medium containing 40  $\mu\text{M}$  NPA, to ensure even and rapid exposure to the inhibitor.

Growth medium contained 0.5 $\times$  MS basal salts (Murashige and Skoog, 1962), 1.5% sucrose and 0.8% agar. Auxin transport inhibitor substances, 1-N-naphthylphthalamic acid (NPA; Chem Service, West Chester, PA, USA), 2-chloro-9-hydroxyfluorene-9-carboxylic acid (HFCA; Sigma), and 2,3,5-triiodobenzoic acid (TIBA; Sigma), were dissolved in dimethylsulfoxide (DMSO) at 500 mM, and added to autoclaved growth medium as well as the top agar used for plating the seeds. The concentration of DMSO was adjusted in all samples to correspond to the amount of DMSO used at the highest concentration of inhibitor in each experiment.

The central vascular cylinder of the root was marked by expression of the *uidA* gene, encoding  $\beta$ -glucuronidase, in the transgenic *Arabidopsis* line 553-643 (van den Berg et al., 1995). We have found that in this line *uidA* gene expression marks phloem in stems and leaves (see Fig. 2). The phloem identity of these regions was confirmed by comparison to the *uidA* expression conferred by the promoter of the *Arabidopsis* sucrose synthase gene *Asus1*, known to be expressed in the phloem of *Arabidopsis* (Martin et al., 1993).

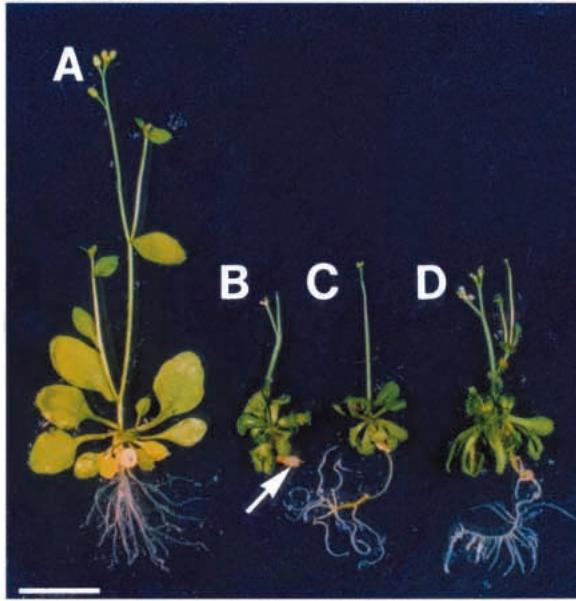
### Microscopy

For light microscopy, plant material was fixed, cleared and mounted according to the method of Berleth and Jürgens (1993). To assay  $\beta$ -glucuronidase activity, dissected organs were incubated in a solution containing 50 mM  $\text{NaH}_2\text{PO}_4$ , pH 7, 1 mM X-glucuronide, 5 mM ferrocyanide, 5 mM ferricyanide and 0.1% (w/v) Triton X-100 overnight at 37°C. Thereafter the samples were fixed as above, embedded in 4% agar and cut into 30-60  $\mu\text{m}$  sections in a vibratome. Sections were cleared and mounted as above. Photomicrographs were taken in a Zeiss Axiophot microscope using either dark-field or differential interference contrast settings.

For confocal laser scanning microscopy (CLSM), the cell content of seedlings was removed by incubation overnight in 5% (w/v) NaOH, 0.1% SDS at 37°C followed by several washes in distilled water. The cell wall of seedlings was stained with a periodic acid-Schiff's reagent (acriflavine, Sigma) and cleared as described by Vollbrecht and Hake (1995). Leaf primordia were dissected and oriented in a droplet of methylsalicylate on a coverslip such that one half of the lamina was approximately parallel to the coverslip. The coverslip was inverted onto a depression slide and sealed with immersion oil. Images were obtained on a Molecular Dynamics Sarastro 1000 confocal laser scanning microscope using a 514 nm excitation line, 520 nm beam splitter and a 535 nm barrier filter at the photomultiplier. The leaf primordia were scanned at a series of focal planes, 2-4  $\mu\text{m}$  apart. The images were saved as TIF files, and the pattern of provascular strands was reconstructed by integration of a series of computer images using Photoshop 4.0 (Adobe Systems Inc.).

## RESULTS

We have explored the role of auxin transport in vascular development by studying the vascular system of plants grown in the presence of auxin transport inhibitors. In the first section we will briefly summarize the morphological effects of auxin transport inhibition and relate them to alterations recorded in the vascular system of *Arabidopsis* inflorescences, roots and rosette leaves. In the subsequent two sections, we will focus on the vascular responses in first-node rosette leaves of *Arabidopsis* in order to assess the effect of dosage and developmental stage of inhibitor application. Finally, we will

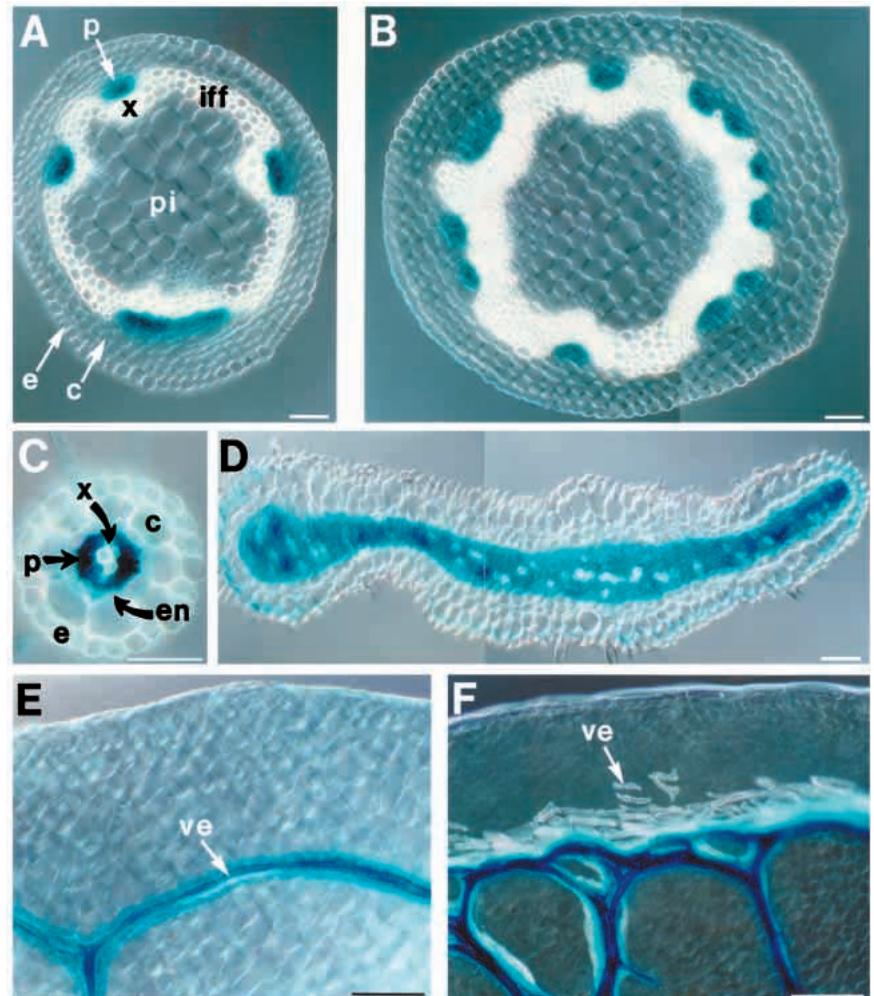


**Fig. 1.** Morphology of auxin transport inhibited *Arabidopsis* plants. Plants were grown for six weeks on medium without inhibitor (A) or medium supplemented with 20  $\mu\text{M}$  NPA (B), 20  $\mu\text{M}$  TIBA (C), or 20  $\mu\text{M}$  HFCA (D). Arrow marks the extremely short root typical of plants grown on medium supplemented with NPA. Scale bar, 10 mm.

compare the effect of auxin transport inhibition on the *Arabidopsis* leaf vascular system to those in three other dicot species and to the vascular phenotype of the *Arabidopsis* auxin transport mutant *pin formed 1* (*pin1*). All our experiments were conducted in sterile culture which by itself could have an influence on plant growth and tissue differentiation. We therefore include brief descriptions of *in vitro* grown control plants.

