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Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth

Karin Ljung, Rishikesh P. Bhalerao and Göran Sandberg*

Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, The Swedish University of Agricultural Sciences, S-901 83 Umeå, Sweden

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*For correspondence (Fax: +46 90 786 5901; e-mail: Goran.Sandberg@genfys.slu.se).

Summary

The distribution and biosynthesis of indole-3-acetic acid (IAA) was investigated during early plant development in *Arabidopsis*. The youngest leaves analysed, less than 0.5 mm in length, contained 250 pg mg^{-1} of IAA and also exhibited the highest relative capacity to synthesize this hormone. A decrease of nearly one hundred-fold in IAA content occurred as the young leaves expanded to their full size, and this was accompanied by a clear shift in both pool size and IAA synthesis capacity. The correlation between high IAA content and intense cell division was further verified in tobacco leaves, where a detailed analysis revealed that dividing mesophyll tissue contained ten-fold higher IAA levels than tissue growing solely by elongation. We demonstrated that all parts of the young *Arabidopsis* plant can potentially contribute to the auxin needed for growth and development, as not only young leaves, but also all other parts of the plant such as cotyledons, expanding leaves and root tissues have the capacity to synthesize IAA *de novo*. We also observed that naphthylphthalamic acid (NPA) treatment induced tissue-dependent feedback inhibition of IAA biosynthesis in expanding leaves and cotyledons, but intriguingly not in young leaves or in the root system. This observation supports the hypothesis that there is a sophisticated tissue-specific regulatory mechanism for auxin biosynthesis. Finally, a strict requirement for maintaining the pool sizes of IAA was revealed as reductions in leaf expansion followed both decreases and increases in the IAA levels in developing leaves. This indicates that leaves are not only important sources for IAA synthesis, but that normal leaf expansion depends on rigorous control of IAA homeostasis.

Keywords: indole-3-acetic acid, auxin, distribution and biosynthesis, feedback inhibition, naphthylphthalamic acid, leaf expansion.

Introduction

During the initial growth phase, a seedling clearly needs to rapidly develop photosynthetic capacity in the leaves and a root system that can provide the organism with water and nutrients. Equally importantly, it must also develop a vascular system to connect the shoot and root tissues. Both intrinsic and extrinsic signals serve as developmental regulators that allow the plant to co-ordinate this developmental sequence. One of the most important intrinsic developmental signals is auxin, indole-3-acetic acid (IAA), which is believed to affect many developmental processes, including cell division, cell expansion and the development of vascular tissue and roots. Much remains unknown about the mechanisms of auxin action, as well as the sites of auxin synthesis and its distribution within the plant. However, recent progress in analytical technology, allowing quantitative analysis of IAA in sub-milligram amounts of plant tissue by mass spectrometry (Edlund *et al.*, 1995; Ribnicky *et al.*, 1998) has opened up new possibilities to analyse in detail the pool sizes and IAA synthesis capacity of specific tissues (Barlier *et al.*, 2000; Casmiro *et al.*, 2001; Gray *et al.*, 1998). IAA contents have previously been analysed in *Arabidopsis*, but seldom in a truly tissue specific manner, which is essential to extend our knowledge about the function of this hormone.

In the study reported here we analysed the temporal and spatial distribution of IAA during early plant development and during vegetative growth. We have also investigated the sites of IAA biosynthesis in young *Arabidopsis* plants,

and found evidence of an IAA biosynthesis feedback-inhibition mechanism that is induced in specific tissues and developmental stages. This, together with IAA transport, conjugation and catabolism, is likely to be an essential part of the homeostatic mechanism that controls free IAA levels in the plant. We have also performed experiments to elucidate whether the changes in IAA concentration that occur during leaf development may be part of a mechanism controlling the transition from cell division to cell expansion, and if expansion growth depends on IAA concentrations being maintained within strict limits.

The data gathered may help to assess whether correlations observed *in vitro* between IAA concentrations and specific developmental processes such as cell division and cell elongation also hold true for *in vivo* conditions. To address these issues we have induced feedback inhibition of IAA biosynthesis to lower the IAA-content in expanding leaves, and used the auxin overproducing mutants *sur1* and *sur2* to study the effect of increased IAA content in expanding leaves. The results obtained provide evidence that strict developmental control of auxin homeostasis is important for normal leaf development.

Results

High IAA levels are strongly correlated with high rates of cell division in Arabidopsis *leaves*

Developing leaves provide an interesting *in vivo* system for investigating cell division/cell expansion, growth and the role of auxin in these processes. We have investigated the IAA concentration in leaves of different sizes during vegetative growth, both under short (SD) (Figure 1a–c) and long day (LD) conditions (Figure 1d). Plants grown under SD conditions were selected for analysis when they had developed 7, 12 or 20 leaves, and plants grown under LD conditions when they had developed eight leaves. We observed an inverse correlation between leaf size and IAA concentration that was independent of growth conditions and developmental stage. When pooled quantifications for SD-grown plants were plotted against leaf weight (Figure 1e), a clear picture emerged of IAA concentrations being extremely high in leaves less than 1 mg in weight, but dramatically dropping when cell division ceased and cell elongation was the sole process causing leaf growth. We also investigated the IAA content of other tissues of young

