The auxin influx carrier is essential for correct leaf positioning

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Summary

Auxin is of vital importance in virtually every aspect of plant growth and development, yet, even after almost a century of intense study, major gaps in our knowledge of its synthesis, distribution, perception, and signal transduction remain. One unique property of auxin is its polar transport, which in many well-documented cases is a critical part of its mode of action. Auxin is actively transported through the action of both influx and efflux carriers. Inhibition of polar transport by the efflux inhibitor *N*-1-naphthylphthalamic acid (NPA) causes a complete cessation of leaf initiation, a defect that can be reversed by local application of the auxin, indole-3-acetic acid (IAA), to the responsive zone of the shoot apical meristem. In this study, we address the role of the auxin influx carrier in the positioning and outgrowth of leaf primordia at the shoot apical meristem of tomato. By using a combination of transport inhibitors and synthetic auxins, we demonstrate that interference with auxin influx has little effect on organ formation as such, but prevents proper localization of leaf primordia. These results suggest the existence of functional auxin concentration gradients in the shoot apical meristem that are actively set up and maintained by the action of efflux and influx carriers. We propose a model in which efflux carriers control auxin delivery to the shoot apical meristem, whereas influx and efflux carriers regulate auxin distribution within the meristem.

Abbreviations: IAA, indole-3-acetic acid; NAA, naphthalene-1-acetic acid; NOA, naphthoxy-1-acetic acid; NPA, *N*-1-naphthylphthalamic acid; NPA-pins, meristems grown on NPA that lack leaf primordia; 2,4-D, 2,4-dichlorophenoxyacetic acid.

Keywords: leaf positioning, auxin influx carrier, auxin, inhibitors of auxin transport.

Introduction

The plant hormone auxin has a wide variety of effects on plant growth and morphogenesis. It is involved in apical–basal and radial pattern formation in developing embryos (Jürgens, 2001; Liu *et al.*, 1993), in tropic growth (Rosen *et al.*, 1999), the formation of vascular tissue (Berleth *et al.*, 2000), the positioning and outgrowth of lateral roots (Casimiro *et al.*, 2001; Laskowski *et al.*, 1995) and of leaf and flower primordia (Berleth and Sachs, 2001). A unique regulatory factor in auxin action is its controlled transport. Auxin is directionally transported from the shoot to the roots, a process called polar auxin transport (Lomax *et al.*, 1995). Disruption of polar auxin transport with specific efflux inhibitors causes severe developmental defects (Celenza *et al.*, 1995; Liu *et al.*, 1993; Okada *et al.*, 1991). When tomato shoot tips were cultured in the presence

of *N*-1-naphthylphthalamic acid (NPA), the meristems grew as pin-like structures (NPA-pins) in which leaf initiation was completely blocked, but stem growth and meristem maintenance proceeded normally. Organ formation could be restored by local auxin application, but not by other effectors (Reinhardt *et al.*, 2000). Similar results were obtained with the *pin1* mutant of *Arabidopsis*, which is defective in a gene that encodes a putative auxin efflux carrier (Gälweiler *et al.*, 1998). Inflorescence axes of the *pin1* mutant lack flowers, but meristem growth and maintenance are not affected (Vernoux *et al.*, 2000). Local application of IAA to the apex of the *pin1* mutant induces the formation of lateral organs (Reinhardt *et al.*, 2000). These experiments suggest that differences in local auxin concentrations set up by regulated auxin transport determine the site of primordium formation.

Whereas auxin efflux carriers are indispensable for organ positioning and outgrowth, the importance of regulated auxin influx is not evident. Putative auxin influx carriers have been found by mutational approaches. Mutations in the *AUX1* gene lead to an agravitropic phenotype in the root, which can be bypassed by growing *aux1* plants in the presence of naphthalene-1-acetic acid (NAA), but not IAA (Marchant *et al.*, 1999). Since NAA transport is influx-carrier independent, this is good evidence that *AUX1* is an import carrier. *AUX1* is expressed in the shoot apical meristem (Marchant *et al.*, 1999, 2002), but no shoot phenotype is apparent in *aux1* mutants (Pickett *et al.*, 1990). The simplest explanation would be that influx carriers have no function in the meristem. However, it is equally possible that one or more of the described *AUX1*-related genes (*LAX 1-3*) mask a defect in *AUX1* (Swarup *et al.*, 2000).

As an alternative to genetic approaches, specific inhibitors can be used to evaluate the function of auxin influx carriers. Such a pharmacological approach can be particularly useful when genetic approaches are complicated by redundancy. Recently, a systematic screen was carried out to identify compounds that interfere with the carrier-mediated 2,4-dichlorophenoxyacetic acid (2,4-D) uptake in suspension-cultured tobacco cells. One compound, naphthoxy-1-acetic acid (NOA) was shown to be a specific inhibitor of the auxin influx carrier with minimal effects on auxin efflux (Imhoff *et al.*, 2000). When tested on whole plants, the addition of NOA to the growth medium was sufficient to phenocopy the *aux1* mutant. Furthermore, NOA was not able to block polar auxin transport activity and did not exhibit auxin-like activity (Parry *et al.*, 2001). Thus, NOA represents a useful tool for physiological studies, addressing the role of auxin influx *in planta*.