### Morphology and anatomy of auxin transport inhibited plants

Six-week old *Arabidopsis* plants grown on media without auxin transport inhibitors produced inflorescences with numerous fertile flowers, an extensively ramified root system and rosettes of seven to nine leaves (Fig. 1). Rosette leaves had narrow petioles and broad blades. Plants grown on media supplemented with 20  $\mu\text{M}$  of either NPA, TIBA or HFCA had shorter inflorescences that produced few or no flowers (Fig. 1). These flowers were grossly distorted and invariably sterile. The root system was reduced, comprising only a very short, swollen primary root with no lateral roots (NPA; arrow in Fig. 1B) or a somewhat longer primary root with a reduced number of lateral roots (TIBA, HFCA; Fig. 1C,D). Root tips were radially expanded, most dramatically in plants grown on NPA. Rosettes were generally composed of fewer (three to eight), irregularly arranged leaves. The petioles of these leaves were broader



**Fig. 2.** Effect of auxin transport inhibition on the internal organization of stems, roots and leaves. Specimens from plants grown on medium without NPA (A,C,E) and supplemented with 20  $\mu\text{M}$  NPA (B,D,F). The xylem bundles and interfascicular fibers are recognizable under differential interference contrast microscopy (DIC) by thick and bright cell walls, the vascular bundles are marked by blue color due to GUS expression of the phloem-specific enhancer-trap line 533-643. (A,B) Stem sections at the level of the first internode. Note the dramatically increased amount of cells with thick secondary cell walls in stems from auxin transport inhibited plants. (C,D) Cross-section through the differentiation zone of the primary root. The root in D is an extreme example from a spectrum of radially distorted roots. (E,F) Marginal region of a second-node rosette leaf. Note the impaired continuity and increased width of phloem as well as xylem strands. c, cortex; en, endodermis; e, epidermis; iff, interfascicular fibers; pi, pith; p, phloem; ve, vessel elements; x, xylem. Tissues are marked only in A and C. Scale bars in A,B,D,E,F,G, 100  $\mu\text{m}$ ; C, 50  $\mu\text{m}$ . DIC microscopy of histological section in A-D, of whole-mount preparation in E,F.

while the laminae were reduced in size. Except for more extreme root abnormalities of plants grown on NPA, similar phenotypical abnormalities were evoked by each of the three inhibitor substances.

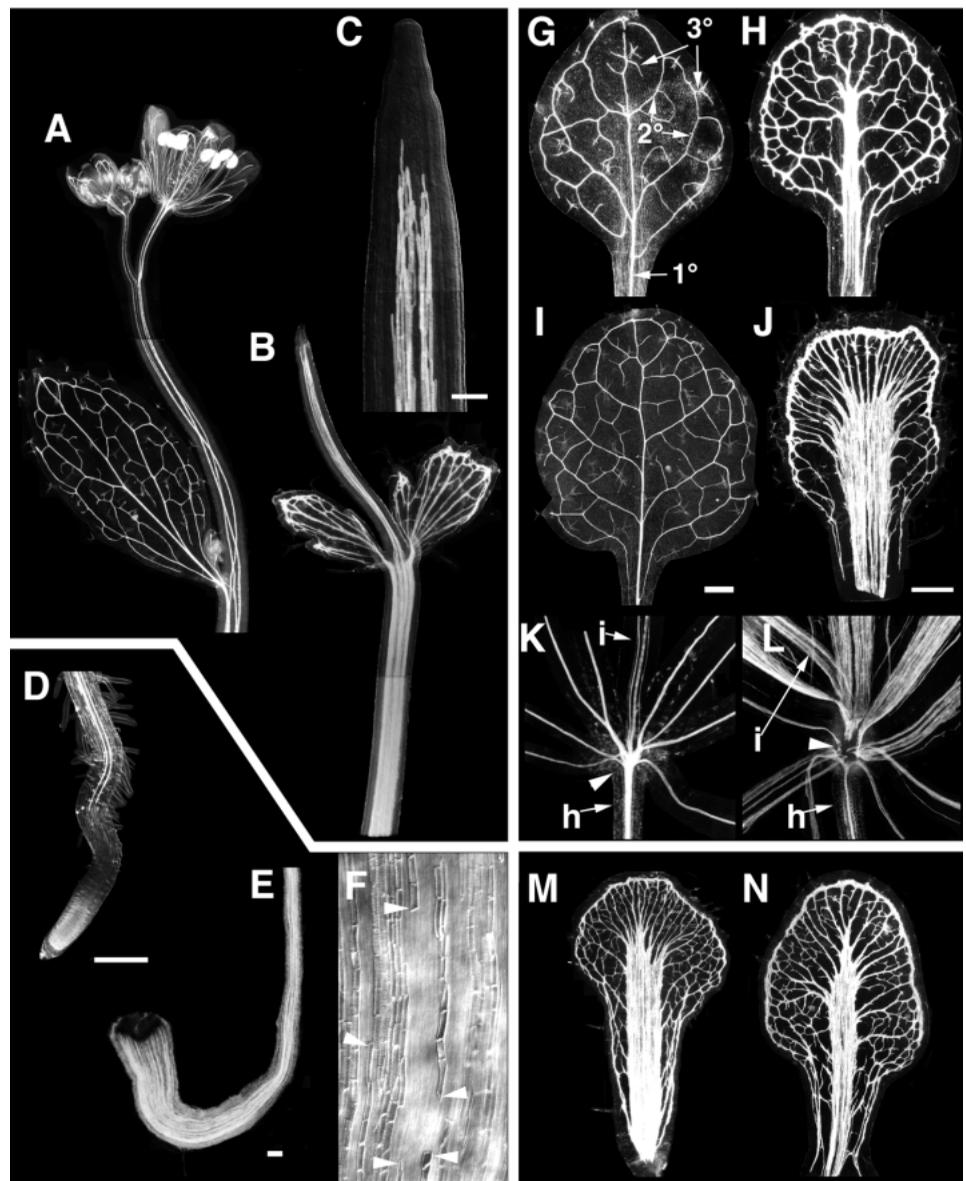
In order to visualize possible defects in the radial and longitudinal organization, the anatomy of inflorescence stems, roots and rosette leaves was analyzed in histological sections as well as in cleared preparations. In the figures usually only the xylem pattern is shown, while the closely related features in the phloem are only shown at selected sites. In the following we summarize characteristic anatomical alterations displayed by at least ten plants that were grown for 6 weeks in the

presence of each of the three auxin transport inhibitors (20  $\mu\text{M}$ ).

### Stem anatomy

Fig. 2A shows a cross section through an inflorescence stem of a 6-week old control plant just below the first node. The inflorescence radial pattern consists of a single layer of epidermis, three to five layers of cortex and four to seven vascular bundles. At this age and position, xylem bundles together with interfascicular fibers formed a ring of cells with thick secondary cell walls that circumscribed the central pith of parenchymatic cells. In the longitudinal dimension, vascular

**Fig. 3.** Vascular organization in auxin transport inhibited plants. Whole-mount preparations of cleared organs from plants germinated and grown on medium without NPA (A,D,G,I,K) and medium supplemented with 20  $\mu\text{M}$  NPA (B,C,E,F,H,J,L), 20  $\mu\text{M}$  TIBA (M) and 20  $\mu\text{M}$  HFCA (N). Mature xylem cells appear bright under dark-field optics due to the refraction properties of their thick secondary cell walls. (A,B) Inflorescence tips of plants grown in the absence of NPA (A) and presence of NPA (B). Note the increased amount of interfascicular fibers and vascular bundles in the stem, the lack of flowers and the reduced size of cauline leaves in B. (C) Close-up picture of the inflorescence tip of a plant grown on NPA. Xylem tissue continuity is somewhat impaired, but vascular strands are formed in the absence of lateral organs. (D,E) Primary root tips of a control (D) and of an auxin transport inhibited plant (E). Numerous parallel vessels extend towards the root apex in auxin transport inhibited roots. (F) Differential interference contrast image of parallel vessels in the root show interruptions, defective interconnection and elongation of vessel elements (arrowheads). (G,H) First-node rosette leaf of a control plant (G) and of a plant grown on NPA (H). Note the contiguous vascular differentiation zone along the leaf margin and the appearance of numerous parallel vessels in the center. (I,J) Second-node rosette leaf of a control (I) and a NPA grown plant (J). Vascular tissue in the center of the leaf is far more abundant than in auxin transport inhibited leaves from the first node (H). (K,L) Junction of inflorescence stem and rosette leaf petioles with the hypocotyl in control (K) and NPA grown plants (L). Arrowheads point at contiguous (K) versus non-contiguous (L) xylem strands in this region. (M,N) Second-node leaves of plants grown on medium supplemented with 20  $\mu\text{M}$  TIBA (M), or HFCA (N). The venation pattern alterations are very similar to those in comparable leaves of plants grown on NPA (J).