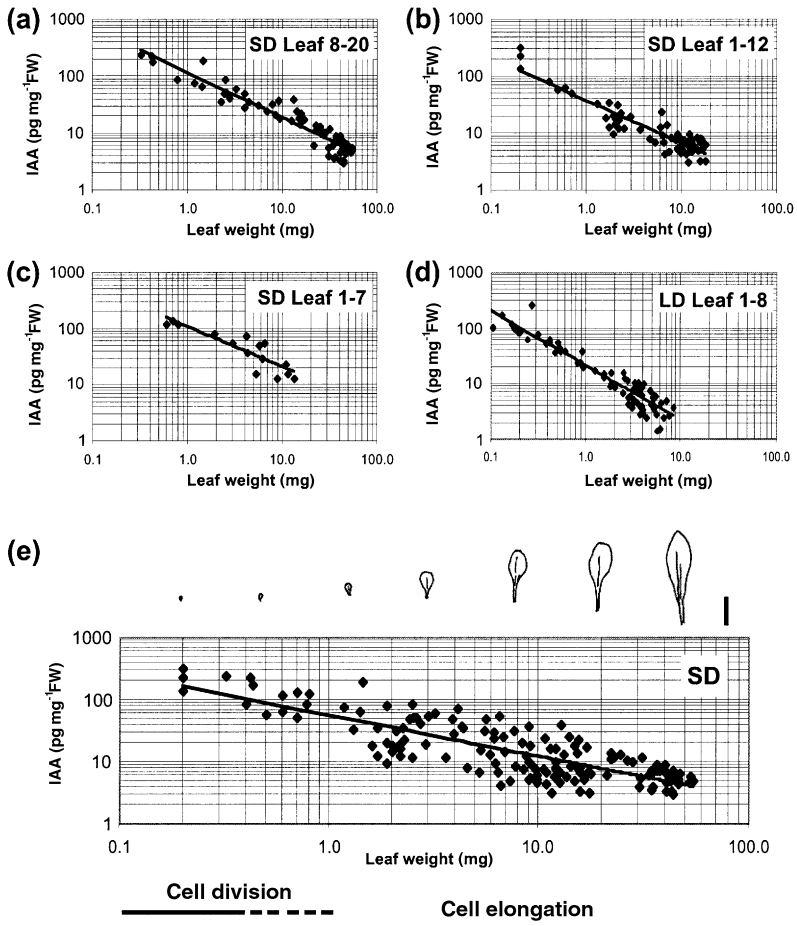
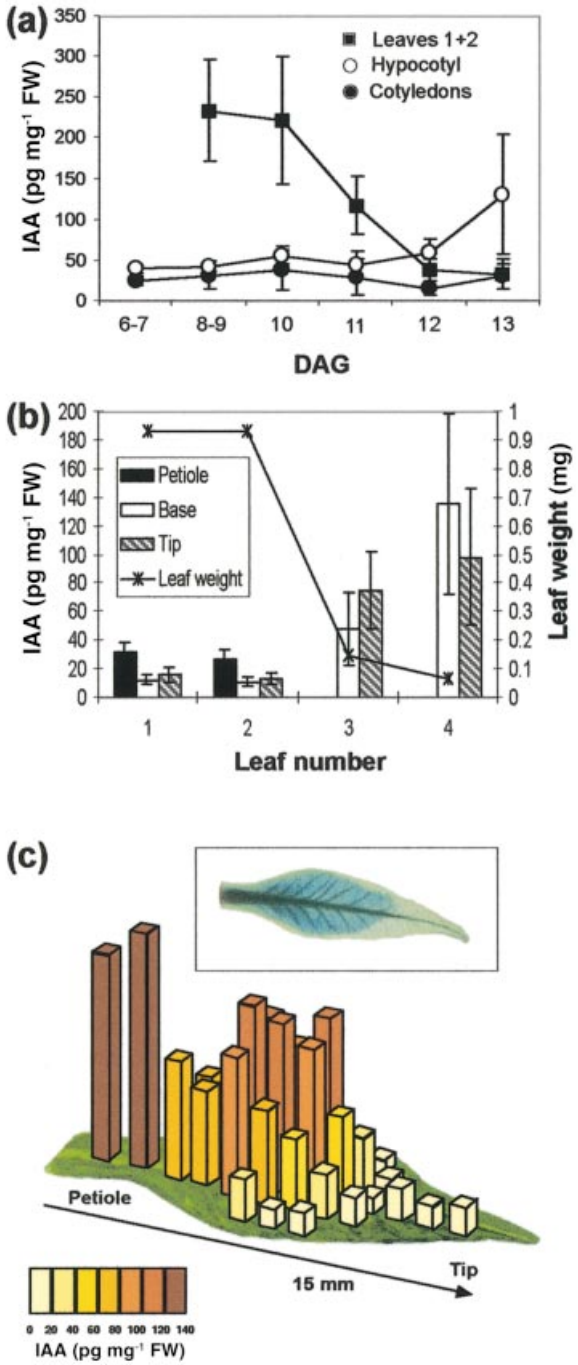


Figure 1. IAA levels in *Arabidopsis* leaves. The IAA concentration was measured in (a) leaves 8–20 from six plants grown for six weeks under SD (b) leaves 1–12 from six plants grown for 4.5 weeks under SD (c) leaves 1–7 from three plants grown for 3 weeks under SD and (d) leaves 1–8 from 10 plants grown for 16 days under LD. (e) Pooled IAA data for all the leaves grown under SD. The data are presented as log log^{-1} plots of the IAA concentration in individual leaves versus leaf weight. Bar in (e) = 1 cm.

developing *Arabidopsis* seedlings. The plants were divided into cotyledons, the first developing leaf pair and the hypocotyl. As can be seen in Figure 2(a) the IAA levels in the first developing true leaves are consistent with those found in the previous study: starting high and falling by approximately 90% when leaf expansion was initiated. In the cotyledons, the levels remained much lower, and constant, throughout the entire study period. The hypocotyls initially contained low amounts of IAA, which started to increase 12–13 Days After Germination (DAG), probably indicating that the leaves and hypocotyl at this stage are achieving full transport capacity for IAA. This experiment was repeated under LD conditions, and exactly the same correlations were found between IAA levels, tissue types and developmental stages, again demonstrating highly reproducible relationships between developmental stages of the tissues and IAA contents (data not shown).