Here, we focus on the distinct function of the influx carrier in leaf positioning and outgrowth. Leaf formation occurs at the shoot apical meristem. The meristem is divided into the central zone, where a group of slowly dividing stem cells is responsible for the self-maintenance of the meristem and the peripheral zone, where leaf primordia are formed (Bowman and Eshed, 2000; Clark, 2001). In tomato, leaves are arranged in a spiral with a divergence angle between successive leaves of approximately 140°.

To evaluate the function of the auxin influx carriers in leaf positioning, we used the influx carrier inhibitor NOA. In addition, the difference in transport properties of the auxins IAA, 2,4-D and NAA were exploited. We show that all three auxins can efficiently induce organogenesis, but that only the natural auxin IAA can induce formation of localized organs. These results indicate that the regulation of organ formation and phyllotaxis requires not only auxin efflux carriers, but also influx carriers.

Results

Localized organ initiation on NPA-pins requires the natural auxin IAA

The work of Reinhardt *et al.* (2000) left us with an important question. If NPA would inhibit all active transport, auxin would move through the meristem by diffusion. Taken into account the low molecular weight of IAA and the minute dimensions of the meristem, any auxin gradient established by local application of the compound would dissipate within minutes. Hence, one would have expected the entire peripheral zone to react to auxin by forming ring-shaped primorida. Since this was not the case, we infer the involvement of non-NPA-inhibitable active transport. To test this hypothesis, we made use of the different transport properties of the synthetic auxins NAA and 2,4-D. Due to its lipophilic nature, NAA can enter cells by diffusion, whereas 2,4-D and IAA uptake requires a predominant contribution of the influx carrier, especially at low external auxin concentrations (Delbarre *et al.*, 1996). If regulated auxin influx is needed for proper leaf initiation, NAA should not be able to substitute for the natural auxin IAA, when applied to NPA-pins, whereas the herbicide 2,4-D should induce leaf formation in a similar way as IAA. Shoot apices of tomato plants were dissected and cultivated on MS medium containing 10 μM NPA. This completely inhibited further formation of leaves. After 14 days of culture, a naked meristem on a pin-shaped stem lacking newly formed leaves was obtained (Figure 1a,b). The addition of a droplet of lanolin paste containing 1 mM IAA at the flank of the NPA-pin induced primordium formation (Figure 1c), whereas control treatments had no effect (Figure 1b), as was shown before (Reinhardt *et al.*, 2000). NPA-pins were treated with lanolin paste containing IAA in a concentration range between 0.1 and 10 mM. At first glance, these may seem artificially high concentrations. However, considering the microscopically small volume of the lanolin paste (estimated as <5% of the volume of the meristem) and considering retention of IAA in the lanolin paste, as well as oxidation and diffusion in the tissue, we can expect to cover the physiological concentration range (Ljung *et al.*, 2001; Uggla *et al.*, 1996). The rate of primordium formation increased with increasing concentrations of IAA (Figure 2a). In contrast to treatments with IAA, after addition of 1 mM NAA to a defined position at the periphery of the NPA-pin, the entire peripheral zone reacted by bulging, and a ring formed around the meristem (Figure 1d). Since the number of cells that are recruited into primordia is higher at high IAA concentrations (Reinhardt *et al.* 2000), we tested whether the induction of rings by NAA was concentration dependent and perhaps only observed at high concentrations. NAA was added to the periphery

of NPA-pins in a concentration range between 0.1 and 10 mM and the rate of primordium formation was compared to rates obtained with the same concentrations of IAA (Figure 2a,b; compare panels non-induced). With NAA, the induction of organogenesis was approximately as efficient as it was with IAA. In contrast, the localization of primordium formation was very different. The formation of primordia was predominantly at the site of application, when pins were treated with IAA (referred to as localized inductions) and only a few pins showed the formation of a ring (referred to as ring inductions). When NPA-pins were treated with NAA, inductions were predominantly ring-shaped (Figures 1d and 2b). A high number of ring inductions were observed already after addition of 0.3 mM NAA, whereas the addition of 10 mM IAA still resulted in localized primordium formation. With increasing IAA

