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# AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues

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**Plants employ a specialized transport system composed of separate influx and efflux carriers to mobilize the plant hormone auxin between its site(s) of synthesis and action. Mutations within the permease-like AUX1 protein significantly reduce the rate of carrier-mediated auxin uptake within *Arabidopsis* roots, conferring an agravitropic phenotype. We are able to bypass the defect within auxin uptake and restore the gravitropic root phenotype of *aux1* by growing mutant seedlings in the presence of the membrane-permeable synthetic auxin, 1-naphthaleneacetic acid. We illustrate that AUX1 expression overlaps that previously described for the auxin efflux carrier, AtPIN2, using transgenic lines expressing an *AUX1* promoter::*uidA* (GUS) gene. Finally, we demonstrate that AUX1 regulates gravitropic curvature by acting in unison with the auxin efflux carrier to co-ordinate the localized redistribution of auxin within the *Arabidopsis* root apex. Our results provide the first example of a developmental role for the auxin influx carrier within higher plants and supply new insight into the molecular basis of gravitropic signalling.**

*Keywords*: *Arabidopsis*/AUX1/auxin/gravitropism/transport

## Introduction

Plant hormones influence almost every aspect of plant growth and development (Davis, 1995). Auxins are considered unique amongst plant hormones in demonstrating a polarity in their movement (reviewed by Goldsmith, 1977). Indole-3-acetic acid (IAA), the major form of auxin in higher plants, is first synthesized within young apical tissues, then conveyed to its basal target tissues employing a specialized delivery system termed polar auxin transport (reviewed by Lomax *et al.*, 1995). IAA is transported into and out of the cell across the plasma membrane through the activities of the auxin influx and efflux carriers, respectively. The identification of auxin transport inhibitors such as 1-*N*-naphthylphthalamic acid (NPA) has greatly facilitated our understanding of the physiological importance of auxin transport (Katekar and Geissler, 1977). Perturbations in gravitropism, lateral root initiation, vascular differentiation and embryonic patterning represent examples of effects reported following treatment of plant tissues with NPA (reviewed by Bennett *et al.*, 1998).

All auxin transport inhibitors described to date target the phytotropin binding site within the auxin efflux carrier (reviewed by Lomax *et al.*, 1995). Morris *et al.* (1991) have proposed that the auxin efflux carrier comprises at least three components: a transmembrane carrier protein, an NPA binding protein and a third, labile regulatory component. Several genes have been identified within the model plant, *Arabidopsis thaliana* which encode putative auxin efflux carrier components. These genes include the bacterial transporter-like family of EIR/PIN/AGR sequences (Chen *et al.*, 1998; Galweiler *et al.*, 1998; Luschnig *et al.*, 1998; Muller *et al.*, 1998; Utsuno *et al.*, 1998); *TIR3*, which encodes (or regulates the activity of) the NPA binding protein based on the reduced rate of polar auxin transport and NPA binding activity of the *tir3* mutant (Ruegger *et al.*, 1997); and *RCN1*, an ortholog of regulatory subunit A of protein phophatase 2A, which represents a putative regulator of efflux carrier activity (Garbers *et al.*, 1996). Many of the morphological changes exhibited by *Arabidopsis* plants that are mutated in one of the putative efflux carrier components can be phenocopied in wild-type plants following NPA treatment (Okada *et al.*, 1991; Galweiler *et al.*, 1998), emphasizing the developmental importance of the auxin efflux carrier.

Auxin influx carrier activity was first described by Rubery and Sheldrake (1974). Physiologists have questioned the importance of carrier-mediated auxin uptake since the protonated form of IAA is capable of diffusing across plant membranes (Goldsmith, 1977). Determining the developmental importance of carrier-mediated IAA uptake has been hampered by the lack of suitable auxin influx-carrier specific inhibitors (Lomax *et al.*, 1995). Molecular genetic studies within *Arabidopsis* have led to the identification of the agravitropic mutant, *aux1* (Maher and Martindale, 1980). The *aux1* mutant of *Arabidopsis* displays an altered growth response to the auxins IAA or 2,4-dichlorophenoxyacetic acid (2,4-D) (Maher and Martindale, 1980). The *AUX1* gene has been cloned and found to encode a highly hydrophobic polypeptide featuring between 10 and 12 transmembrane spanning domains (Bennett *et al.*, 1996). Co-linearity with a family of plant amino acid permeases (Bennett *et al.*, 1996; Fischer *et al.*, 1998) has prompted suggestions that AUX1 performs a transport function, facilitating the uptake of the amino acid-like signalling molecule, IAA. The *aux1* mutant therefore provides a promising experimental tool with which to address the function of the auxin influx

carrier during *Arabidopsis* development. We report that mutations within AUX1 impair auxin influx carrier activity. Furthermore, we demonstrate that AUX1 regulates root gravitropism by facilitating auxin uptake within the root apical tissues.