Cell division is intense in developing leaf primordia and in all parts of a very young *Arabidopsis* leaf. When the leaf reaches a length of 1–2 mm, cell division ceases, first at the apex, then progressively further down the leaf. Division then gradually becomes restricted to the basal part of the leaf blade, before ceasing completely (Donnelly *et al.*, 1999; Pyke *et al.*, 1991; Van Lijsebettens and Clarke, 1998). After cell division is terminated, leaf growth is restricted to expansion growth. Cell division has also been shown to cease in a basipetal fashion in leaves of both sunflower and tobacco (Granier and Tardieu, 1998; Poethig and Sussex, 1985). To investigate if the basipetal cessation of cell division is mirrored by a similar reduction in IAA content, the first four developing leaves of 10-day-old *Arabidopsis* plants were divided into base and tip, at the middle of the leaf blade. The petioles were also harvested, and significantly higher IAA levels were always observed in them than in the leaf blades (Figure 2b, leaves 1 and 2), indicating that accumulation and active IAA transport occurs in this tissue. No significant differences in IAA concentrations were detected between leaf base and leaf tip. However, this experiment was too technically demanding to perform on *Arabidopsis* leaves smaller than 1–2 mm in length. The technical difficulties are also reflected in the large standard deviations observed in the results of these experiments (Figure 2b, leaves 3 and 4).



High IAA levels correlate spatially with tobacco leaf areas exhibiting high rates of cell division

To test whether high IAA levels are correlated with high rates of cell division in leaves we analysed tobacco rather

Figure 2. IAA levels in developing wild type *Arabidopsis* seedlings and in a developing tobacco leaf.
(a) IAA concentration in leaves 1 + 2 (the first true leaves, not including the shoot apex), hypocotyl (cut under the cotyledon/hypocotyl junction) and cotyledons of *Arabidopsis* seedlings grown under SD. Tissues from five to 10 seedlings were pooled for each sample, and three to six samples were analysed for each data point.
(b) Leaf weight and IAA concentration in the tip, base and petiole of leaves from 10-day-old *Arabidopsis* seedlings grown under LD. Tissues from five seedlings were pooled for each sample, and five samples were analysed for each data point. FW, fresh weight; error bars indicate standard deviations.
(c) IAA concentration in 2 mm tissue discs cut from a 1.5-cm long tobacco leaf. Mesophyll tissue was sampled except for the petioles, where the samples also contained vascular tissue. GUS staining of a tobacco leaf carrying the CYC1–1::GUS construct.

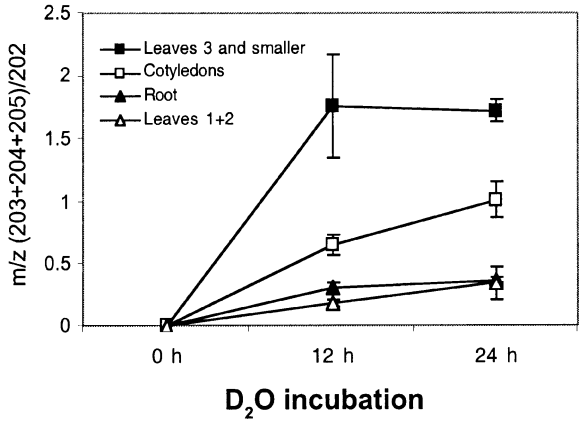


Figure 3. IAA biosynthesis in different plant organs excised from developing wild type *Arabidopsis* seedlings.
Ten-day-old seedlings grown under LD were divided into leaves 3 and smaller (black squares), cotyledons (white squares), roots (black triangles) and leaves 1 + 2 (white triangles) prior to incubation in media containing 30% deuterated water. The incorporation of deuterium into IAA was measured by GC-HR-MS. Each sample contained pooled plant material from 10 seedlings. Samples were measured in triplicates, and corrections were made for background and natural isotope abundances. Error bars indicate standard deviations.

than *Arabidopsis* leaves, to avoid having to dissect out parts of very small leaves, and to enable us to separate areas of cell division and cell expansion in single leaves. Based on the staining patterns of leaves carrying cell cycle markers fused to GUS (CYC1–1::GUS and CDC2–6::GUS), we were able to select leaves for IAA analysis that were in transition from division to elongation growth, with intense cell division occurring at the middle and base of the leaf blade (Figure 2c). From these leaves we sampled 2 mm discs of mesophyll tissue, allowing truly high-resolution analysis of IAA levels. We observed that the central and basal parts of 1.5 cm long tobacco leaves contained high levels of IAA (Figure 2c) compared to the tip and margins of the leaf blade. The highest IAA levels were found in the petiole, supporting our earlier observations in *Arabidopsis* (Figure 2b). Our results show that high levels of IAA in the mesophyll are strictly correlated to zones with intense cell division. In tobacco leaves of this specific size, the relative growth rate of different transverse sections of the leaf is constant (Poethig and Sussex, 1985), indicating that cell expansion is more or less uniform throughout the leaf blade. During the rapid growth phase that follows, cell expansion progresses basipetally, starting at the tip of the leaf. Later in development the relative growth rate of the base of the leaf becomes higher than closer to the tip, so the basal part of the leaf expands more rapidly, thus forming the characteristic tobacco leaf shape. It is noteworthy that the very high levels of IAA that we observe very early in leaf development in both *Arabidopsis* and tobacco are temporally correlated with the onset of vascular differentiation in the developing leaves, as well as with stages of leaf development in which rates of cell division are high.

