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Novel auxin transport inhibitors phenocopy the auxin influx carrier mutation *aux1*

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Summary

The hormone auxin is transported in plants through the combined actions of diffusion and specific auxin influx and efflux carriers. In contrast to auxin efflux, for which there are well documented inhibitors, understanding the developmental roles of carrier-mediated auxin influx has been hampered by the absence of specific competitive inhibitors. However, several molecules that inhibit auxin influx in cultured cells have been described recently. The physiological effects of two of these novel influx carrier inhibitors, 1-naphthoxyacetic acid (1-NOA) and 3-chloro-4-hydroxyphenylacetic acid (CHPAA), have been investigated in intact seedlings and tissue segments using classical and new auxin transport bioassays. Both molecules do disrupt root gravitropism, which is a developmental process requiring rapid auxin redistribution. Furthermore, the auxin-insensitive and agravitropic root-growth characteristics of *aux1* plants were phenocopied by 1-NOA and CHPAA. Similarly, the agravitropic phenotype of inhibitor-treated seedlings was rescued by the auxin 1-naphthaleneacetic acid, but not by 2,4-dichloro-phenoxyacetic acid, again resembling the relative abilities of these two auxins to rescue the phenotype of *aux1*. Further investigations have shown that none of these compounds block polar auxin transport, and that CHPAA exhibits some auxin-like activity at high concentrations. Whilst results indicate that 1-NOA and CHPAA represent useful tools for physiological studies addressing the role of auxin influx *in planta*, 1-NOA is likely to prove the more useful of the two compounds.

Keywords: Arabidopsis, AUX1, auxin transport, auxin influx carrier, auxin transport inhibitors, root gravitropism.

Introduction

Auxins represent a group of naturally occurring indole molecules that act as hormones in plants (Davies, 1995). The major form of auxin in higher plants, indole-3-acetic acid (IAA), regulates the fundamental processes of cell division, elongation and differentiation. IAA is first synthesized within young apical tissues (Bartel, 1997), then conveyed to basal target tissues using a specialized delivery system termed polar auxin transport (PAT). The polarized movement of auxin has been demonstrated to be critical for many developmental processes, including embryo patterning, vascular differentiation and root gravitropism (reviewed by Bennett *et al.*, 1998).

Molecular genetic strategies within the model plant *Arabidopsis thaliana* are being employed to dissect the molecular basis of auxin transport. Several auxin transport mutants have been isolated to date (Bennett *et al.*, 1998) and, in some cases, mutated genes were identified (Bennett *et al.*, 1996; Chen *et al.*, 1998; Gälweiler *et al.*, 1998; Luschnig *et al.*, 1998; Müller *et al.*, 1998; Utsuno *et al.*, 1998). Genes of the *PIN* family have homology with bacterial genes coding for membrane transporters. Proteins encoded by the *AtPIN1* and *AtPIN2* genes, the latter also termed *AGR* (Chen *et al.*, 1998; Utsuno *et al.*, 1998) or *EIR1* (Luschnig *et al.*, 1998), display asymmetrical cellular distribution within the elongation zones of root and shoot tissues. This suggests that *PIN* gene products might support directional auxin efflux in transport-competent cells (Gälweiler *et al.*, 1998; Müller *et al.*, 1998). It has been proposed that AtPIN1 mediates PAT throughout the stem and root *in planta* (Gälweiler *et al.*, 1998), whereas

AtPIN2 contributes to localized auxin redistribution in the root tip (Müller *et al.*, 1998).

The *AUX1* gene of *A. thaliana* encodes a protein with similarities to plant and fungal amino acid permeases, and there is evidence that the AUX1 protein might have a role in the uptake of auxin into the cell (Bennett *et al.*, 1996; Marchant *et al.*, 1999). Mutations within the *AUX1* gene confer an auxin-insensitive phenotype. The altered root-growth response is specific to auxins requiring carrier-mediated uptake, and can be bypassed by growing *aux1* plants in the presence of the auxin 1-naphthalene acetic acid (1-NAA) which enters plant cells in a carrier-independent manner (Marchant *et al.*, 1999; Yamamoto and Yamamoto, 1998). Mutations in *AUX1* and *AtPIN2* confer an agravitropic root-growth phenotype. Marchant *et al.* (1999) have proposed that AUX1 regulates gravitropic curvature in unison with the auxin efflux carrier to co-ordinate the localized redistribution of auxin in the root apex, providing the first example of a developmental role for the auxin influx carrier in higher plants.