(K,L) Junction of inflorescence stem and rosette leaf petioles with the hypocotyl in control (K) and NPA grown plants (L). Arrowheads point at contiguous (K) versus non-contiguous (L) xylem strands in this region. (M,N) Second-node leaves of plants grown on medium supplemented with 20  $\mu\text{M}$  TIBA (M), or HFCA (N). The venation pattern alterations are very similar to those in comparable leaves of plants grown on NPA (J). h, hypocotyl; i, inflorescence axis; Scale bars, 100  $\mu\text{m}$  (C); 200  $\mu\text{m}$  (D,E); 500  $\mu\text{m}$  in I, J. A,B,G,H,M,N are of the same magnification as J. Nomenclature for leaf vascular strands: Vascular strands, commonly referred to as 'veins', are distinguished by hierarchical degrees according to Hickey (1979) as illustrated in G. Veins branching from the single midvein (primary vein, 1 $^\circ$ ) are denoted secondary (2 $^\circ$ ), from which tertiary (3 $^\circ$ ) veins emanate. Occasionally, quaternary veins attached to tertiaries are observed (not visible in G).

bundles were continuous connecting developing flowers, cauline leaves, or axillary buds (Fig. 3A). NPA-treated plants had more vascular tissues (Figs 2B, 3B). The width of the layer of interfascicular fibers and vascular bundles was approximately doubled, and supernumerary, often very broad, vascular bundles were observed (Fig. 2B). The longitudinal continuity of the vascular bundles was generally impaired, but relatively ordered vascular bundles were observed even in the pin-shaped portions of inflorescence stems (Fig. 3C). The latter observation is intriguing as it demonstrates that ordered vascular differentiation in the stem does not depend strictly on lateral organ derived signals (see Discussion).

### Root anatomy

The radial organization of the *Arabidopsis* root consists of single-cell layers of epidermis, cortex and endodermis enclosing the central vascular cylinder with a diarch organization of the xylem and two opposite poles of phloem (Dolan et al., 1993). We analyzed the radial pattern of tissues in the differentiation zone of auxin transport-inhibited plants. The radial sequence of tissue layers was normal and even in dramatically distorted roots the cortex consisted of no more than two cell layers (Fig. 2C). By contrast, the central vascular cylinder could be dramatically enlarged and misshapen (Fig. 2D). The oversized vascular cylinder contained a large number of xylem strands randomly distributed in the central cylinder (Fig. 2D). Individual vessel elements were only partly elongated and improperly aligned to each other and vessels were frequently interrupted (Fig. 3F).

### Leaf anatomy

In the following descriptions of leaf vascular patterns, we will use the term 'vein' for leaf vascular strand and adopt the traditional nomenclature of hierarchical orders of vein classes (Hickey, 1979) that has also been applied to *Arabidopsis* leaves (Kinsman and Pyke, 1998). We further introduce the terms 'central' and 'marginal' to refer to regions in the leaf irrespective of the appearance of vascular strands at these sites (for details see legend of Fig. 3). The first-node rosette leaves of control plants had a narrow primary vein, consisting of up to six parallel vessels (data not shown), and two to three even narrower curved secondary veins per lamina half (Fig. 3G). The secondary veins formed a series of prominent arches in which the distal end of a secondary vein joined the neighboring secondary vein. This basic pattern is referred to as pinnate brochidodromous venation (Hickey, 1979). In addition, a network of tertiary veins formed bridges between secondary veins in the intercostal areas as well as small arches close to the leaf margin. Quaternary veins can extend from tertiary veins and end blindly in the areoles.

In the corresponding leaves of plants germinated and grown in the presence of auxin transport inhibitors a number of distinct alterations in vascular organization were observed. First, veins were broader, consisting of a greater number of cells, and the cells were frequently improperly aligned (Fig. 2F). Second, instead of individual arches of secondary veins, a continuous broad zone of vascular differentiation extended along the entire margin of the lamina ('marginal vascularization zone' in the following; Figs 2F, 3H). Third, the number of parallel vascular strands in the central regions and in the petioles was increased and these strands were often

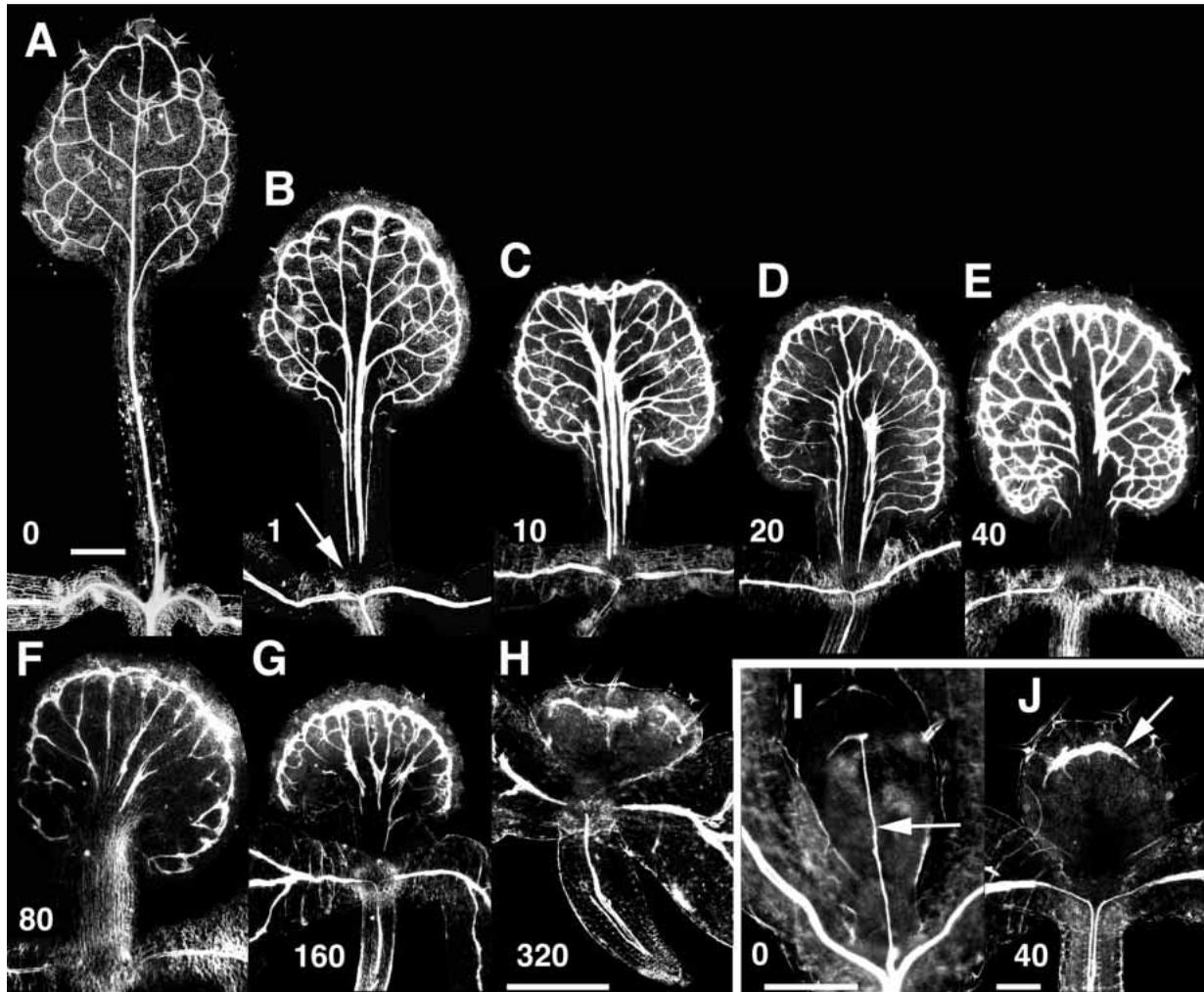
separated from each other (Fig. 3H). Fourth, the marginal and central regions were connected by an increased number of secondary veins (four to six per lamina half as opposed to two or three in normal first-node rosette leaves). These veins were often branched close to the marginal differentiation zone (Fig. 3H). Fifth, an increased number of tertiary veins connected secondary veins, or ended blindly in the areoles (Fig. 3H). Sixth, the leaf-traces, the vascular strands normally connecting the rosette leaves with the vascular system in the hypocotyl, were usually not detectable (Fig. 3L). The vascular patterns of second-node rosette leaves (as well as later rosette leaves, data not shown) displayed very similar alterations, except that their central regions and petioles were further broadened and even more extensively vascularized (Fig. 3J). In experiments described in a later section (Fig. 5Q,R), this seemingly distinguishing feature could, however, be attributed to the relatively earlier exposure of the leaf primordia to NPA rather than heteroblastic differences.