concentrations, primordia were induced that were wider than normal (Reinhardt *et al.*, 2000), but they never surrounded the entire meristem. When the herbicide 2,4-D was locally applied to NPA-pins, primordium induction rates were comparable to results obtained with IAA (Figure 2a,c). Optimal primordium induction rates were obtained at concentrations between 0.3 and 1 mM 2,4-D, whereas 10 mM 2,4-D had toxic effects on NPA-pins (data not shown). However, localization of primordia was severely compromised. Primordia were often ring-shaped (Figures 1f and 2c) and strikingly, primordia also formed opposite or next to the place of auxin application, especially at low concentrations of 2,4-D (Figures 1e and 2c; mislocalized inductions). Ring-shaped primordia, induced by either NAA or 2,4-D, developed further into tubular leaves (Figure 1g,h).
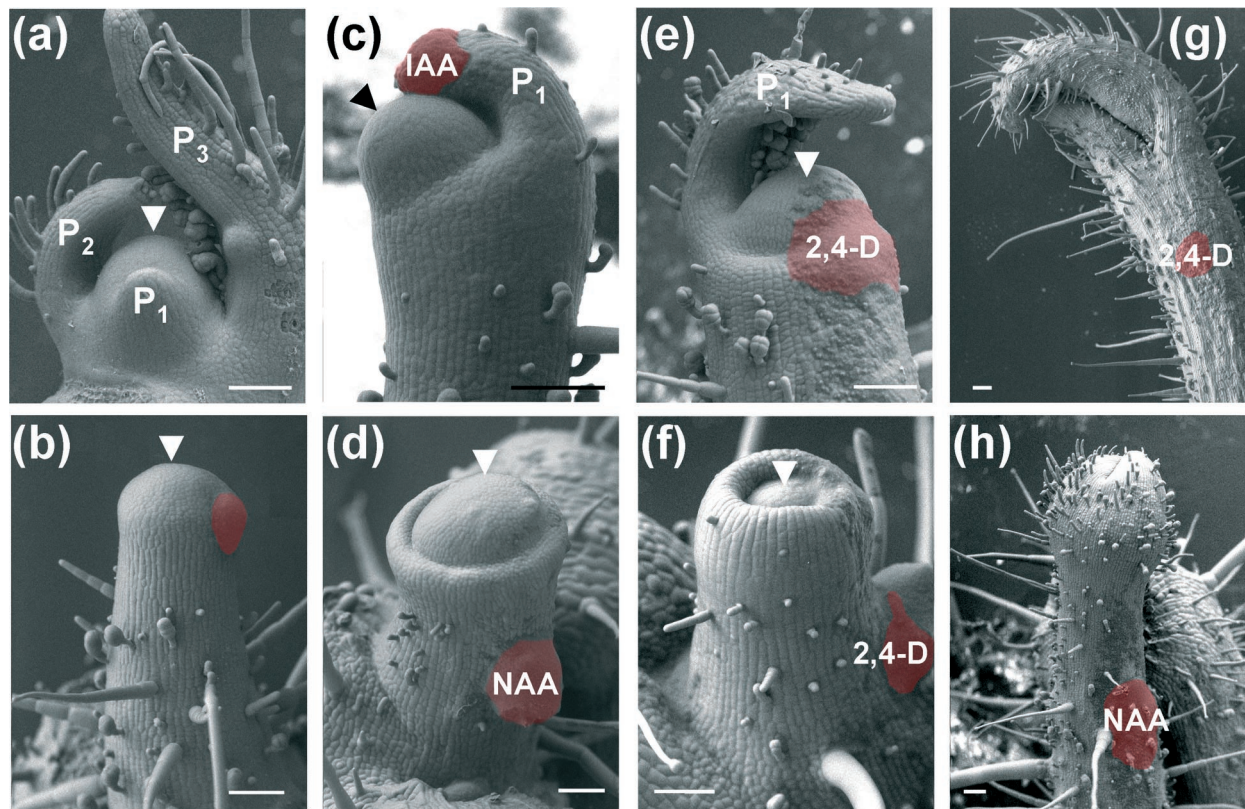

Figure 1. Inhibition of leaf primordium initiation by the auxin transport inhibitor NPA and induction of primordia by the natural auxin IAA and the synthetic auxins NAA and 2,4-D.
(a) Control tomato apex with the meristem (arrowhead) and the three youngest primordia (P_1–P_3 with P_1 being the youngest).
(b) NPA-pin treated with control paste.
(c) Induction of a leaf primordium (P_1) on an NPA-pin treated with lanolin paste containing 1 mM IAA.
(d) Ring-shaped induction on an NPA-pin treated with lanolin paste containing 1 mM NAA.
(e) Induction of a leaf primordium on an NPA-pin opposite to the treatment with 0.3 mM 2,4-D ('*mislocalized*').
(f) Ring-shaped induction on an NPA-pin treated with lanolin paste containing 0.3 mM 2,4-D.
(g) Ring-shaped induction on an NPA-pin treated with lanoline paste containing 1 mM 2,4-D. Inductions grow as tubular leaves that split up at the tip and occasionally form leaflets.
(h) Ring shaped induction on an NPA-pin treated with lanoline paste containing 0.3 mM NAA. Inductions grow as tubular leaves that split up at the tip and occasionally form leaflets.
Pictures were taken 5 (a–f) and 8 days (g–h) after the local auxin treatments. Arrowhead, center of the meristem; red spots, lanoline–paraffin paste; bars = 100 μM.
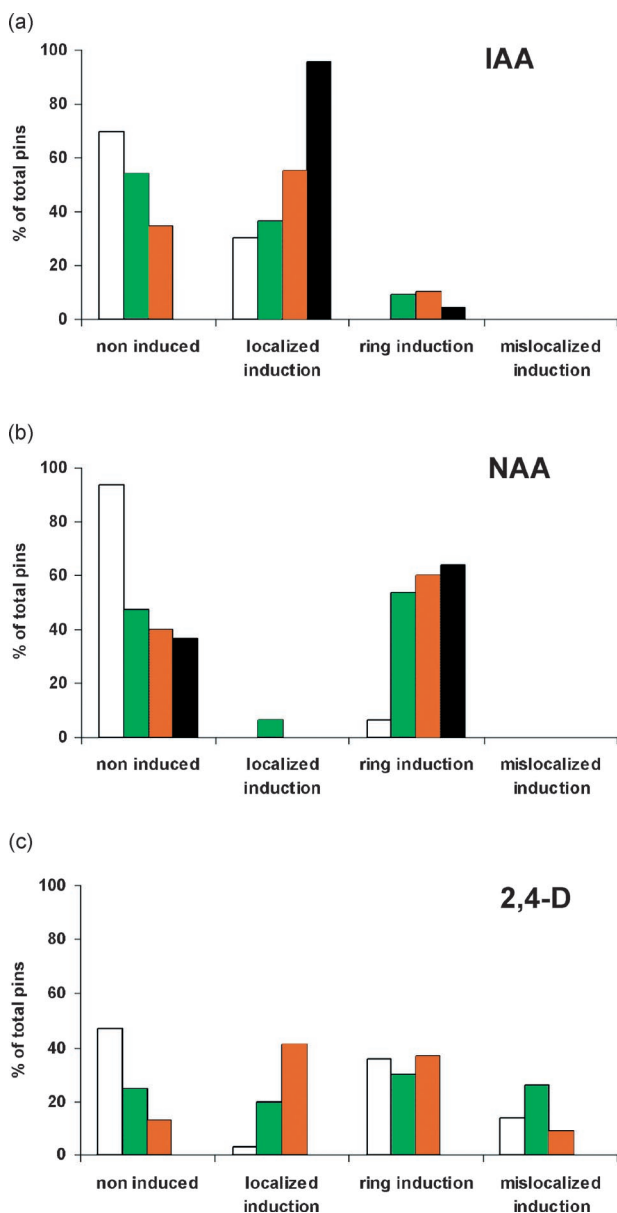
(a)