The youngest leaves have the highest biosynthesis capacity for IAA, but all parts of a young Arabidopsis *seedling can synthesize IAA*

There are several indications that the seedlings of different plant species rely on stored forms of IAA conjugates as the main sources of free IAA during the first days of germination, and that *de novo* synthesis is initiated later during seedling growth (Bialek *et al.*, 1992; Epstein *et al.*, 1980; Ljung *et al.*, 2001). Considerable progress has been made in recent years towards increasing our understanding of IAA biosynthesis (Bandurski *et al.*, 1995; Bartel, 1997; Normanly *et al.*, 1993; Ouyang *et al.*, 2000). There is evidence, for instance, for the existence of at least two different biosynthetic pathways of IAA synthesis (the 'tryptophan dependent' and 'non-tryptophan dependent' pathways), which are believed to be developmentally regulated (Ljung *et al.*, 2001; Michalczuk *et al.*, 1992; Normanly *et al.*, 1993; Sitbon *et al.*, 2000). However, our understanding of the specific pathways is still not precise enough to allow us to monitor total biosynthetic fluxes in all pathways simultaneously. An alternative way to monitor biosynthesis is to use deuterium oxide. This has several advantages: it is easily taken up into the plant, it enters all cellular compartments and it has little effect on growth when used in concentrations below 40% (Mitra *et al.*, 1976). It is also a general label, allowing all IAA biosynthesis to be monitored, regardless of the pathway(s) being used by the plant during the study (Jensen and Bandurski, 1996; Pengelly and Bandurski, 1983). By keeping the incubation times with deuterated water relatively short and by avoiding high temperatures and extreme alkaline or acidic conditions during extraction and purification, the risk of incorporation of deuterium into the IAA molecule via non-specific exchange reactions is greatly reduced (Cooney and Nonhebel, 1991). Alkali treatment to remove deuterium from exchangeable positions at the IAA molecule has been used to specifically monitor deuterium incorporation via *de novo* synthesis of IAA, as opposed to deuterium incorporation via non-specific exchange reactions into IAA precursors such as IAA esters and tryptophan (Pengelly and Bandurski, 1983). In a study where maize seedlings were grown for several days on 30% deuterated water, alkali treatment of the IAA samples prior to analysis showed no incorporation of deuterium into the IAA molecule that could be of non-enzymatic origin (Jensen and Bandurski, 1996). By omitting the alkali treatment procedure it is possible to measure the pool size of free IAA at the same time as measuring deuterium incorporation into the IAA molecule. This is important

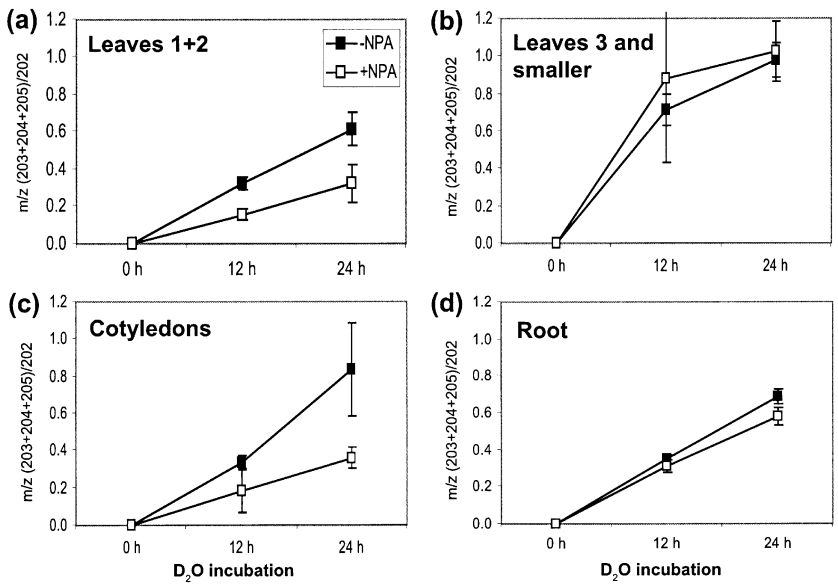


Figure 4. IAA biosynthesis in intact developing wild type *Arabidopsis* seedlings. *De novo* synthesis of IAA was measured in leaves 1 + 2 (a), leaves 3 and smaller (b), cotyledons (c), and roots (d) from 10-day-old seedlings grown under LD. The plants were incubated with media containing 30% deuterated water with (white squares) or without (black squares) NPA. The incorporation of deuterium into IAA was measured by GC-HR-MS. Each sample contained pooled plant material from 10 seedlings. Samples were measured in triplicates, and corrections were made for background and natural isotope abundance. Error bars indicate standard deviations.

when only small amounts of tissue are available for analysis.

In this investigation we have demonstrated that high IAA levels are correlated with high rates of cell division in developing leaves. Young leaves are believed to be the major source of IAA, but no thorough investigation has yet been performed in any plant species to elucidate if IAA can be synthesized in other organs or other developmental stages. We therefore decided to pinpoint the sites of IAA biosynthesis by feeding dissected organs of *Arabidopsis* seedlings with 30% deuterated water, and the *de novo* synthesis of IAA was then measured by mass spectrometry isotopomer analysis. Incorporation of deuterium was measured after 12 and 24 h of incubation of 10-day-old seedlings and the results, shown in Figure 3, indicate that all analysed parts of the seedling are capable of synthesizing this hormone. To avoid possible interference from IAA transport between different plant organs the plants were dissected, prior to the deuterium feeding experiment, into leaves that were mainly undergoing expansion growth (leaves 1 + 2, the first true leaves, approximately 10 mm in length), young developing leaves with high rates of cell division as well as expansion (leaves 3 and smaller, less than 4 mm in length, including all leaf primordia and the shoot apical meristem), cotyledons and the root system. The highest relative synthesis capacity was observed in the young developing leaves, but the cotyledons were also capable of synthesizing IAA at a high rate. Lower but still significant rates of IAA biosynthesis were detected in expanding leaves and in the root system.