In summary, the similar morphological and anatomical responses with each of the three inhibitor substances suggest that the recorded defects represent consequences of reduced auxin transport rather than drug specific responses. The anatomical effects in stems, roots and leaves shared three characteristics. First, individual vascular strands were less well aligned and often formed diffuse regions of obliquely interconnected and/or misshapen cells. Second, at most sites more vascular tissue was formed. Third, organ shape changes were observed and were largely attributable to reduced longitudinal expansions and enlarged central regions which in stems and roots are the location of undifferentiated provascular tissue (see Discussion).

### The effect of NPA dosage on the leaf vascular pattern

Leaf development tolerates fairly high concentrations of NPA and the complex vascular system of leaves displays a number of discernible responses. We therefore chose the vascular system of a leaf from the first node to study the development of the vascular pattern at different levels of residual auxin transport. To this end, germinating seedlings, which contain minute primordia of the first-node leaves (Medford et al., 1992), were grown on a wide range of NPA concentrations. Differentiated vessel elements of the xylem can be studied most easily and were scored in at least ten plants for each NPA concentration. The pattern of phloem and the provascular strands displayed qualitatively similar responses (data not shown).

NPA concentrations as low as 1  $\mu\text{M}$  caused a considerable change in the venation pattern. The number and width of veins of all orders increased. Vascularization at the leaf margin was enhanced, especially at the tip of the leaf (Fig. 4B). Secondary veins approached the primary vein at a more acute angle, and often did not fuse with the primary vein. As a result, several parallel veins could frequently be seen in the petiole (Fig. 4B). At 10  $\mu\text{M}$  NPA, vascularization was enhanced along the entire margin of the lamina and additional vascular strands were formed in the central region (Fig. 4C). Even more secondary veins were observed, many of which were bifurcated close to the margin. In virtually all plants observed, leaf traces did not connect with the central vascular bundle in the hypocotyl. Exposure to all higher NPA concentrations resulted in the



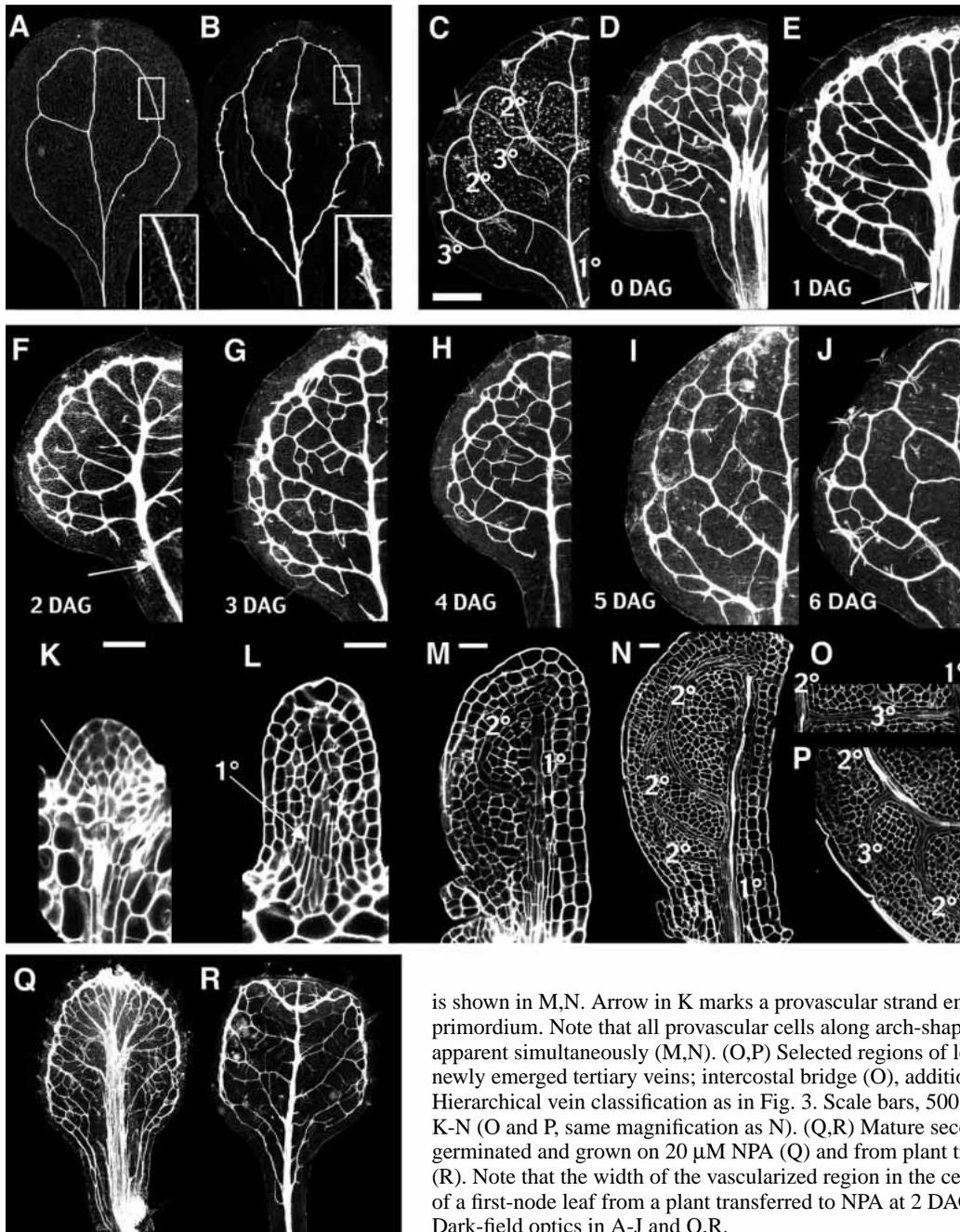
**Fig. 4.** The effect of NPA dosage on vessel differentiation in first-node leaves. (A-H) Vessel differentiation recorded 10 days after germination. A leaf from the first node of plants germinated and grown on medium supplemented with NPA. NPA concentrations, in  $\mu\text{M}$ , are indicated in the figure. Arrow in (B) indicates a gap between the xylem vessels in the petiole and the vascular bundle of the hypocotyl. Note the dose-dependent restriction of differentiated xylem tissue towards the leaf margin in A-H. For clarity, all other rosette leaves have been removed. (I,J) Vessel differentiation recorded 5 days after germination on medium without NPA (I) and supplemented with  $40 \mu\text{M}$  NPA (J). Note the presence of continuous vessels at disparate positions in I and J: continuous with the vascular system of the hypocotyl in I (arrow) while restricted to the apical leaf margin in J (arrow). Size bars,  $500 \mu\text{m}$  in A,H (B-G same magnification as A),  $200 \mu\text{m}$  in I,J. Whole-mount preparations, dark-field optics.

marginal vascular zone being very pronounced, while progressively less vascular differentiation was observed in the basal regions of the leaf. At  $20 \mu\text{M}$  NPA, vascularization in the midvein region was usually less extensive than at  $10 \mu\text{M}$  and the vascular gap at the base of the leaf trace was further enlarged (Fig. 4D). At  $40 \mu\text{M}$  NPA, barely any differentiated vessels were found in the central region or in the petiole (Fig. 4E) and at  $80 \mu\text{M}$ , leaves often had no midvein at all (Fig. 4F). At  $160 \mu\text{M}$  NPA, the majority of secondary veins were rather faint. These remaining secondary veins were sometimes connected to each other, but ultimately ended blindly within the leaf (Fig. 4G). At  $320 \mu\text{M}$  NPA, the venation was reduced to a marginal crescent from which few short secondary veins emanated (Fig. 4H).

In summary, increasing NPA concentrations resulted in broadened vascular differentiation zones in the marginal region and an apparent reduction of vascular differentiation at more

basal positions. This observation is indicative of an apically derived signal that upon inhibition of its transport becomes progressively confined to the source region (see Fig. 7).