Biochemical and physiological studies of auxin transport have addressed mainly the mechanisms underlying auxin efflux, as most known auxin transport inhibitors target the phytotropin-binding site in the auxin efflux complex (Rubery, 1990). Equivalent pharmacological studies of carrier-mediated auxin uptake have been hampered by the absence of suitable auxin influx carrier inhibitors. Several workers have employed 2-naphthalene acetic acid (2-NAA) to inhibit auxin influx carrier activity in tissue fragments (Sussman and Goldsmith, 1981) and isolated membrane vesicles (Benning, 1986; Jacobs and Hertel, 1978). However, 2-NAA also perturbs auxin efflux (Delbarre *et al.*, 1996) and exhibits weak auxin activity (Katekar, 1979).

Recently, Imhoff *et al.* (2000) have screened a large number of aryl and aryloxyalkylcarboxylic acids for selective reduction of carrier-mediated 2,4-dichlorophenoxyacetic acid (2,4-D) uptake in suspension-cultured tobacco cells. 1-naphthoxyacetic acid (1-NOA) and 3-chloro-4-hydroxyphenylacetic acid (CHPAA) have been selected, based on their capacity to inhibit auxin influx carrier activity at micromolar concentrations. We have evaluated the effects of both compounds at the plant level using a variety of molecular and physiological bioassays. We have focused our interest mainly on root responses in order to explore possible similarities with the phenotype of the *aux1* mutant. We discuss the ability of both compounds to phenocopy the *aux1* mutation, and also the utility and limits of the two inhibitors to further study the role of the auxin influx carrier.

Results

Effect of 1-NOA and CHPAA on root elongation

We first investigated the effect of 1-NOA and CHPAA on elongation responses using a root-elongation bioassay on intact *Arabidopsis* seedlings (Figure 1). Seedlings grown on media supplemented with concentrations of these compounds, ranging from 0.01 to 30 μM, were compared with seedlings grown on the efflux carrier inhibitor NPA or the synthetic auxins 2,4-D and 1-NAA. Auxin concentrations >0.3 μM for 2,4-D and >1.0 μM for 1-NAA exhibited maximum inhibition of elongation. The efflux carrier inhibitor NPA caused a less severe retardation of root elongation at concentrations >1 μM (as described by Ruegger *et al.*, 1997). 1-NOA showed no significant inhibition of root elongation at concentrations up to 30 μM (Student's *t*-test, $P = 0.88$), whereas CHPAA caused a retardation of root elongation at concentrations of ⩾10 μM ($P = 0.0008$).

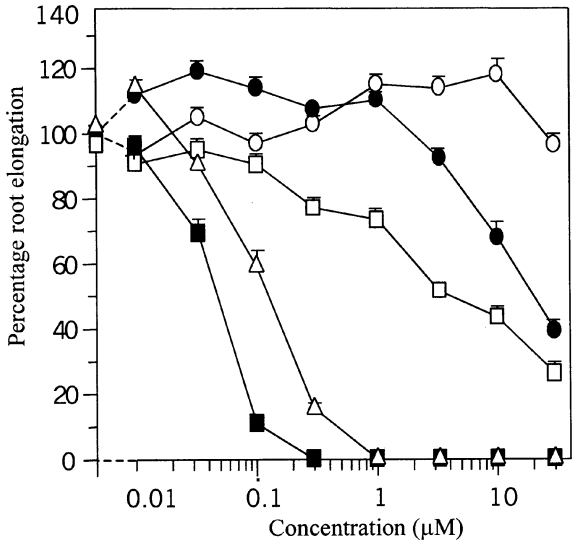

Figure 1. 2,4-D, 1-NAA, 1-NOA, CHPAA and NPA exhibit different effects on root elongation in *Arabidopsis*.
Arabidopsis seedlings were grown for 3 days on MS agar before being transferred to agar containing a range of concentrations of the auxins 2,4-D (■), 1-NAA (△) or the auxin transport inhibitors NPA (□), 1-NOA (○) and CHPAA (●). New growth was measured after a further 3 days and expressed as a percentage of root elongation on non-supplemented media. Values were calculated from three independent experiments using at least 16 seedlings per data point. Error bars represent SE values.