NPA decreases the IAA content in specific tissues, probably by feedback inhibition of IAA biosynthesis

The plants were dissected into different parts prior to deuterium feeding to prevent normal vascular transport of IAA, however, this can also cause wound-related effects that might disturb IAA biosynthesis. We therefore conducted a second series of feeding experiments with intact plants to compare their responses with those of excised plant parts. In these experiments we included a set of trials in which 40 μM NPA was added to the medium, to block polar IAA transport and thus trap IAA in the tissue where it had been synthesized (Figure 4). For young leaves, the addition of NPA did not cause any significant changes in IAA biosynthetic rates compared to intact plants (Figure 4b), but relative to excised young leaves the amount of IAA incorporation detected was lower. Based on this result, it is tempting to speculate that there is a non-polar transport pathway that is more important than the polar transport pathway for IAA export from these small leaves. Excision lowers the amount of newly synthesized IAA detected in the root compared to intact plants, but it is important to note that the root system at this developmental stage is capable of synthesizing its own IAA. The decrease in the amount of newly synthesized IAA detected in the root after excision can be explained either by a general decrease in metabolism due to a lack of assimilates transported from the shoot to the root system, or by the loss of polar IAA transport. NPA treatment caused only a minor decrease in the observed IAA biosynthesis (Figure 4d), again supporting the hypothesis that there might be an NPA-independent mechanism that

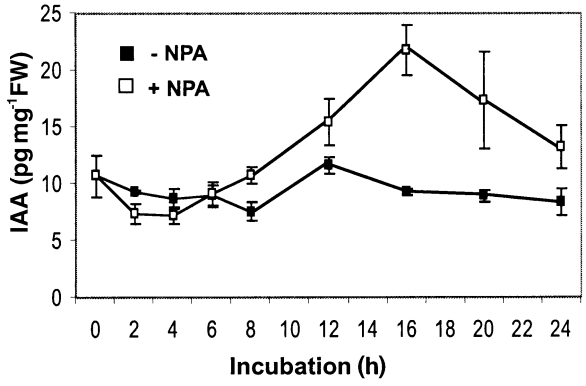


Figure 5. IAA levels in expanding leaves of wild type *Arabidopsis* seedlings treated with NPA, showing feedback inhibition of IAA biosynthesis.
Ten-day-old wild-type *Arabidopsis* seedlings were incubated for 2–24 h in liquid media with (white squares) or without (black squares) 40 μM NPA. Leaves 1 + 2 from five seedlings were pooled for each sample, and three samples were analysed for each data point. FW, fresh weight; error bars indicate standard deviations.

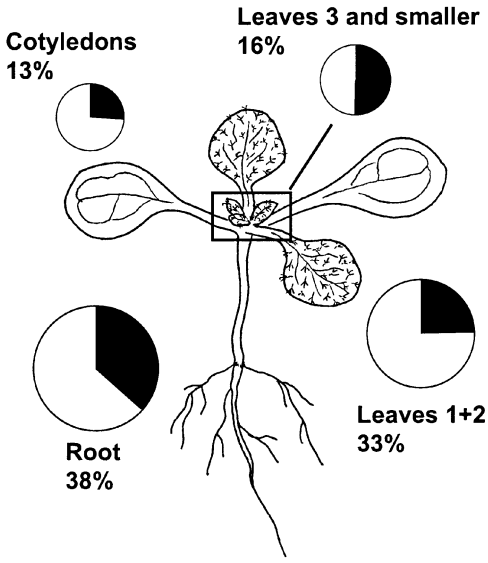


Figure 6. Total IAA pool sizes in different parts of a 10-day-old wild-type *Arabidopsis* seedling grown under LD.
The circles show the absolute pool sizes of IAA in different organs of the seedling. Black parts of circles show the amount of IAA that had been synthesized *de novo* in different tissues after 24 h of incubation in 30% deuterated water and 40 μM NPA.

transports IAA from the shoot down to the root. Similar analysis of expanding leaves and cotyledons from intact plants revealed that there was a decrease in IAA biosynthetic rates in these organs after NPA treatment (Figure 4a,c). Our finding that NPA treatment lowers IAA biosynthesis in these tissues is a novel observation, as NPA is expected to block polar transport of newly synthesized IAA out of these putative source tissues, thus leading to accumulation rather than depletion. One possible explanation for these results is that the treatment with NPA creates an initial increase in IAA concentration that induces feedback inhibition of IAA biosynthesis, and that this subsequently leads to lowered IAA levels in the leaves. In order to test this hypothesis we conducted a series of experiments with 10-day-old seedlings that were incubated for 2–24 h in liquid media containing 40 μM NPA, and we then monitored the IAA content in both young and expanding leaves for the duration of the experiment. Initially, after the addition of NPA, an increase in IAA content in the expanding leaves (leaves 1 + 2) was detected that peaked after 16 h (Figure 5). Thereafter the levels dropped dramatically, also indicating the existence of a feedback inhibition mechanism. This type of regulation was not observed in the young leaves (leaves 3 + 4), where the levels were constant for the duration of the experiment (data not shown), indicating that in young leaves NPA does not block transport of IAA out of the tissue. This does not exclude the possibility that the NPA treatment might also activate mechanisms for IAA degradation and conjugation that together with the feedback inhibition of IAA biosynthesis lowers the IAA level in these tissues, maintaining the IAA concentration at a level that is optimal for growth and development.