To determine whether auxin transport inhibition permanently reduced vascular differentiation in the basal part of the leaf or only delayed the progression of vascular differentiation towards the leaf base, we analyzed the effect of NPA on the temporal sequence of vessel differentiation in first-node leaf primordia at consecutive days after germination. In leaves of control plants, the maturation of vessels in the primary vein started in the hypocotyl and proceeded acropetally towards the leaf tip (5-day old primordium in Fig. 4I). Concentrations of  $1 \mu\text{M}$  NPA and higher reversed the direction of vessel maturation. The maturation of vessels started along the leaf margin and the vessel elements differentiated in the basipetal direction towards the center/base of the leaf (6 day old primordium in



**Fig. 5.** Vascular responses to staged application of NPA. (A,B) Vessel patterns of cotyledons from plants germinated and grown for 15 days on medium without (A) and with the addition of 20  $\mu\text{M}$  NPA (B). Inserts show a higher magnification of the framed areas. (C-J) Venation patterns of mature first-node rosette leaves after different exposure times to NPA. Leaf from control plant not exposed to NPA (C) and leaves from plants transferred to 40  $\mu\text{M}$  NPA-containing medium at successive stages (D-J): at germination (D) and at 1 (E), 2 (F), 3 (G), 4 (H), 5 (I) 6 (J) days after germination. Arrows in E and F indicate the gradual determination of primary vein width. Note that the pattern of tertiary and quaternary veins in I is still more extensive than in the control. (K-P) Confocal laser scanning microscopy images of first-node leaf primordia at different DAG. Stages in DAG correspond to those in F-J: 2 (K), 3 (L), 4 (M), 5 (N) and 6 (O,P) and show the state of provascular differentiation when NPA was added. Since the laminae of young leaf primordia are curved, only one half of each primordium

is shown in M,N. Arrow in K marks a provascular strand emerging at the base of the young leaf primordium. Note that all provascular cells along arch-shaped secondary veins become apparent simultaneously (M,N). (O,P) Selected regions of leaf primordium at 6 DAG showing newly emerged tertiary veins; intercostal bridge (O), additional arch at leaf margin (P). Hierarchical vein classification as in Fig. 3. Scale bars, 500  $\mu\text{m}$  in C, applies to C-J; 20  $\mu\text{m}$  in K-N (O and P, same magnification as N). (Q,R) Mature second-node leaves from plants germinated and grown on 20  $\mu\text{M}$  NPA (Q) and from plant transferred to 20  $\mu\text{M}$  NPA at 3 DAG (R). Note that the width of the vascularized region in the center of the leaf in R is similar to that of a first-node leaf from a plant transferred to NPA at 2 DAG (F). Whole-mount preparations. Dark-field optics in A-J and Q,R.

Fig. 4J). Furthermore, inspection of older leaves (25 days) revealed that basal vascular differentiation was permanently blocked only at extremely high NPA concentrations (160 and 320  $\mu\text{M}$ ), while at all lower concentrations it proceeded slowly beyond what is shown in Fig. 4 (data not shown). Thus, NPA application caused an inverse, basipetal direction of vessel differentiation. With higher NPA concentrations, the basipetal progression of vessel differentiation was progressively slowed down and finally completely blocked. The temporal patterns of vascular differentiation could be explained by assuming that vascular differentiation occurs at a critical auxin concentration which in normal leaves are reached at points of auxin flow convergence in the center, but upon inhibition of auxin transport are shifted towards source

regions at the margin of the leaf primordia (see Discussion and Fig. 7).

#### NPA-sensitive stages of leaf vascular development

In contrast to rosette leaves, cotyledons of plants germinated and grown on medium supplemented with NPA displayed a normal venation pattern (a midvein and one to two secondary veins per lamina half; Fig. 5B). This observation suggests that auxin transport influences vascular patterning only in early stages of primordia development, which for cotyledons occurred during embryogenesis prior to the exposure to NPA. In order to identify the developmental stages at which auxin transport influences the leaf vascular pattern, first-node leaf primordia were exposed to NPA at progressively later stages

of leaf primordium development. This was accomplished by transferring seedlings from medium without NPA to medium containing 40  $\mu\text{M}$  NPA at successive days after germination (DAG). Seedlings were then grown in this medium until 18 DAG, when the resulting vascular pattern was recorded (see Materials and Methods). To monitor the stage of vascular ontogeny at the onset of NPA exposure, parallel samples were fixed for microscopy.

The effect of NPA was very pronounced in plants exposed to it early in leaf development. The vascular patterns in first-node leaves from plants that had been transferred to NPA-containing medium at 1 DAG (Fig. 5E) were largely similar to those of plants that had germinated on NPA medium (Fig. 5D), except that the vessels in the midvein region were less separated and extended further basally. In plants exposed to NPA from 2 DAG, those vessels formed a singular midvein, while the vascular zone at the leaf margin was still pronounced and there were still supernumerary secondary veins (Fig. 5F). These features indicate that the width of the midvein region could be influenced by modulation of auxin transport only up to 2 DAG. The vascular system of leaves from plants transferred at 3 DAG was further normalized, but there was still extensive vasculature close to the margin and more secondary and tertiary veins (Fig. 5G). Transfer to NPA at 4 DAG resulted in the restoration of normal, curved and unbranched secondary veins, but the vasculature at the leaf margin still appeared more extensive than in untreated leaves (Fig. 5H). Transfer at 5 DAG resulted in a pattern of primary and secondary veins similar to untreated leaves, but there were still more tertiary veins than in control leaves (Fig. 5I). Transfer to NPA at 6 DAG did not result in any marked deviation from the normal vascular pattern in a first-node leaf, however, xylem strands still appeared somewhat irregular due to improper alignment and shape of individual vessel elements (Fig. 5J; similar to those seen in Fig. 5B). This effect is reminiscent of the strand abnormalities observed in cotyledons exposed to NPA at germination and suggests that auxin transport promotes oriented cell differentiation beyond the stages at which it influences the sites of vascular differentiation.

Rosette leaves of later nodes appeared to react similarly to staged application of transport inhibitor. However, as shown in Fig. 5Q central regions and petioles are even broader and highly vascularized in rosette leaves of the second node when plants were germinated and grown on NPA. In order to distinguish between effects due to true heteroblastic differences among rosette leaves and effects brought about by different stages at which leaf primordia are at any given time of NPA exposure, we analyzed the vascular phenotypes of rosette leaves of the second node in plants exposed to NPA at 3 DAG. At this stage their primordia have attained a size roughly similar to those of first-node leaves at germination. As shown in Fig. 5R leaf shape alterations and enhanced vascularization in the central region of second-node leaves disappeared upon later exposure to NPA, while the vascular pattern in the leaf blade remained clearly responsive to inhibition of auxin transport. Thus, first- and second-node rosette leaves appear to react similarly to auxin transport inhibition, if the inhibitor is applied at roughly equivalent stages of primordium development.

In order to correlate stages of NPA sensitivity to the development of the early provascular pattern, parallel samples,

were analyzed by CLSM. We used CLSM to generate series of optical sections through leaf primordia. That enabled us to trace and visualize provascular strands within at least one half of a leaf primordium (Fig. 5). Results described below are based on ten independent observations for each stage. Provascular strands were identified as parallel files of narrow cells and assigned to veins as 'provascular veins', of different orders as described for the mature venation pattern. At 2 DAG, leaf primordia were recognizable as small bulges of no more than 40  $\mu\text{m}$  length (Fig. 5K). A provascular strand extended from the base of the primordium to the vascular bundle of the hypocotyl, but no provascular strand was apparent within leaf primordia of this stage (Fig. 5K). At 3 DAG, the primordium had attained a length of approximately 100  $\mu\text{m}$  and a three to four cell wide provascular strand could be seen in its center (Fig. 5L). Provascular secondary veins were not present at this stage. At 4 DAG, leaf lamina and petiole regions could be discerned (Fig. 5M). At this stage the provascular primary vein extended to the tip of the primordium and a pair of curved provascular secondary veins had regularly formed in the apical region. Less well-defined, continuous strands were found further basally, likely reflecting the emergence of another provascular secondary vein (Fig. 5M). At 5 DAG, two to three curved provascular secondary veins were seen in each lamina half and vessel elements had differentiated along the route of the primary vein (Fig. 5N). At 6 DAG, provascular tertiary veins forming intercostal bridges and marginal loops were seen in CLSM images (Fig. 5O,P). At this stage, vessel elements had begun differentiating along the routes of the first secondary veins (Fig. 5P).

In summary, different orders of leaf veins become unresponsive to inhibition of auxin transport in the same sequence as their provascular counterparts appeared. Furthermore, a correlation of the NPA sensitivity of veins to the appearance of provascular strands indicated that anatomically recognizable provascular strands are unresponsive to further manipulations of auxin transport (see Discussion).

### Auxin transport inhibition in other dicotyledonous plants

To determine whether inhibition of auxin transport would have similar effects in other dicotyledonous plants we exposed seedlings of *Aurinia saxatilis* (alyssum), *Antirrhinum majus* (snapdragon) and *Nicotiana tabacum* (tobacco) to NPA and analyzed the effect on the xylem system in the second-node leaves after 18 days. In all three species, the marginal and central zones of vascular differentiation were broadened and connected by an increased number of secondary veins. Tertiary and quaternary veins were also more abundant (Fig. 6). Thus, the vascular responses of alyssum, snapdragon and tobacco closely resemble those described in *Arabidopsis*, suggesting that similar auxin flows play similar roles in determining the vascular pattern of a wide range of dicotyledonous plants.