Protective effect of 1-NOA and CHPAA against root growth inhibition by 2,4-D

We have exploited the observation, made using short-term uptake assays (Delbarre *et al.*, 1996), that the synthetic auxins 2,4-D and 1-NAA enter plant cells primarily by carrier-mediated transport and by diffusion, respectively, to create a novel auxin transport inhibitor assay. Inhibition of influx carrier activity would be predicted to selectively block the uptake of 2,4-D but not 1-NAA. Three-day-old wild-type *Arabidopsis* seedlings grown on MS agar were transferred onto media containing either 0.1 μM 2,4-D or

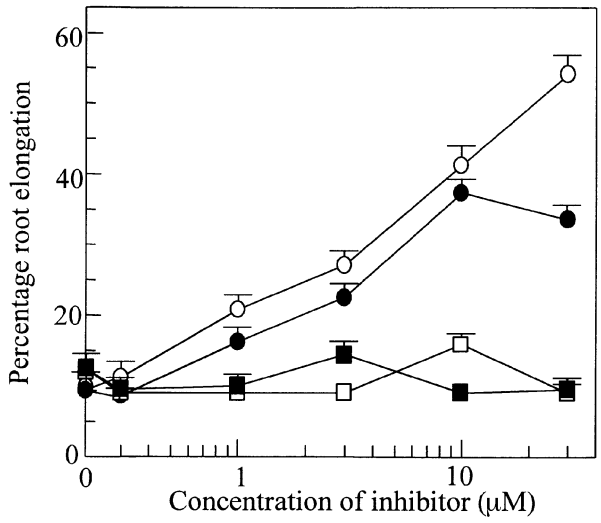

Figure 2. Auxin influx carrier inhibitors selectively restore root elongation in the presence of 2,4-D but not 1-NAA.
Arabidopsis seedlings were grown on MS agar for 3 days before being transferred to agar containing 0.1 μM 2,4-D supplemented with increasing concentrations of 1-NOA (○) or CHPAA (●) or containing 0.5 μM 1-NAA supplemented with increasing concentrations of 1-NOA (□) or CHPAA (■). New growth was measured after a further 3 days and expressed as a percentage of root elongation on non-supplemented media. Values were calculated from three independent experiments using at least 16 seedlings per data point. Error bars represent SE values.

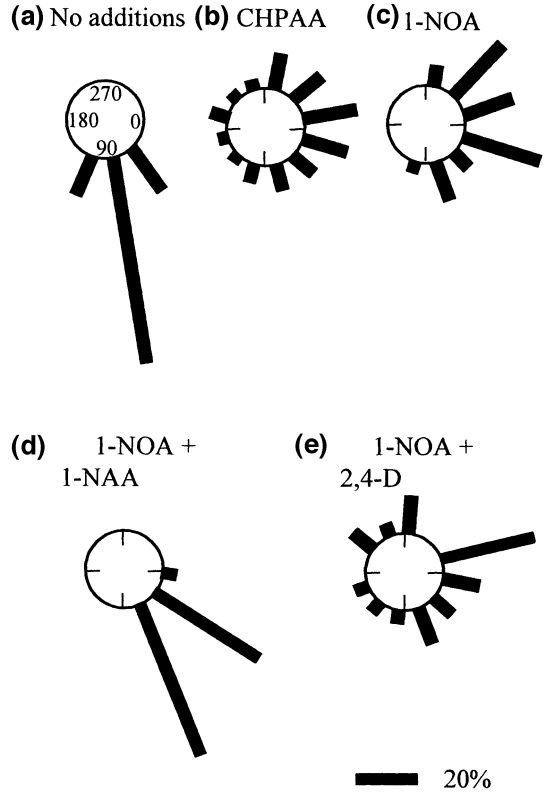

Figure 3. Auxin influx carrier inhibitors disrupt root gravitropism.
Arabidopsis seedlings were germinated in a vertical orientation on media supplemented with influx carrier inhibitors 1-NOA or CHPAA, auxins 1-NAA or 2,4-D, or a mixture of inhibitor and auxin. Three days after germination, seedlings were rotated 90° to provide a gravitropic stimulus. After a further 24 h the direction of growth of the root tips was determined. The number of roots in each of the 12 30° sectors between 0 and 360° from horizontal were counted and expressed as a percentage of the total number of roots. Vertical position represents normal gravitropic response. Values were calculated using ≈50 seedlings per experiment. (a) untreated seedlings; (b) 10 μM CHPAA; (c) 30 μM 1-NOA; (d) 30 μM 1-NOA plus 0.1 μM 1-NAA; (e) 30 μM 1-NOA plus 0.03 μM 2,4-D. Roots treated with 1-NAA alone behaved as untreated seedlings and re-oriented normally.