(b)

(c)

Figure 2. Induction of primordium formation by different concentrations of IAA, NAA and 2,4-D. Apices were cultured on 10 μM NPA for 14 days. Then NPA-pins were treated with different concentrations of IAA (a), NAA (b) or 2,4-D (c) at the peripheral zone and rates of non-induced meristems, localized, ring-shaped or mislocalized inductions of primordia were determined 5 days after the local treatments (White = 0.1 mM, green = 0.3 mM, red = 1 mM, black = 10 mM of the auxin indicated). Values are calculated as percentage of pins per experiment. $n = 23$ (0.1 mM IAA), 33 (0.3 mM IAA), 93 (1 mM IAA), 22 (10 mM IAA), 31 (0.1 mM NAA), 47 (0.3 mM NAA), 30 (1 mM NAA), 22 (10 mM NAA), 36 (0.1 mM 2,4-D), 73 (0.3 mM 2,4-D), 46 (1 mM 2,4-D).

In summary, the three auxins tested induced leaf organogenesis at comparable rates. The localization of organ formation, however, was lost when NAA was applied to NPA-pins and was variable when 2,4-D was used as the external auxin source.

Inhibition of auxin influx in the shoot apex affects leaf initiation and positioning

The results obtained so far (Figure 1; Reinhardt *et al.*, 2000) suggest a role for the influx carrier in regulating auxin distribution in the meristem for proper leaf positioning. If this were correct, it would be expected that inhibition of auxin influx in the shoot apex would affect leaf positioning. We used the *in vitro* culture system to determine the influence of the specific influx inhibitor NOA on primordium formation. Tomato shoot apices were dissected and grown in culture as described before (Fleming *et al.*, 1997; Reinhardt *et al.*, 2000), and primordium formation was observed during 14 days of culture in the presence of different concentrations of NOA (Table 1). In control plants, five primordia had formed after a period of 14 days. Primordia initiated at the periphery of the meristem and approximately one-sixth of the peripheral zone was integrated into an initiating primordium (Figure 3a,b). In the presence of NOA, leaf primordia were not only initiated more slowly, but they also developed as much wider structures and often surrounded more than half of the meristem (Figure 3c–e). The size of the meristem decreased (Table 1), whereas the primordia were larger than in control apices. Furthermore, the gap between successive leaves was smaller in the presence of NOA, and primordia often developed next to each other (Figure 3c) or were initiated simultaneously (Figure 3e). Primordia expanded, but unlike control plants, no leaflets formed (Figure 3f,h). These observations were clearly evident at 10 μM NOA and became more extreme at 50 μM NOA. Thus, whereas inhibition of auxin efflux with NPA caused the cessation of lateral organ formation, inhibition of auxin influx led to the formation of organs that occupied a larger segment of the meristem.

Localized organ induction on NPA-pins is perturbed by NOA

The results obtained above suggest that the auxin influx carrier has regulatory functions, primarily in localizing primordium formation. To control the source of auxin and to exclude auxin diffusion from primordia into the meristem, NPA-pins lacking primordia were used to further characterize the function of the influx carrier in leaf positioning. If the influx carrier is relevant for the distribution of auxin in the meristem, treatments of NPA-pins with IAA should not result in localized primordium formation when influx is inhibited with NOA. Tomato meristems grown on NPA-containing medium for 14 days were transferred to medium containing 10 μM NPA and 10 μM NOA to block the activities of both auxin influx and efflux carriers and were incubated for another 10 days. The presence of both carrier inhibitors in the culture medium had no influence on the shape of the pins ($n = 35$). As an additional control, NPA-pins were

Table 1 Primordium formation of tomato apices cultured on NOA

NOA in medium	Number of primordia			Meristem after 14 days (µM)
	3 days	7 days	14 days	
0 µM	2.0 ± 0.65	4.0 ± 0.0	5.0 ± 0.47	198 ± 10.6
10 µM	1.0 ± 0.38	2.5 ± 0.71	3.6 ± 0.52	not done
50 µM	1.35 ± 0.49	1.85 ± 0.67	2.6 ± 0.5	156 ± 24.0

Dissected tomato apices consisting of the meristem and the youngest forming primordium were cultured on MS medium with different concentrations of NOA and primordia were counted at the indicated timepoints. Meristem size was measured after 14 days of culture. $n = 10$ (0 µM NOA, 10 µM NOA), 20 (50 µM NOA) (Values $\pm$ SD). All values are significantly different from the values obtained at 0 µM NOA (t-test).