### Vascular defects in *Arabidopsis* mutants

Mutations in three genes, *PIN FORMED1* (*PIN1*), *MONOPTEROS* (*MP*) and *LOPPEDI* (*LOP1*) have been reported to result in reduced auxin transport in *Arabidopsis* stem segments (Okada et al. 1991; Przemeck et al., 1996; Carland and McHale, 1996). While the vascular system seems

to be reduced in *mp* and *lop1* mutants (Przemeck et al., 1996; Carland and McHale, 1996), enhanced xylem formation in *pin1* mutant stems has recently been observed (Gälweiler et al., 1998). In order to determine whether mutations in *PIN1* result in defects similar to those induced by chemical inhibition of auxin transport, we analyzed *pin1* mutant inflorescence tips, rosette leaves and roots. As shown in Fig. 6G-I the vascular system in *pin1* mutant inflorescence tips and rosette leaves showed defects very similar, though somewhat weaker than those in our auxin transport inhibited plants. Vascular strands were discontinuous, particularly near the inflorescence tip (Fig. 6G) and rosette leaves often displayed enhanced vascularization near the margin as well as multiple strands in the center (Fig. 6H). By contrast, *pin1* mutant roots were not markedly affected and the central cylinders were not expanded (Fig. 6I). Together, these findings support the notion that the *PIN1* product could be directly involved in auxin efflux, but further suggest that *PIN1* acts in concert with other genes of similar function (see Discussion).

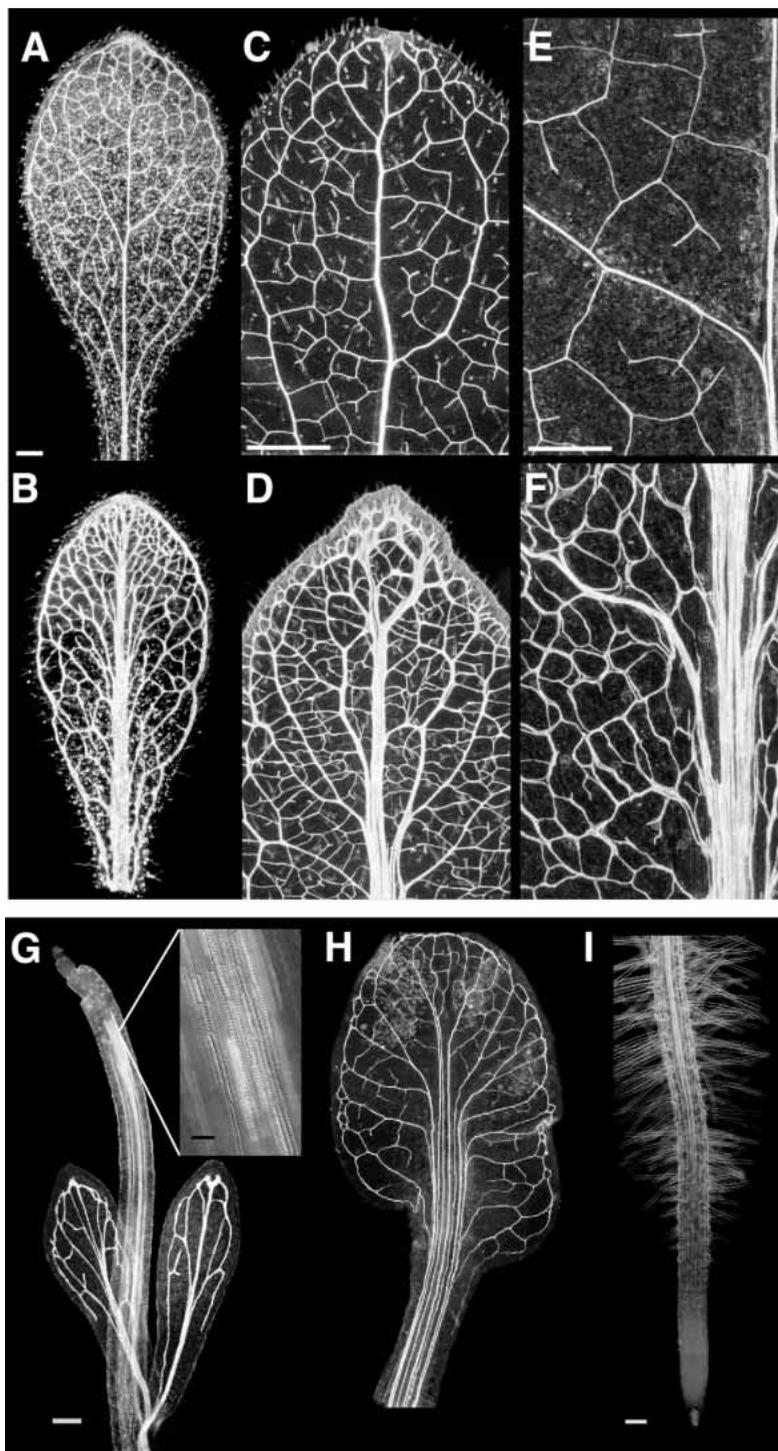
Two further genes, *ETHYLENE INSENSITIVE ROOT1 (EIR1)* [alternative names *AGRAVITROPIC1 (AGRI)*, *PIN FORMED2 (PIN2)*] and *AUX1* encode membrane proteins and have been implicated in auxin efflux and influx, respectively (Bennett et al., 1996; Luschnig et al., 1998). Consistent with the assumed specialized function of these genes in the root elongation zone, we have not detected vascular defects in organs of either *eir1* or *aux1* mutants (data not shown).

## DISCUSSION

A large number of experiments demonstrate the considerable adaptive capacities of the vascular system (Sachs, 1989), raising the possibility that vascular patterning in normal organ development could be a partially self-organizing process linked to organ development by rather coarse cues. Several models propose that auxin and/or its apical-basal flow could be involved in specifying the locations of vascular strands (summarized in Nelson and Dengler, 1997). We investigated the effects of auxin transport inhibition on the continuity of vascular strands and the pattern of vascular connections in plant organogenesis. We found highly reproducible alterations in response to auxin transport inhibition that indicate a role for auxin signals in restricting vascular differentiation to narrow zones, promoting vascular continuity, and specifying the venation pattern of leaves.

### Development of the vascular system under conditions of reduced auxin transport

In a first set of experiments we assessed the morphological and anatomical abnormalities evoked by application of three auxin transport inhibitors.



**Fig. 6.** Leaf vascular organization in three dicotyledonous plant species and vascular system in *Arabidopsis pin1* mutants. Leaf of alyssum plant grown without NPA (A) and in the presence of 2 μM NPA (B). Leaf tip of a snapdragon plant grown without NPA (C) and in the presence of 10 μM NPA (D). Midvein region of a tobacco leaf from a plant grown without NPA (E) and in the presence of 40 μM NPA (F). Pictures of control and NPA-treated leaves are of identical magnification. (G-I) Inflorescence tip (G), rosette leaf (H) and primary root (I) of an *Arabidopsis pin1* mutant 4 weeks after germination. Note the presence of abundant but improperly aligned vessel strands in inflorescence tips and pronounced vascularization at the margins and in the center of *pin1* mutant rosette leaves. Scale bars, 500 μm in A,C,E; 200 μm in G; 100 μm in I; 25 μm in detail of G. Whole-mount preparations, dark-field optics, DIC in detail of G.

The common responses can be summarized as follows. First, continuity of the vascular tissues is by far the most sensitive anatomical feature; vascular cells were abnormally shaped and improperly aligned at inhibitor concentrations that had no marked effect on the integrity of other tissues. Second, at many locations, reduced auxin transport resulted in more cells undergoing vascular differentiation. Thus, auxin transport appears to be important in promoting vascular tissue continuity and limiting vascular differentiation at virtually all analyzed sites.

It is interesting to note that there is remarkable agreement between our observations and the predictions of the auxin canalization model (summarized by Sachs, 1981, 1991). According to this model, vascular differentiation occurs along preferred routes of flow of a signal, IAA itself and/or closely associated substances. Canalization of this flow towards narrow routes is thought to result from a positive feed-back mechanism that enhances the conductivity of cells functioning as conduits (for details see Fig. 7A). Our observations are consistent with the proposed role of auxin flows in orienting cell differentiation and thereby promoting vascular continuity. Broadening zones of vascular differentiation would be the likely outcome of local auxin accumulation and lateral auxin spread under conditions of impaired drainage (Fig. 7B,C). The canalization model was deduced from studies of vascular regeneration after wounding. Our results suggest that similar feed-back mechanisms occur in vascular strand formation during normal organ development.