The relationships between IAA pools in different plant organs

Analysis of relative synthesis capacity will not entirely reveal how important a certain tissue is as a source of IAA on a whole seedling basis, as the size of different organs differs enormously. In Figure 6, the absolute sizes of the IAA pools in different parts of a 10-day-old seedling are presented in relation to the amounts of newly synthesized IAA detected after a 24 h incubation period with NPA and 30% deuterated water. The largest single IAA pool is located in the root system (38%) and the expanding leaves (33%). However, due to their small size, the smallest leaves contain only a minor part of the total pool, even though the synthesis capacity and the IAA content per unit mass in these leaves is very high. The data presented in Figure 6 describe the absolute pool sizes of IAA and the time it takes the plant to replace the entire pool in the specific organs with newly synthesized IAA. To evaluate the importance of the individual pools on a whole plant level, transport fluxes to and from different tissues also have to be taken into account.

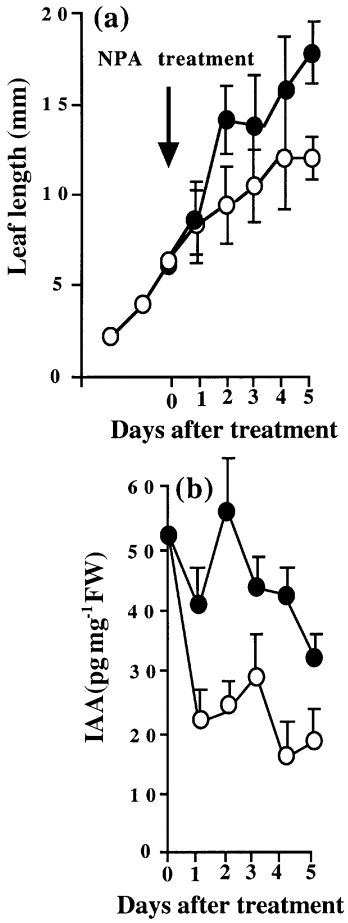


Figure 7. Effects of feedback inhibition induced by NPA treatment on leaf expansion and IAA levels in expanding *Arabidopsis* leaves.
Leaf length (a) and IAA concentration (b) was investigated in plants grown first for 10 days under LD on agar without NPA, and then for 1–5 days on agar containing 100 μM NPA ○. Control plants grown without NPA were analysed in the same way ●. FW, fresh weight; error bars indicate standard deviation.

Reductions in IAA levels due to feedback inhibition of IAA biosynthesis reduce leaf expansion

Our next set of experiments was designed to evaluate whether the correlation between IAA content and leaf size reflects not only that a leaf of a certain size has a specific capacity to synthesize this hormone, but also that IAA is an important factor in the overall control of leaf growth. For this, we made use of our recent finding that NPA treatment reduces IAA levels in expanding leaves when feedback inhibition of IAA biosynthesis is induced. We conducted a series of experiments in which 10-day-old seedlings grown on soft agar were transferred to and incubated up to 5 days on agar containing 100 μM NPA. Both the IAA levels in the leaves and the size of the leaves were measured daily (Figure 7). As expected from our previous findings, the IAA contents in the expanding leaves dropped significantly even the first day after transition to NPA (Figure 7b), and remained lower in the NPA-treated plants for the duration of the experiment. The rapid reduction in IAA pool size observed after just 24 h of NPA treatment was mirrored by an equally rapid decrease in leaf expansion (Figure 7a).

Increased IAA contents in the sur1 *and* sur2 *mutants also reduce leaf expansion*

In a complementary experiment we investigated whether up-regulation of the IAA pool in expanding leaves had the same detrimental effect on expansion growth as the decreased pool size in the previous experiment. For this purpose we used seedlings of the mutants *superroot1* (*sur1*) and *superroot2* (*sur2*), which had already been shown to contain increased IAA levels. The *sur1* mutants show a large increase in the content of both free and conjugated IAA early in development (Boerjan *et al.*, 1995), whereas *sur2* mutants show a similar early increase of IAA in all parts of the seedling, but revert to wild-type phenotype 12–15 DAG (Barlier *et al.*, 2000). Both mutants show phenotypic traits typical for auxin overproduction such as elongated hypocotyls, small and epinastic cotyledons and an increased number of lateral roots. The phenotype of the *sur1 sur2* double mutant is additive. We analysed the IAA levels as well as the relative expansion rates of developing leaves from *sur1* and *sur2* seedlings (Figure 8). Compared to wild type, the first expanding leaves of *sur1* and *sur2* have significantly higher levels of this hormone, which are closely correlated to reductions in relative leaf expansion rates. Our analysis shows that the extremely high level of IAA in the first leaves of *sur1* is correlated with a dramatic reduction in leaf expansion, whereas the more moderate increase in the first leaves of *sur2* is accompanied by an intermediate reduction of expansion. Interestingly, the second leaf pair of *sur2* reverts to normal IAA levels and also has an almost normal rate of leaf expansion. These results, in combination with those previously presented, demonstrate that any alterations in the optimal pool size of IAA during the expansion phase have detrimental effects on the expansion rate of the leaf. Thus there is good reason to believe that the IAA pool in the leaf is under strict homeostatic control.

Discussion

We have used advanced mass spectrometry to analyse the distribution of IAA in *Arabidopsis* seedlings from germination and during the entire period of vegetative growth. We have observed that actively dividing young leaves contain the highest concentrations of IAA, and that the hormone levels drop dramatically as the leaves expand.