Figure 3. Primordium formation on tomato apices cultured in the presence of the auxin influx carrier inhibitor NOA. Tomato apices were grown for 14 days on 50 µM NOA and the shape of newly formed primordia was visualized by low-vacuum scanning electron microscopy.

(a) Side view and (b) top view of control apices cultured on medium for 3 days.

(c) Side view and (d), (e) top view of apices cultured for 14 days on medium containing 50 µM NOA. Primordia are enlarged and the size of the meristem is decreased.

(f) Leaf of control plant with leaflet (arrow).

(g) Schematic representation of a tomato apex with the youngest and the second youngest primordia (P$_1$ and P$_2$, respectively). The sites of incipient primordium formation (I$_1$) and the site of the following primordium (I$_2$) are indicated as yellow areas.

(h) Inhibition of leaflet formation (arrow) in the presence of 50 µM NOA.

Arrowhead, center of the meristem; I$_1$ and I$_2$, newly formed primordia with I$_2$ being the younger primordium; bars = 100 µM. In (b) and (d) I$_1$ was excised prior to visualization.
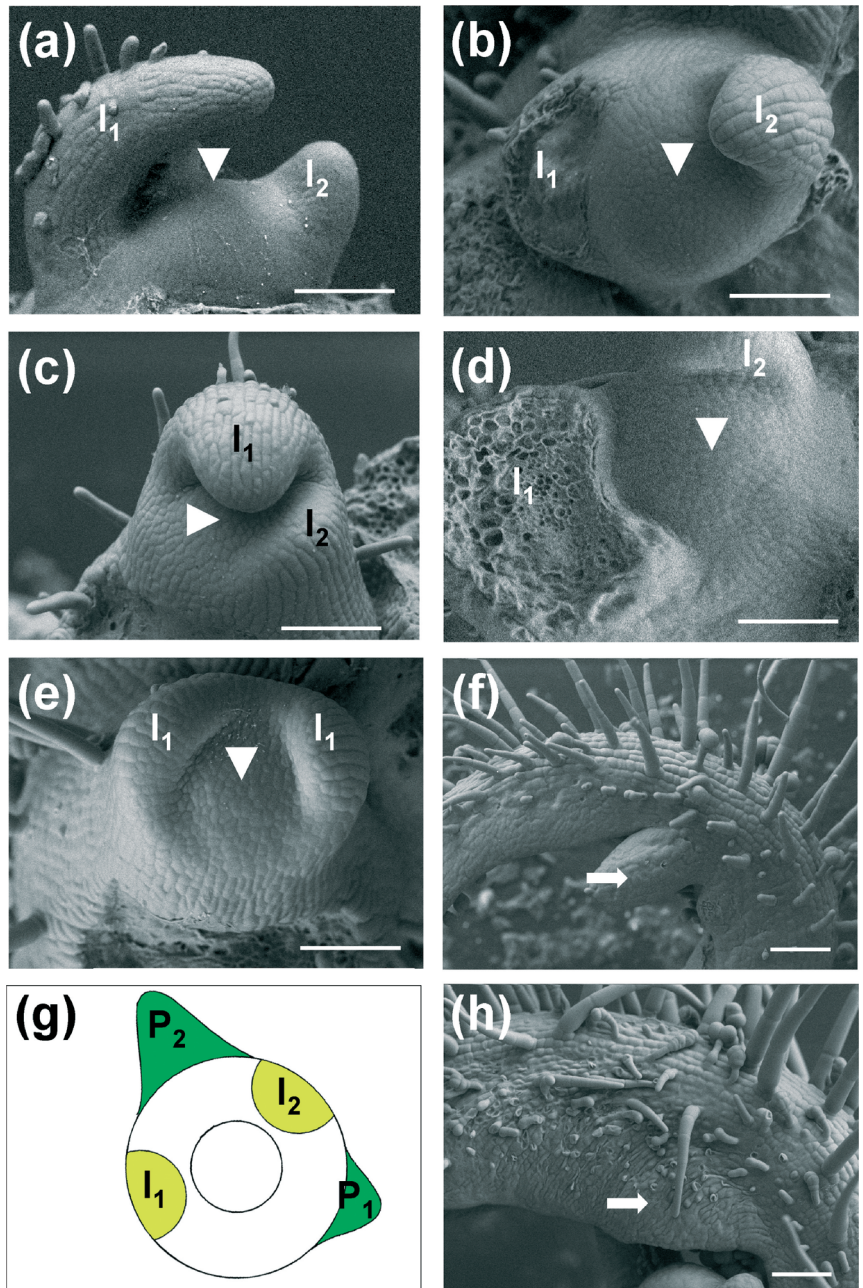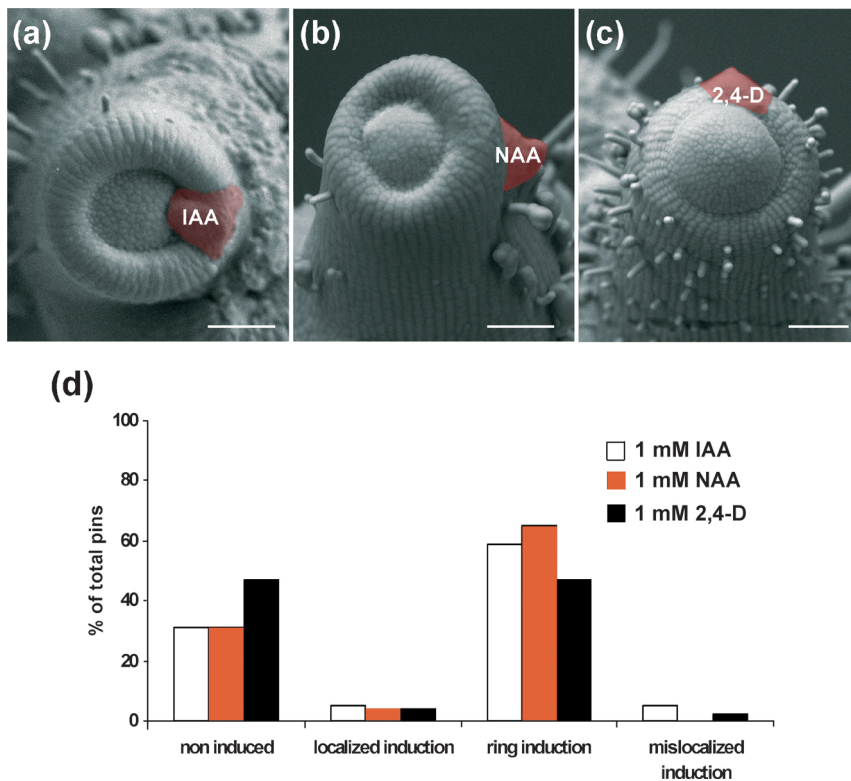
Figure 4. Primordium formation on NPA-pins treated with auxin when auxin influx carriers are bypassed.