Auxin transport has other well established functions (Davies, 1995 and references therein). For example, we confirmed the observation that lateral organ formation in the inflorescence meristem is very sensitive to inhibition of auxin transport (Okada et al, 1991). The resulting naked inflorescence stems contain ordered arrays of vascular bundles (Fig. 3C), indicating that position and internal organization of stem vascular bundles can be specified during stem ontogeny irrespective of the presence of recognizable lateral organs, which had been controversial in the literature (Ma and Steeves, 1992 and references therein). In stems and roots, inhibition of auxin transport resulted in organ shape changes and we show that these are largely due to broader vascular regions (vascular bundles in stems, central cylinder in roots, Fig. 2). Moreover, in mature organs nearly all cells within these oversized regions have further differentiated into vascular cell files suggesting that auxin transport is essential not only for delimiting provascular regions in organ primordia (and thereby affecting organ shape) but also for restricting zones of further vascular differentiation later in organ development.

### Polar effects of auxin transport inhibition

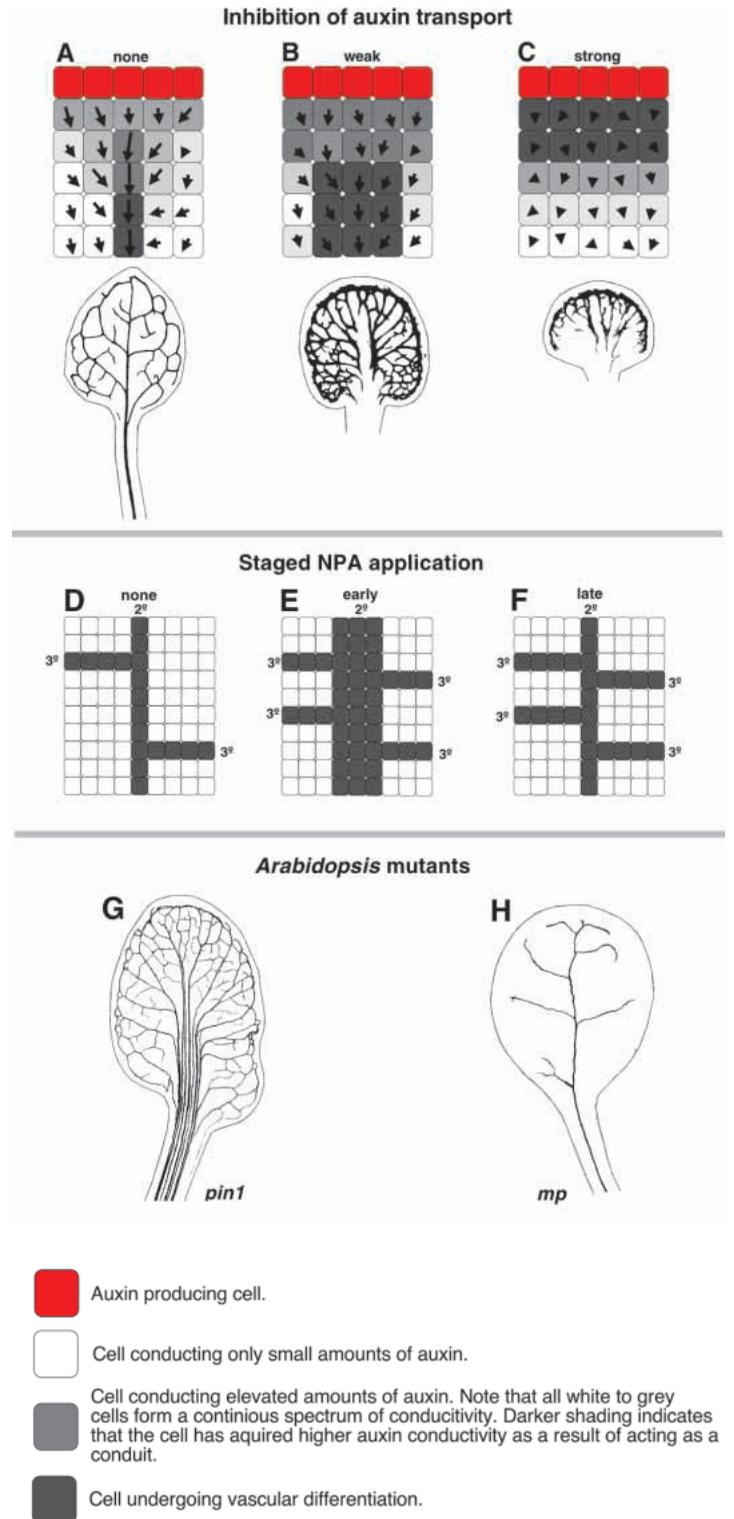
In a second set of experiments we assessed the effect of progressively reduced auxin transport on the vascular patterns of leaves. Application of 1  $\mu$ M NPA (Fig. 4B) resulted in the formation of pronounced veins along the leaf margin. This was not accompanied by detectable anatomical alterations and could therefore reflect a direct effect of auxin flows on leaf vascular patterning. Higher NPA concentrations further increased vascular differentiation along the margin, also accompanied at basal positions by an inverse effect, the decrease, and eventual disappearance, of vessel differentiation. The increased vascular differentiation along the leaf margins

could be due to local auxin accumulation close to an auxin source in this region (Fig. 7A). The gradual reduction of vessel differentiation at basal positions likely reflects insufficient auxin supply to the basal regions due to impaired transport (Fig. 7B,C). This interpretation implies that major auxin sources are located along the margins. Several studies have identified young leaf primordia as sources of free IAA (Davies, 1995; Wallroth-Marmor and Harte, 1988), however the sublocalization of auxin sources within leaf primordia is unknown. It should be emphasized that our interpretation does not exclude auxin production in other parts of the leaf primordium and that marginal auxin sources would be required only in fairly young leaf primordia (see below). Inhibitor mediated uncoupling of basal responses from apical auxin sources is not without precedent. Application of auxin transport inhibitor to the root/hypocotyl junction in *Arabidopsis* reduced levels of free IAA in the roots and decreased the number and density of lateral roots (Muday and Reed, 1997). In a similar way, high concentrations of NPA could delay and eventually block auxin drainage to basal regions in the leaf.

### Stage-specific effects of auxin transport inhibition

*Arabidopsis* leaf vascular development has been described in detail (Telfer and Poethig, 1994; Nelson and Dengler, 1997; Kinsman and Pyke, 1998). In a third series of experiments we assessed the stages in leaf development at which differentiation of individual classes of veins are dependent on normal auxin transport. To this end, first-node leaf primordia were exposed to NPA at consecutive days after germination and in parallel samples leaf primordia were analyzed by CLSM. Our results indicate that specific parts of the leaf vascular pattern become unresponsive to NPA in a reproducible sequence. The width of primary veins became unresponsive to auxin transport inhibition at 2 DAG, prior to the emergence of provascular strands in the primordia. Secondary veins became unresponsive approximately 2 days later at the stage of early lamina formation, while tertiary and quaternary vein formation remained NPA sensitive up to 5 DAG. This sequence of NPA-sensitive periods is colinear with the sequence of emergence of the respective provascular strands as determined by CLSM. These correlations entail three interesting conclusions. First, established provascular strands appear to be insensitive to NPA and their presence seems to influence the response of adjacent cells to NPA. When provascular primary and secondary veins become visible in CLSM their width and position can no longer be influenced by NPA, while the formation of emerging intercalating tertiary/quaternary vascular strands within the same primordium remains sensitive to NPA (Fig. 7D,E). Unresponsiveness of provascular strands to experimental manipulation has been reported by others. Wounding of pea leaf primordia could influence the position of major veins only when performed very early in development. When the primordia were wounded after provascular strands had emerged, unsevered strands continued to differentiate and influenced the orientation of subsequently formed vascular strands in a way that suggested that they could act as auxin drainage canals (Sachs, 1989). In our experiments vascular strands remain narrow, if a single provascular file has been formed prior to NPA application. A simple explanation could be the expression of NPA-insensitive auxin carrier proteins in