0.5 μM 1-NAA supplemented with various concentrations of 1-NOA or CHPAA. The roots of seedlings grown in the presence of either auxin at these concentrations exhibited 90% inhibition of elongation (Figures 1 and 2). Root elongation was partly restored in 2,4-D-treated seedlings supplemented with increasing concentrations of 1-NOA or CHPAA (Figure 2). In contrast, neither influx carrier inhibitor exhibited a protective effect on roots co-incubated with 1-NAA (Figure 2). Hence both molecules prevented inhibition of elongation caused by 2,4-D but not 1-NAA.

Perturbation of root gravitropism by 1-NOA and CHPAA

Localized redistribution of auxin at the root tip is thought to be required to maintain the gravitropic response of the root (Marchant *et al.*, 1999; Müller *et al.*, 1998). The observation that *Arabidopsis* seedlings carrying a mutation in the *AUX1* gene display agravitropic root growth prompted an investigation into whether 1-NOA or CHPAA could disrupt root bending in gravistimulated seedlings. *Arabidopsis* seedlings were grown vertically on agar plates in the presence of either inhibitor at various concentrations up to 30 μM, then given a gravitropic stimulus by rotation through 90° (Figure 3). Seedlings grown on agar without any addition displayed normal root bending (Figure 3a). Plant roots began to grow randomly in the presence of 5 μM CHPAA, and were fully agravitropic at 10 μM inhibitor (Figure 3b). The effect of 1-NOA was not as pronounced as that shown by CHPAA, as seedlings treated with 1-NOA exhibited a largely normal root-bending response at concentrations up to 10 μM. However, roots became agravitropic in the presence of 30 μM 1-NOA (Figure 3c).

Following the same idea as in the previous experiment (Figure 2), we investigated whether 1-NAA or 2,4-D could rescue the agravitropism caused by the inhibitors. Seedlings were grown in the presence of either inhibitor (1-NOA or CHPAA) together with 1-NAA or 2,4-D. Experiments were designed such that, under all auxin treatments, roots elongated at an equivalent rate (≈65% of wild type; Figure 1), ensuring reliable measurement of the gravitropic response. Seedlings grown in the presence of

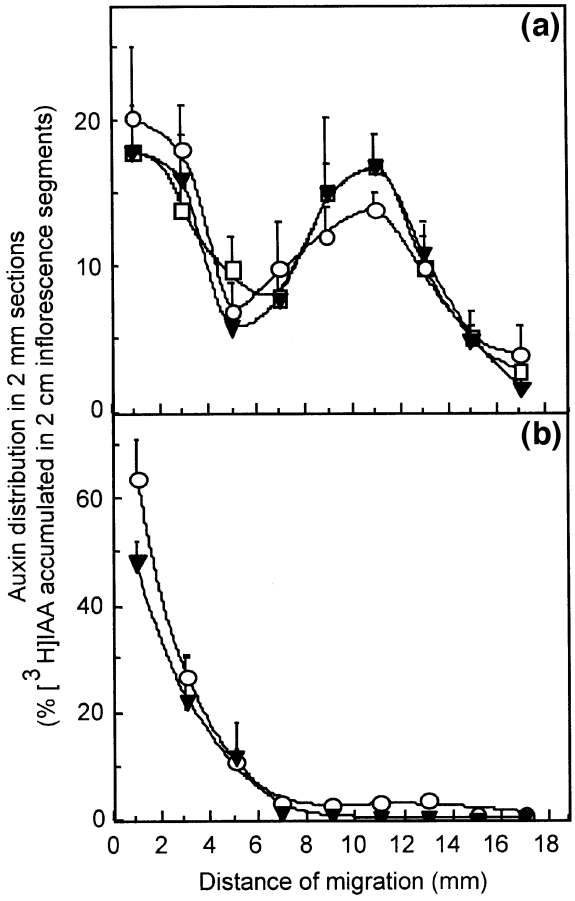

Figure 4. Auxin influx carrier inhibitors do not block [^{3}H]IAA transport within *Arabidopsis* inflorescence tissues.
Segments of inflorescence axes (2 cm) were pre-incubated in buffer containing 1.5 μM IAA with or without 25 μM 1-NOA, CHPAA or NPA for 10 min. The distribution of radiolabelled IAA was measured within segments after a 5 min pulse of [^{3}H]IAA followed by a 90 min chase.
(a) Basipetal migration of a pulse of [^{3}H]IAA measured in the absence (▼) and presence of 1-NOA (○) or CHPAA (□).
(b) Acropetal migration of a pulse of [^{3}H]IAA measured in the absence of inhibitor (▼) and basipetal migration measured in the presence of NPA (○). Data expressed as mean (±SD) of values calculated from two independent experiments using three segments per experiment.