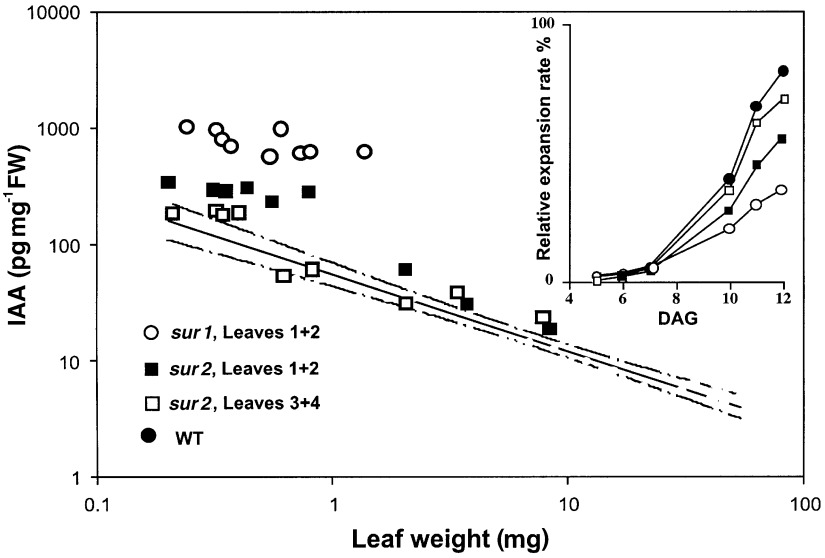


Figure 8. Leaf expansion and IAA levels in young leaves from the auxin overproducing mutants *sur1* and *sur2* compared with wild-type.
IAA concentration in developing leaves from homozygous *sur1* and *sur2* seedlings. The regression line with 99% confidence intervals was calculated from wild-type data (see Figure 1e). FW, fresh weight. Relative leaf expansion rates were calculated as percentage of final leaf length.

These observations apply to plants of different ages and appear to be independent of photoperiod.

We have also investigated the *de novo* synthesis of IAA in different parts of young *Arabidopsis* seedlings, and although the youngest leaves have the highest synthesis capacity, all parts of a young *Arabidopsis* plant can synthesize IAA. We have shown, for instance, that roots of young *Arabidopsis* plants are capable of synthesizing their own IAA, and that the IAA pool in the root system comprises a large proportion of the total IAA in the plant. The experiments with excised roots demonstrate that this root-localized pool is not derived from auxin transported from source tissues in the aerial part of the plant. However, it is still not clear when IAA synthesis is initiated in the root, and what specific tissues of the root system have the capacity to synthesize IAA.

The IAA levels are always much lower and constant in the cotyledons than in the true leaves. The cotyledons are formed in the embryo, prior to germination, and serve as nutrient storage organs for the growing seedling. The vascular tissue is less highly developed in cotyledons than in the true leaves, and they are probably important to the plant mainly during the early stages of seedling growth. However, the cotyledons do have a relatively high capacity for IAA synthesis, and it is therefore possible that they can serve as an important IAA source during the early stages of development or under circumstances when other IAA sources such as the leaves are not developing normally.

We also used NPA to trap IAA in the plant organs where it was synthesized. This had dramatic effects on IAA levels as well as leaf expansion. When 10-day-old plants were incubated with NPA for 2–24 h we first observed a more than two-fold increase in IAA content in the expanding leaves, followed by a sharp decrease, demonstrating the existence of feedback inhibition of IAA biosynthesis. This regulatory mechanism was not seen in the youngest leaves, indicating that the leaves must reach a specific developmental stage before the feedback mechanism is turned on. The general opinion in the literature is that NPA treatment causes an accumulation of IAA in all source tissues (Berleth and Mattsson 2000). We have shown that this is not the case, at least not in expanding leaves and cotyledons. This does not exclude the possibility that there might be local accumulation of IAA in specific parts of the organs that possess a mechanism of feedback inhibition. It is well documented that plants grown on high concentrations of NPA for long periods display major changes in vascular patterning, with vascular tissue forming close to the leaf margin (Mattsson *et al.*, 1999). This observation is consistent with our findings, as the alterations in vasculature observed by Mattsson *et al.* were induced in leaves of a size that did not show feedback inhibition in our experiments.

NPA treatment for 1 to 7 days caused IAA levels in the leaves to drop and leaf expansion to cease. Interestingly, the auxin overproducing *sur1* and *sur2* mutants, which contain higher amounts of free IAA than wild type early in development, also had reduced rates of leaf expansion. These experiments clearly show that normal leaf expansion is dependent on optimal IAA concentrations. Our experiments also clearly show that the plant needs to maintain stringent control over the IAA pool in the leaves not only for optimal expansion growth, but also to ensure that they can fulfil their role as important IAA sources for the rest of the plant.

In this study we have shown that all parts of the seedling can synthesize IAA, and even if the leaves probably are the most important sources, the dynamic nature of auxin

biosynthesis and tissue specific regulatory mechanisms indicate that the supply of IAA to the different growth zones of the seedling is much more complex than previously thought. The existence of mechanisms that control the pool size of IAA in a tissue-specific manner also means that molecular markers for auxin contents should be used with caution, as such markers can only detect large differences in concentration, and in many cases show tissue-specific sensitivity for auxin.

Experimental procedures

Chemicals and isotopically labelled substrates

[$^{13}C_6$]IAA and 70% deuterium oxide were from Cambridge Isotope Laboratories (Andover, MA, USA). Murashige-Skoog medium (MS-medium) and X-GlcA-cyclohexylammonium salt (X-Gluc) were from Duchefa (Haarlem, The Netherlands), and all other chemicals were from Sigma (St Louis, MO, USA) if not stated otherwise.