Apices were cultured on 10 μM NPA for 14 days, then transferred to medium containing 10 μM NPA + 10 μM NOA and after 2 days, the flank of the meristem was treated with 1 mM IAA (a, white in d), 1 mM NAA (b, red in d) or 1 mM 2,4-D (c, black in d). Rates of localized, ring-shaped or mislocalized induction of primordia were determined 5 days after the auxin treatments. The pins were visualized by low-vacuum scanning electron microscopy and the lanolin–paraffin paste was colored in red for clarity. Values are calculated as percentage of the total amount of pins per experiment. $n = 22$ (1 mM IAA), 26 (1 mM NAA) and 40 (1 mM 2,4-D).

treated locally with lanolin paste containing 0.1 or 1 mM NOA ($n = 25$). None of these treatments had any effect on NPA-pins, indicating that NOA had no auxin-like effects at the concentrations used (data not shown). In contrast, when NPA-pins grown on medium containing 10 μM NPA + 10 μM NOA were treated with 1 mM IAA in lanolin paste, leaf formation was induced. Organogenesis was efficiently induced, irrespective of whether NOA was present in the incubation medium or not (compare Figure 2a with Figure 4d). However, organ formation was not restricted to the site of IAA application. From the NPA-pins that reacted, 85% formed a localized primordium in the absence of NOA (Figure 2a), whereas in the presence of NOA, 93% were ring inductions (Figure 4a,d). The ring inductions obtained in the presence of NOA were very similar to ring inductions obtained after treatment of NPA-pins with NAA (Figures 1d and 4a). Only in one case out of 22, a primordium formed locally and in one case the induction of a primordium was observed away from the place of the treatment. NAA induced ring-shaped organs in the presence as well as in the absence of NOA (compare Figure 2b with Figure 4d). When 2,4-D was added to the pins, the rate of organogenesis was reduced by 34% in the presence of NOA (compare Figure 2c with Figure 4d). However, developing primordia were predominantly ring shaped in the presence of NOA (Figure 4c,d). Thus, primordium formation was ring shaped when the influx carrier was inactivated regardless of the auxin applied externally, whereas

ring-shaped primordia were obtained particularly with NAA (which requires no influx carrier) when NOA was absent. Consequently, localized primordium formation requires regulated auxin import.

Discussion

The value of pharmacological approaches

The shoot meristem is the source of all aerial postembryonic structures, and most mutations affecting it are highly pleiotropic, leading to severely aberrant structures already at the embryo stage (Reinhardt and Kuhlemeier, 2001). This complicates the interpretation of phyllotactic phenotypes by genetic approaches, as phyllotaxis is, by definition, a postembryonic reiterative phenomenon. The basis of the experiments described here is a robust *in vitro* culture system in which tomato shoot apices grow at a normal rate and form normal tissues and organs. This culture system enabled us to apply various effectors and study their influence on leaf initiation and positioning (Fleming *et al.*, 1997; Fleming *et al.*, 1999; Reinhardt *et al.*, 2000). Such a pharmacological approach allows the application of effectors in a manner that is precisely restricted in time and space, and thus the starting point is a perfectly developed mature shoot tip. Needless to say, application experiments are not without drawbacks. In particular, they should be scrutinized for specificity and biological relevance of the

added compounds. Fortunately, the action and specificity of the auxin transport inhibitors used in this study, NPA and NOA, has recently been documented in a series of carefully executed studies (Delbarre *et al.*, 1996; Imhoff *et al.*, 2000; Lomax *et al.*, 1995; Parry *et al.*, 2001).