30 μM 1-NOA plus 0.1 μM 1-NAA (Figure 3d) exhibited a largely gravitropic root growth. In contrast, 2,4-D at a concentration of 0.03 μM did not rescue the agravitropism induced by 1-NOA (Figure 3e). Similar results were also obtained using CHPAA (data not shown). These results suggest that 1-NAA was able to bypass the agravitropic root growth mediated by both influx carrier inhibitors.

Effect of 1-NOA and CHPAA on efflux carrier mediated polar auxin transport

The auxin influx carrier inhibitors 1-NOA and CHPAA have been identified using short-term auxin uptake assays in tobacco suspension cell cultures (Imhoff *et al.*, 2000). However, the influx carrier inhibitor 1-NOA was also reported by Imhoff *et al.* (2000) to exhibit an inhibitory effect on efflux carrier activity in tobacco cultured cells under long-term experiments.

In order to address whether auxin influx carrier inhibitors could also interfere with efflux carrier activities *in planta*, we checked their effect on a classical PAT assay performed on *Arabidopsis* inflorescence segments given a pulse-chase treatment with [^{3}H]IAA. This material was selected for the PAT assay as it allows reliable and highly reproducible results (Figure 4). Tissue segments were pre-incubated with 1.5 μM unlabelled IAA or 1.5 μM IAA plus 25 μM solutions of either 1-NOA, CHPAA or NPA, then given a 5 min pulse of [^{3}H]IAA followed by a 90 min chase with unlabelled IAA. The distribution of [^{3}H]IAA was determined by cutting sequential 2 mm sections from the basal ends of the segments. Control tissues that had not been treated with any auxin transport inhibitor exhibited a basipetal rather than acropetal movement of [^{3}H]IAA (Figure 4), illustrating the polarity of auxin transport within *Arabidopsis* inflorescence segments. Pre-incubation with either 1-NOA or CHPAA failed to modify the distribution of [^{3}H]IAA, whereas NPA treatment effectively abolished the basipetal transport of [^{3}H]IAA (Figure 4). These results suggest that neither 1-NOA nor CHPAA are able to block PAT activity.

All inhibitors were applied on the cut section of inflorescence segments, providing direct access for these compounds to the whole cross-section. We have addressed whether the inability of 1-NOA to reach internal tissues represents a practical explanation for its absence of effect on PAT, by treating *Arabidopsis* inflorescence segments from an auxin-inducible *IAA2:GUS* reporter line with its isomer, 2-NOA (a known auxin, Katekar, 1979). Fluorimetric assays confirm that 2-NOA is able to induce GUS activity within inflorescence segments, suggesting that naphthoxyacetic acids do penetrate into the tissue segments (data not shown).

Effect of 1-NOA and CHPAA on the expression of early auxin-responsive genes

In addition to the use of the *IAA2:GUS* reporter line, we have determined whether the influx carrier inhibitors might influence the expression of early auxin-responsive genes *in planta*. Northern hybridization assays were used to monitor the ability of either compound to induce the expression of auxin-responsive genes. Tobacco seedlings were grown in liquid culture for 6 days, then exposed to either auxins or auxin transport inhibitors for 1 h at the concentrations indicated (Figure 5). Total RNA prepared from seedling samples was probed with *Nt-gh3*, which was reported to be selectively induced by auxins (Roux