Plant material and growth conditions

Seeds of wild-type *Arabidopsis thaliana* ecotype Columbia, and the mutants *sur1–3* and *sur2–1* were cold-treated (4°C) for 3–4 days before sowing on soil (Simontorp Blomjord, Svalöf Weibull Torv AB, Sweden) or on agar plates, to break dormancy and to synchronize germination. Seeds grown on agar plates were first sterilized. The plants were put in growth chambers under cool white fluorescent lights at a temperature of 22 ± 2°C and a relative air humidity of approximately 50%. The light intensity was 70–120 μmol m^{-2} s^{-1} and the light cycles were 9 h light : 15 h dark (short day, SD) and 18 h light : 6 h dark (long day, LD). Seedlings germinated on soil were replanted after 1–2 weeks in 7 cm × 7 cm plastic trays using the same soil, where they could continue to grow until harvesting. Seeds of wild-type *Nicotiana tabacum* (SR1) and the CYC1–1::GUS and CDC2–6::GUS lines were grown under standard greenhouse conditions.

Collection of plant material

Seedlings homozygous for *sur1–3* were selected for IAA and leaf expansion measurements. Plants were dissected under a Stemi SV 11 stereomicroscope equipped with a MC 80 DX camera (Carl Zeiss Jena GmbH, Jena, Germany) using a pair of soft tweezers and a scalpel. The plant tissue was immediately transferred to pre-weighed Eppendorf tubes, the tubes were then re-weighed, immersed in liquid nitrogen and stored at –80°C. The balance used was an AT 20 6-digit analytical model (Mettler-Toledo GmbH, Greifensee, Switzerland) and precautions were taken to avoid errors in weighing caused by evaporation from the plant material or by static electricity. For very small plant parts, material from several plants were pooled so that the weight of the final sample was never less than 0.1 mg. This was done to minimize the effect of weighing error on the estimation of sample weight, and to get enough plant material for IAA measurements. The length and width of leaves from *Arabidopsis* seedlings grown on soil and agar plates were also measured under the stereomicroscope. Dissected and/or measured leaves were assigned a number starting with the first true leaves (leaves 1 and 2) and the younger leaves were then given number 3 and higher, following the phyllotaxis of the plant. Leaves from 8- to 10-week-old tobacco plants in the vegetative growth phase were collected for IAA analysis and stained with X-Gluc (Van Lijsebettens and Clarke, 1998). Leaf discs, 2 mm in diameter, were cut out from points representing the whole area of 1.5 cm long leaves, weighed and frozen in liquid nitrogen.

Analysis of endogenous IAA content

For IAA measurements, samples were extracted, purified and analysed by gas chromatography–selected reaction monitoring–mass spectrometry (GC–SRM–MS) as previously described (Edlund *et al.*, 1995). Calculation of isotopic dilution factors was based on the addition of 50 pg [$^{13}C_6$]IAA/mg tissue.

Deuterium feeding experiments

Arabidopsis seedlings were grown in Petri dishes containing soft agar (1 × MS-medium, 1% sucrose, 0.5% agar, pH 5.7) under LD for 10 days, and were then transferred to liquid culture medium (1 × MS-medium, 1% sucrose, pH 5.7) with or without 40 μM NPA and incubated for 4 h. They were then transferred to media with or without 30% deuterated water and 40 μM NPA, and incubated with gentle shaking under constant light for 12 and 24 h. After incubation, the plants were dissected into the expanding first true leaves (leaves 1 + 2, approximately 10 mm in length), young leaves (leaves 3 and smaller, less than 4 mm in length, including all leaf primordia and the shoot apical meristem), cotyledons and the root system. Experiments were also performed on seedlings that had been dissected in the same way before incubation in liquid culture media (1 × MS-medium, 1% sucrose, pH 5.7) with or without 30% deuterated water for 12 and 24 h. The different plant parts were then weighed and frozen in liquid nitrogen. The extraction and purification was carried out as described elsewhere (Edlund *et al.*, 1995) with the addition of an extra purification step before analysis by GC-High Resolution (HR)-MS; namely, after purification on XAD-7, the samples were methylated and purified on 50 mg C18 SPE columns (Bond Elut, Varian, Harbor City, CA, USA). Calculation of isotopic dilution was based on the addition of 50 pg [$^{13}C_6$]IAA/mg tissue. Analysis by GC-HR-MS was done at a resolution of at least 10 000, and isotopomers of the base peak of methylated and trimethylsilylated IAA were measured with m/z ratios of 202.105, 203.112, 204.118, 205.124 and 208.125. Corrections were incorporated for the contribution of natural isotopic abundances to m/z 203–205. The incorporation of deuterium into the IAA molecule was then calculated, and corrections for background were made by analysing samples from control plants grown without deuterated water.

NPA treatment

Twenty-four hour incubation experiments. Wild-type *Arabidopsis* seeds were placed on soft agar plates (1 × MS-medium, 1% sucrose, 0.5% agar, pH 5.7) and the seedlings were then grown under LD conditions as previously described for 10 days. Intact seedlings were then transferred to liquid media (1 × MS-medium, 1% sucrose, pH 5.7) with or without 40 μM NPA. Leaves 1 + 2 (the first true leaves) and leaves 3 + 4 were then harvested for IAA measurements after 2–24 h incubation in constant light conditions.

Five-day incubation experiments. Wild-type seedlings were grown under LD on soft agar plates (1 × MS-medium, 2% sucrose, 0.5% agar, pH 5.7) covered with a net to avoid the aerial parts coming into contact with the agar. When the seedlings were 10 days old they were transferred, together with the net, to new plates with or without 100 μM NPA, and were kept growing on this medium for 1–5 days. Leaves 3 + 4 and leaves 5 + 6 were harvested for IAA measurements every day, and at the same time the lengths and widths of the leaves were measured.

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