Correct leaf positioning requires active efflux and influx of auxin

Application of auxin polar transport inhibitors completely inhibited leaf initiation with no discernible effect on stem growth and meristem maintenance (Figure 1a; Reinhardt *et al.*, 2000). Local application of IAA, but not of non-auxin effectors, could restore organ formation. In addition, the size of the newly formed primordia was dependent on the concentration of IAA added to the periphery of the meristem. These experiments point to a role of auxin both in the initiation and the positioning of lateral organs.

The most striking observation in the present work is the effect of synthetic auxins after local application to the flanks of NPA-pins. Both NAA and 2,4-D induced leaf primordia with a frequency comparable to that observed with the naturally occurring auxin IAA, indicating that they were competent inducers of organogenesis. However, in contrast to IAA, the positioning of the primordium and the delineation of its boundaries were severely compromised.

Although NAA and 2,4-D are both potent auxins, they differ in their physical and chemical properties. In particular, NAA was shown to enter into cells by diffusion without need for the import carrier (Delbarre *et al.*, 1996). Thus, it was conceivable that the different effects of IAA and NAA are related to their different uptake properties and, consequently, to their different distribution within the meristem. Support for this notion came from experiments in which IAA was applied in the presence of NOA, an inhibitor of the import carrier (Figure 4). In the presence of NOA, IAA mimicked the action of NAA. This is a strong evidence that indeed aberrant localization is caused by a defect in auxin import and that the import carrier is required to restrict organogenesis in the circumferential dimension and to establish primordium boundaries.

High concentrations of 2,4-D to NPA-pins could elicit localized induction, but this effect was negated by NOA (compare Figure 2c with Figure 4d). This implies that with 2,4-D too, the import carrier is involved in proper localization and delineation of the primoridum. However, it must be noted that the effects of 2,4-D are more complex and cannot be explained exclusively by the differences in transport properties. In particular, the mislocalization of primordia may be caused by the lack of turnover of 2,4-D, which can cause considerable accumulation within cells. How this leads to mislocalization remains a matter of speculation.

When NPA-treated meristems were treated with IAA, the size of the primordia was correlated with the concentration of the applied auxin (Reinhardt *et al.*, 2000). A similar correlation may hold for NOA-treated meristems (Figure 3). We suggest that in the absence of active influx, a shallower auxin gradient is established, leading to more cells being exposed to elevated auxin and thereby being recruited into new primordia.

A model for the regulation of phyllotaxis by auxin gradients

In numerous models, the control of phyllotaxis is based on gradients of inhibiting substances emanating from the existing primordia (Schwabe, 1984; Steeves and Sussex, 1989). Our work implicates auxin as a regulator of phyllotaxis, and it is appropriate to speculate about auxin concentration gradients as part of the signaling mechanism.

The indirect evidence outlined above suggests a simple model for the role of auxin in organogenesis (Figure 5a). The first part of the model posits that auxin is not synthesized in the meristem, but is imported from more mature tissues via an acropetal, NPA-inhibitable, transport pathway. We deduce this from the observation that organ formation ceases after the inhibition of auxin transport with NPA and is reversible by the addition of auxin to the meristem (Figure 5b). In contrast, if the meristem were a source of auxin, NPA would cause auxin accumulation within the meristem, which we would expect to lead to the formation of large or ring-shaped primordia.

The second part of the model posits that once auxin has reached the meristem, it is redistributed within the meristem by active transport. We propose that the youngest pre-existing primordia P_2 and P_1 are not yet synthesizing auxin and act as sinks for auxin, thereby creating a local auxin maximum at I_1, the site of incipient primordium formation (Reinhardt and Kuhlemeier, 2001). Thus, rather than an inhibitor emanating from the primordia, auxin is proposed to be a positive effector moving in the opposite direction.

While the efflux carrier may well play a role in the redistribution of auxin in the meristem, five lines of reasoning point to a function of the influx carrier in organ positioning and delineation. (i) Application of IAA to NPA-pins led to localized primordia (Reinhardt *et al.*, 2000). Since auxin would equilibrate within minutes over the short distances of the meristem through simple diffusion, it follows that NPA pins are still capable of carrying out active transport, presumably by a non-NPA-inhibitable carrier. (ii) When tomato apices were cultured on NOA, the newly initiated primordia grew as wider structures. This observation is compatible with the presence of a shallower auxin gradient within the meristem (Figure 5c). (iii) When NOA was applied to NPA-pins, local application of IAA did not cause localized primordia to form but instead led to the induction of ring-shaped organs encompassing the entire peripheral zone
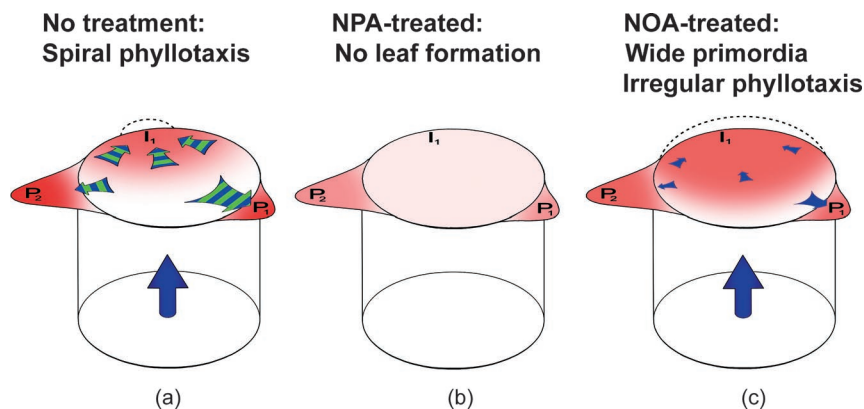
No treatment:
Spiral phyllotaxis