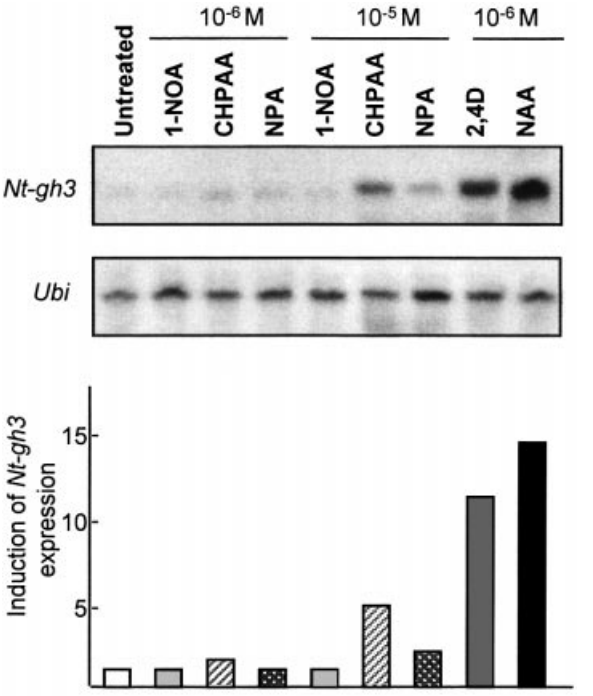

Figure 5. Auxin-responsive Nt-*gh3* mRNA is detectable after treatment with NPA and CHPAA but not 1-NOA.
Six-day-old tobacco seedlings were exposed to auxins 2,4-D and 1-NAA, the auxin influx carrier inhibitors 1-NOA and CHPAA, or the efflux carrier inhibitor NPA at the indicated concentrations for 1 h. Total RNA prepared from each sample was electrophoresed, Northern blotted and probed with either a radiolabelled *Nt-gh3* cDNA probe or ubiquitin-loading control probe.

and Perrot-Rechenmann, 1997), and with the ubiquitin probe (Figure 5a). The *Nt-gh3* mRNA, which was detected only at a basal level in the untreated control, was clearly elevated more than 10-fold following auxin treatment using 1 μM 2,4-D or 1-NAA (Figure 5). *Nt-gh3* mRNA accumulated weakly after either 10 μM CHPAA or 10 μM naphthylphthalamic acid (NPA) treatment. In contrast, tobacco seedlings incubated with 1-NOA did not accumulate *Nt-gh3* mRNA above its basal level of expression at concentrations up to 10 μM, confirming that 1-NOA has no auxin-like activity.

Discussion

Polar transport inhibitors such as the phytotropin NPA have proved invaluable in demonstrating the involvement of the auxin efflux carrier during polar auxin transport-mediated developmental processes (Lomax *et al.*, 1995). We report the phenotypic characterization of a new class of auxin transport inhibitors that targets the auxin influx carrier. Two novel inhibitors, 1-NOA and CHPAA, were selected for their capacity to block the carrier-mediated influx of auxin in tobacco suspension cells in culture, exhibiting IC_{50} values of 2.2 and 2.4 μM, respectively (Imhoff *et al.*, 2000). The influx carrier inhibitors were initially selected using short-term experimental incubations on cultured cells, so it was important to explore their long-term effects *in planta*.

1-NOA and CHPAA phenocopy the aux1 mutant

The first objective was to explore whether these compounds could generate phenotypes in *Arabidopsis* seedlings or could alter the plants' responses to auxins such as 2,4-D or 1-NAA, known to be specific substrates for the influx carrier or the efflux carrier, respectively. The second objective was to compare the effects of both inhibitors with the characteristics of the *aux1* mutant, proposed to be defective in carrier-mediated auxin uptake (Marchant *et al.*, 1999). First we showed that 1-NOA and CHPAA selectively impaired the growth inhibitory effects of the influx carrier substrate 2,4D, yet neither compound had any effect on growth inhibition induced by the highly diffusible auxin 1-NAA (Figure 2). This differential effect of both inhibitors towards the two synthetic auxins is in favour of a selective inhibition of the auxin uptake mediated by the influx carrier *in planta*. Secondly, both inhibitors disrupted the bending response of gravity-stimulated *Arabidopsis* roots (Figure 3b,c). The effect of the influx carrier inhibitors 1-NOA and CHPAA in inducing agravitropic root growth appeared to phenocopy the *aux1* mutant, supporting the genetic evidence that the auxin influx carrier plays a role in root gravitropism (Marchant *et al.*, 1999). Thirdly, the auxin 1-NAA was able to bypass inhibition of the influx carrier by either compound and rescue the agravitropic phenotype of inhibitor-treated seedlings (Figure 3). Previous studies have determined that the agravitropic root growth phenotype of *aux1* mutants can be rescued by 1-NAA (Marchant *et al.*, 1999; Yamamoto and Yamamoto, 1998). Marchant *et al.* (1999) have also demonstrated that 1-NAA requires auxin efflux carrier activity in order to rescue the *aux1* agravitropic defect. These observations, together with the fact that neither compound is able to block polar auxin transport over a time scale of 90 min (Figure 4), suggest that 1-NOA and CHPAA are unlikely to block auxin efflux carrier activity *in planta*. Hence 1-NOA and CHPAA must cause an agravitropic phenotype by selectively blocking auxin influx carrier activity. Employing an auxin influx carrier during root gravitropism would overcome the biophysical limitations caused by auxin having to diffuse across the plant plasma membrane. Indeed, our ability to rescue *aux1* root gravitropism using 1-NAA (Figure 3; Marchant *et al.*, 1999) reflects, in part, the ability of a membrane-permeable auxin to overcome the limited diffusion of endogenous IAA in the absence of a functional auxin influx carrier such as AUX1.