**Fig. 7.** Auxin transport inhibition responses and *Arabidopsis* auxin transport mutants: model and interpretation. (A-C) Auxin transport inhibition responses of individual vascular strands (upper diagrams) and leaf venation patterns (lower diagrams). (A) Auxin transport not inhibited: Efficient auxin transport drains away enough auxin to keep overall auxin concentrations below the threshold for vascular differentiation (model modified from Sachs, 1991). A positive feed-back mechanism renders conducting cells more conductive, thereby stabilizing auxin routes into preferred auxin canals. The most conductive canals drain auxin from all of the neighboring cells restricting the canalizing zone to a narrow cell file. Only cells within these files are exposed to inductive levels of auxin over extended periods of time and therefore undergo overt vascular differentiation. The canalization model further proposes that auxin flow has an orienting influence on cell differentiation and thereby promotes cell alignment (Sachs, 1981). In leaves, most of the vascular system form a branched pattern, draining auxin basally through veins of higher orders and eventually through the midvein. Cells within individual veins are properly aligned. (B) Weak inhibition of auxin transport: Upon inhibition of auxin transport auxin accumulates to inductive levels closer to the source. Hence more cells are exposed to auxin for longer periods and vascular differentiation occurs in broader zones and at more locations. In leaves, three effects are observed. First vascular strands are broader and increased in number. This effect would occur if a mechanism such as that depicted in the upper diagram were in operation. Second, marginal veins become relatively more pronounced. We propose the existence of auxin sources close to the leaf margin at stages critical for vascular patterning (see text for details). Auxin could be trapped in the marginal region for longer periods before inducing efficient drainage canals, thereby inducing excess vascular tissue at the margins. Third, vascular differentiation towards the base of the leaf is delayed and may not be completed. If auxin is trapped in the marginal regions for longer periods, and basal vascular differentiation is dependent on this signal, it is expected to be delayed. (C) Very strong inhibition of auxin transport nearly abolishes auxin flow. This leads to auxin accumulation and differentiation of vascular cells in the immediate vicinity of the auxin source. In leaves, very strong inhibition of auxin transport restricts vascular differentiation to the leaf margin, suggesting major auxin sources in this region. Leaf schemes shown correspond to Fig. 4A,E and G. Early effects of auxin transport on leaf shape are discussed in the text. (D-F) Differential responses of secondary and tertiary veins to staged application of auxin transport inhibitor (NPA). (D,E) The formation of more and broader vascular strands in response to auxin transport inhibition has two effects (compare Fig. 7B). First, more parallel files of differentiated vessels along secondary veins ( $2^\circ$ , two extra files in E as compared to the control in D) and, second, a general increase in the number of tertiary veins ( $3^\circ$ ). (F) If auxin transport inhibition is delayed by 2 days these effects are separated: Secondary veins are no longer affected, while the later developing lower order veins are still increased in number (compare Fig. 5). The experiment demonstrates that cells in the entire region are exposed to NPA, but that cells flanking an earlier formed vascular strand are no longer affected by NPA. Differentiating vascular cells could transport auxin through NPA resistant mechanisms and would therefore efficiently drain auxin from adjacent cells irrespective of NPA exposure. (G,H) Two classes of leaf venation patterns in auxin transport inhibited *Arabidopsis* mutants. (G) *pin1* mutant (Fig. 6G) has abundant continuous veins along the leaf margin as well as additional secondary and primary veins. These features are very similar to those produced by inhibition of auxin transport by low concentrations of NPA, suggesting similar primary defects. (H) *mp* mutants have no veins along the leaf margin and fewer secondary veins than in wild-type leaves (Fig. 7A). This venation pattern could result from reduced sensitivity to vascular inducing auxin signals. In this case vascular differentiation should be restricted to central and basal regions, the focus of auxin produced anywhere in the lamina. Auxin transport capacities of most cell types can be normal, but the reduced number of auxin transporting provascular cells would nevertheless result in reduced auxin transport, if measured in organ segments.



provascular cells. These provascular strands would act as efficient drainage canals even after NPA application, preventing the formation of broad, discontinuous vascular differentiation zones. This simple interpretation does not preclude alternative or additional stabilizing interactions among differentiating vascular cells. Second, the pattern of primary and secondary veins is established very early and could therefore be influenced by the cellular architecture of the young primordium. It has previously been observed that secondary veins in *Arabidopsis* leaves arise as rejoining lobes rather than as isolated branches (Kinsman and Pyke, 1998). CLSM micrographs visualize provascular cells as soon as they are distinguishable by narrower shape and show them in the context of the overall cellular architecture of the primordium. In these micrographs provascular strands of secondary veins emerge as continuous arches of nearly simultaneously differentiating cells. An arched structure cannot easily be explained by canalized auxin flows where each canal should contribute to apical-basal drainage. The emergence of vascular strands that do not conform to the assumed direction of auxin transport has spurred many discussions as to what extent a canalization concept can account for vascular patterns in general (Mitchison, 1980; Sachs, 1989) and alternative models have been devised (Kull and Herbig, 1995; Meinhardt, 1996; summarized by Nelson and Dengler, 1997). Our confocal micrographs show that at the early stages at which the lobes of elongated provascular cells emerge, they are aligned with the outer subepidermal cell layers of primordia (Fig. 5M,N). It is intriguing to speculate that cells within layers of early organ primordia could share a common orientation, as it is indicated by selective interconnection of cells within cell layers in the shoot meristem (Rinne and van der Schoot, 1998). Under conditions of impaired auxin transport, these cells oriented parallel to the margin could be exposed to accumulating auxin, thereby forming more vascular tissues, while the auxin-flow mediated formation of veins toward the base of the leaf would be reduced (Fig. 7B,C). Staged application of NPA shows that all signals involved in the specification of the pattern of primary and secondary veins have to operate at early stages of lamina formation. Finally, late application of NPA to leaf primordia separates vascular responses in the leaf blade from NPA-mediated effects on leaf shape. Although leaves can develop in the presence of very high concentrations of NPA, continuous NPA exposure resulted in broadening of the petiole and of the central region. When application of NPA was delayed (application at 2 DAG on first-node leaf primordia, Fig. 5F; application at 3 DAG on second-node leaf primordia, Fig. 5R), the overall shape of the leaf remained close to normal, while vascular responses in the leaf blade could still be studied. Thus, the leaf blade constitutes a suitable structure to investigate vascular responses to auxin transport inhibition in the absence of interfering organ shape effects.

### Relative contribution of auxin signals in vascular patterning

As a first step to exploring the generality of these results we investigated the rosette-leaf responses in three other dicot species: alyssum, snapdragon and tobacco. Although the three species differ in NPA sensitivity, their leaf venation alterations were qualitatively similar, indicating related functions of auxin flows in the specification of the venation patterns in all three

species. It should be emphasized that an important influence of auxin flows on vascular patterning is not in conflict with the existence of other, auxin-independent mechanisms. These might be identified by the isolation of specific vascular mutants and their molecular genetic analysis. In this context auxin transport inhibition studies can elucidate the relative contributions of auxin transport-dependent mechanisms even in more complex vascular patterns, and can thereby assist the evaluation of vascular mutant phenotypes.

We have compared the vascular features of *Arabidopsis* mutants that have been reported to be impaired in auxin transport. The *PIN1* gene is expressed in all major organs and encodes a presumptive membrane protein product that is located at the basal end of auxin transport competent cells (Gälweiler et al., 1998). Furthermore, cross sections of *pin1* mutant stems revealed increased xylem formation at the insertion points of cauline leaves and the PIN1 protein has therefore been proposed to be part of the general auxin efflux mechanism that is inhibited by NPA. Our vascular analysis of *pin1* mutant leaves, inflorescence tips and roots supports this notion in that vascular features correspond in detail to those observed in NPA-treated plants (Figs 3, 6). However, our data also suggest the existence of gene functions overlapping with those of *PIN1*. The likely lack-of-function mutant *pin1::EN134* is viable throughout development (Gälweiler et al., 1998) and the morphologically similar *pin1-1* mutant used in this study displayed vascular defects corresponding to those evoked by relatively low concentrations of NPA. Thus, judged by vascular defects, critical amounts of auxin efflux could be mediated by unknown gene products and *PIN1* gene activity appeared dispensable in roots.

Mutations in the genes *MP* and *LOP1* result in vascular abnormalities nearly reciprocal to those of *pin1* mutants, indicating unrelated defects (Przemeck et al., 1996; Carland and McHale, 1996). We speculate that *MP* could affect the response of vascular cells to auxin signals (Fig. 7F,G). This interpretation is consistent with the reduced vascular system in *mp* mutants and with the molecular identity of the MP product as a transcription factor implicated in auxin signal transduction (Hardtke and Berleth, 1998). Reduced auxin transport has been measured in *mp* and *lop1* mutant stem segments, but could result from distorted cellular continuity and reduced number of developing vascular strands rather than from impaired auxin transport capacities of individual cells. Thus, at least two primary defects resulting in reduced polar auxin transport appear to be distinguishable by vascular features, which may therefore assist future characterizations of auxin transport mutants in *Arabidopsis* and other species.

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