NPA-treated:
No leaf formation

NOA-treated:
Wide primordia
Irregular phyllotaxis

(a)   (b)   (c)

Figure 5. Model for the regulation of phyllotaxis by auxin gradients. Shown are representations of a meristem decapitated at the site of leaf initiation.
(a) Auxin (red) reaches the meristem via acropetal transport. P_1 and P_2 act as sinks for auxin, leading to redistribution and to the establishment of an auxin gradient with a maximum at I_1. Acropetal auxin transport into the meristem is controlled by the efflux carrier (blue arrow), whereas redistribution of auxin within the meristem involves both, the influx (green) and the efflux carrier (blue).
(b) Inhibition of the efflux carrier by NPA stops the acropetal auxin transport as well as auxin export from the meristem to the primordia leading to decreased auxin contents and the loss of an auxin gradient in the meristem. Therefore, primordium formation ceases.
(c) Inhibition of the influx carrier by NOA reduces directed auxin transport resulting in a shallower auxin gradient and therefore, primordia develop as wider structures.
Red, auxin; blue, auxin transport controlled by the efflux carrier; green, auxin transport controlled by the influx carrier; I_1, site of incipient primordium formation; P_1 and P_2, pre-existing primordia with P_1 being the younger; dashed lines, shape of the future primordium at I_1.

(Figure 4a). (iv) This effect of NOA could be mimicked by using the influx carrier-independent synthetic auxin NAA rather than IAA. (v) Organ induction on NPA-pins by localized application of 2,4-D became largely ring-shaped when NOA was also present in the medium.

In the *Arabidopsis aux1* mutant, which is defective in auxin import, gravitropism was restored by immersing the root in NAA (Marchant *et al.*, 1999). This was taken as evidence that the auxin importer facilitates auxin uptake. This experiment shows that the efflux carrier can establish a functional auxin gradient in the absence of *aux1*, but in our view it does not exclude an auxiliary instructive role for the influx carrier. Similarly, in the shoot apical meristem, efflux and influx carriers may co-operate in setting up correctly spaced and dynamically changing auxin gradients.

The model of Figure 5 is simple, compatible with the data, and provides a useful aid to direct further experiments. We emphasize that it is largely based on indirect evidence. The elaboration of molecular models for auxin action in the shoot meristem is complicated by the minute size of the meristem and the resulting lack of information on key aspects of auxin physiology in this organ. Most importantly, it is not known how and where in the shoot tip auxin is synthesized and how it is redistributed. Technical advances in GC/MS instrumentation will make it possible to measure auxin concentrations and rates of auxin synthesis in minute pieces of tissue (Ljung *et al.*, 2001). Furthermore, the localization of the auxin influx and efflux carriers within the meristem can be determined (Gälweiler *et al.*, 1998; Müller *et al.*, 1998; Swarup *et al.*, 2001). Finally, localized misexpression of genes involved in auxin

synthesis, transport, perception or action should lead to a deregulation of leaf positioning.

Experimental procedures

Plant growth and *in vitro culture*

Tomato plants (*Lycopersicon esculentum* cv Moneymaker) were grown on soil (23°C at 60% humidity, 16 h light, 8 h dark) until the fifth to seventh plastochron stage was reached. Shoot apices were dissected and cultured according to Fleming *et al.* (1997). The auxin transport inhibitor NPA (Interchim, Montluçon, France) was added to the medium from a 100-mM stock solution in DMSO to a final concentration of 10 μM. Dissected apices were cultured for 14 days on 10 μM NPA in order to obtain pin-shaped meristems. Apices were trimmed at the bottom and transferred to fresh medium every 3 days. Where indicated, NOA (Imhoff *et al.*, 2000), kindly provided by A. Delbarre, was added to the medium from a stock solution of 100 mM in DMSO.

Treatments of plant apices

For local treatments of apices, IAA (Fluka, Buchs, Switzerland), NAA (Sigma-Aldrich, Switzerland), 2,4-D (Sigma-Aldrich, Switzerland) 100 mM stock solutions in DMSO were dissolved in a lanolin–paraffin paste and the paste was applied locally on apices (Reinhardt *et al.*, 2000). After treatment, apices were further cultured on the auxin transport inhibitors indicated, for 5 days, and the formation of primordia was evaluated by observation with a binocular microscope (model SMZ-U; Nikon, Japan). Apices were viewed with a variable-pressure scanning electron microscope (model S-3500 N; Hitachi, Tokyo, Japan). The lanolin–paraffin paste was colored red in digital images for clarity. In control experiments, to test for effects of the lanolin–paraffin paste on

leaf induction, 25 NPA-pins were treated with paste, where only DMSO was dissolved. A slight bulging of the meristem at the site of application was observed only in one case.

Acknowledgements

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