These results demonstrate that the influx carrier inhibitors 1-NOA and CHPAA, applied to *Arabidopsis* seedlings, are able to phenocopy important characteristics exhibited by the *aux1* mutant (Marchant *et al.*, 1999).

We have reported that 1-NOA treatment is able to disrupt root gravitropism (Figure 3), yet does not affect the rate of root elongation (Figure 1). One simple explanation for the ability of 1-NOA to uncouple the regulation of root growth from gravitropic curvature is that distinct populations of root apical cells mediate each developmental process. This model is supported by the observation that maximal root growth occurs within the central elongation zone (CEZ), whilst gravitropic curvature is initiated close to the root apex, within a region termed the distal elongation zone (DEZ) (Baluska *et al.*, 1994; Ishikawa and Evans, 1995; Mullen *et al.*, 1998). Our results suggest that carrier-mediated auxin uptake is not needed to maintain the basal rate of root elongation in the CEZ, whereas DEZ cells require high rates of auxin influx to facilitate gravitropic root curvature. Significantly, CEZ and DEZ tissues have been observed to differ in their response to auxin (Baluska *et al.*, 1994; Ishikawa and Evans, 1995). Further insight into the role of the auxin influx carrier during the various phases of root cell elongation awaits immunolocalization of the auxin influx carrier component, AUX1.

Other features of 1-NOA and CHPAA

These two compounds were both shown to phenocopy the *aux1* mutant; however, some differences have been observed between 1-NOA and CHPAA, which require discussion.

CHPAA altered root elongation above 10 μM (Figure 1) and was responsible for an increased accumulation of *Nt-gh3* mRNA (Figure 5). These effects can result either from a weak auxin activity of CHPAA, or from an inhibition of the efflux carrier. NPA also gave a weak auxin-like response (Figures 1 and 5), as observed before (Kutt and Baker, 1966). Whether the slight auxin-like activity of NPA was a primary effect or resulted from increased endogenous auxin accumulation was not determined. CHPAA was not reported to inhibit auxin efflux on tobacco cell suspension (Imhoff *et al.*, 2000); has no effect on polar auxin transport in the *Arabidopsis* inflorescence (Figure 4); and its effect on root gravitropism can be bypassed by 1-NAA (Figure 3), which is not the case for NPA (Marchant *et al.*, 1999). In the absence of evidence for CHPAA-mediated inhibition of auxin efflux carrier activity *in planta* (Figures 4 and 5), it is likely that the induction of *Nt-gh3* gene expression and inhibition of root elongation by CHPAA is a result of its intrinsic auxin-like activity.

Up to the highest concentration assayed, 1-NOA did not inhibit root growth (Figure 1) and has no effect on the accumulation of *Nt-gh3* mRNA (Figure 5) or expression of *IAA2:GUS* in *Arabidopsis* (not shown), indicating that 1-NOA does not exhibit auxin-like activity. This result confirms previous data using the coleoptile straight-growth bioassay which demonstrated that 1-NOA had no auxin activity, in contrast to its isomer 2-NOA which has been demonstrated to show auxin activity in the same bioassay (Katekar, 1979). In tobacco cell suspensions, some inhibitory effect of 1-NOA was reported on the efflux carrier under prolonged treatments. However, 1-NOA was shown to be unable to block polar auxin transport in the *Arabidopsis* inflorescence (Figure 4). As discussed for CHPAA, the capacity of 1-NAA to rescue 1-NOA-mediated defects in root gravitropism suggests that the inhibitor acted selectively on the influx carrier. Considering the effect of NPA on root growth and gene activation, the absence of an effect of 1-NOA on these responses reinforces the idea that 1-NOA does not disturb the activity of the efflux carriers *in planta*.

In summary, we have demonstrated that the two influx carrier inhibitors 1-NOA and CHPAA are both able to phenocopy the main features of the *aux1* mutant. The data also support earlier genetic evidence showing that auxin influx carrier activity is required for *Arabidopsis* root gravitropism (Marchant *et al.*, 1999). We conclude that 1-NOA and, to a lesser extent, CHPAA provide useful tools to manipulate auxin influx carrier activity *in planta*, and will facilitate further studies on the physiological function(s) of auxin influx carriers.

Experimental procedures

Growth of Arabidopsis *seedlings*

Experiments were conducted using *Arabidopsis thaliana* seedlings ecotype Columbia (Col). Seeds were surface-sterilized (Forsthoefel *et al.*, 1992) and plated onto MS agar: 4.3 g l^{-1} MS salts (Sigma, Poole, Dorset, UK), 1% sucrose, 1% bacto-agar, pH to 6.0 with KOH. The plates were placed at 4°C for 48 h and then in constant light (50 mol m^2 sec^{-1}) at 22°C.

Assay for auxin sensitivity of root elongation

Seedlings were germinated on vertical plates of MS agar and grown for 3 days under constant light at 22°C. Seedlings were then individually transferred to a fresh plate containing test compounds at the required concentration, and the position of the primary root tip was marked. The plates were placed vertically under the same growth conditions as previously for a further 3 days. After this time the length of the root that had grown following transfer was measured, and the percentage inhibition calculated relative to a control with no test compound.

Assay for root gravitropism

Seedlings were sown on MS agar and placed vertically in the light for 16 h, after which time they were transferred to constant

darkness for a further 2 days. The plates were then turned through 90° maintaining a vertical orientation. After a further 24 h period the angle of growth of the root tip was measured relative to the horizontal where a completely gravitropic root forms a 90° angle.

Assay of IAA transport in inflorescence axes of A. thaliana

The procedure was adapted from Goldsmith (1982). Transport experiments were performed in a buffer containing 10 mM sucrose, 0.5 mM calcium sulfate, 10 mM 2-(*N*-morpholino)ethane sulfonic acid (Mes), and adjusted to pH 5.7 with KOH. *Arabidopsis thaliana* plants were grown in a greenhouse for 4–5 weeks under a 16 h daylight regime at 22°C. Segments (2 cm) were cut from the tip of each primary inflorescence. The segments were soaked for 10 min in 20 ml 1.5 μM IAA, with or without 25 μM 1-NOA, CHPAA or NPA. Segments were blotted dry, then placed in a 1.5 ml Eppendorf tube containing 20 μl 1.5 μM [^{3}H]IAA (234 TBq mole^{-1}; Amersham Pharmacia Biotech, Bucks, UK), in normal (acropetal transport) or inverted (basipetal transport) orientations. After a pulse of 5 min the segments were rinsed and returned to the original pre-incubation solutions. The total period of transport was 105 min. At the end of this period segments were cut from their basal ends into 2 mm pieces and [^{3}H]IAA was extracted using ethanol. Radioactivity was measured by liquid scintillation counting.

Preparation of tobacco seedling RNA

Nicotiana tabacum L. cv. Xanthi, wild-type line XHFD8 seeds (Muller *et al.*, 1985), were sterilized for 5 min in a solution of sodium hypochlorite (1.8% active chlorine), washed several times with sterile water, and incubated for 12 h at 4°C. Seeds were then dark-grown for 6 days in Gamborg's medium (Sigma) supplemented with 2% sucrose under moderate shaking (100 rpm) at 26°C. Auxins and auxin transport inhibitors were directly added in cultures at the concentrations indicated (Figure 5). Seedlings were collected after 1 h treatment in the dark. Total RNA was extracted from seedlings as described by Logemann *et al.* (1987). Total RNA (30 μg per track) was loaded, then electrophoresed through a 1% agarose gel containing 6% formaldehyde. RNA samples were transferred onto Hybond-N nylon membrane (Amersham Pharmacia Biotech) in 10 × SSC and fixed by UV cross-linking. The Megaprime DNA labelling system (Amersham Pharmacia Biotech) was used to label the full length *Nt-gh3* cDNA probe and the ubiquitin probe with α^{32}P-dCTP (Roux and Perrot-Rechenmann, 1997).

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