## Results

### *The aux1 mutant is defective in carrier-mediated auxin uptake*

We have obtained several lines of evidence indicating that AUX1 regulates auxin uptake carrier activity within *Arabidopsis* roots. First, mutations within the *AUX1* gene selectively confer an altered root growth response towards auxins which require carrier-mediated uptake. Delbarre *et al.* (1996) have observed previously that the influx carrier facilitates the uptake of IAA and the synthetic auxin 2,4-D, but not the lipophilic auxin, 1-naphthaleneacetic acid (1-NAA), which enters the cell via diffusion (Figure 1A). We have examined whether there was any alteration in the response of *aux1* roots towards exogenously applied 2,4-D, IAA and 1-NAA using a root elongation bioassay. *Arabidopsis* wild-type and *aux1* seedlings were germinated in the presence of either 2,4-D, IAA or 1-NAA (Figure 1B, C and D, respectively). All three auxins inhibit wild-type *Arabidopsis* root elongation at the illustrated concentrations. In contrast, *aux1* root growth continues in the presence of either 2,4-D or IAA (Figure 1B and C) but is selectively inhibited by 1-NAA (Figure 1D). A reduced rate of auxin influx within *aux1* roots should impair the inhibitory properties of IAA and 2,4-D, but would fail to attenuate the effects of the membrane permeable auxin, 1-NAA. Dose–response curves confirm that *aux1* root growth exhibits a wild-type level of sensitivity towards 1-NAA (Figure 1E) but has at least a 10-fold increase in resistance to IAA and 2,4-D compared with the wild-type (Pickett *et al.* 1990). The selective response of *aux1* roots towards IAA and 2,4-D versus 1-NAA is therefore diagnostic of impaired hormone uptake.

We have further tested the auxin influx carrier model for AUX1 function by directly assaying the transport properties of *aux1* roots. Root segments from 3- to 5-week-old *Arabidopsis* plants grown in sterile liquid culture were incubated with radiolabelled auxins. As a substrate for both auxin influx and efflux carriers, IAA exhibits biphasic titration curves when incubated with plant tissues, leading to several reported experimental anomalies including increased levels of apparent IAA uptake in the presence of saturating concentrations of unlabelled IAA (Edwards and Goldsmith, 1980; Morris and Robinson, 1998). In the light of these observations we have used the synthetic auxin 2,4-D since it represents a substrate for the auxin influx carrier alone and therefore exhibits a monophasic titration curve (Delbarre *et al.*, 1996). Short-term auxin accumulation assays were performed in the presence or absence of excess unlabelled 2,4-D in order to measure diffusion versus total uptake, respectively (Figure 2A), and hence calculate the saturable, carrier-mediated uptake value (Figure 2B). The values illustrated within Figure 2 represent data collected from 20 independent uptake experiments using [$^{14}$C]2,4-D, each performed in triplicate. In summary, wild-type *Arabidopsis* roots accumulated >2-fold more [$^{14}$C]2,4-D than *aux1* (Figure 2). In contrast,

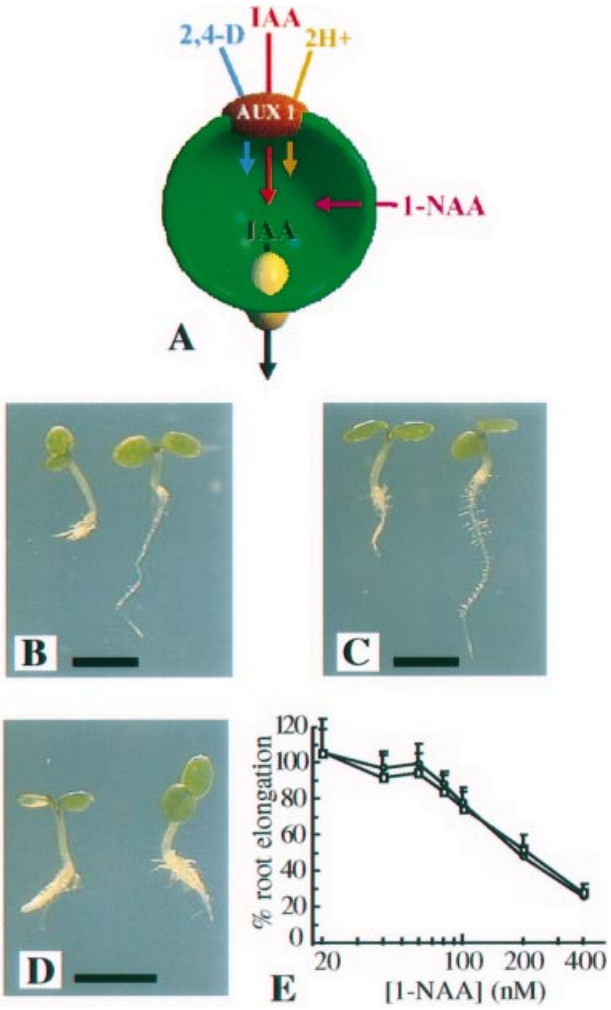


**Fig. 1.** (**A**) The auxins IAA, 2,4-D and 1-NAA adopt either carrier-mediated or diffusion-based modes of entry into plant cells, respectively. (B–E) The growth of the *aux1* mutant is less sensitive than wild-type to auxins requiring carrier-mediated uptake. Wild-type *Arabidopsis* (left) and *aux1-7* mutant seedlings (right) were germinated on MS agar containing either $10^{-7}$M 2,4-D (**B**), $2\times10^{-7}$M IAA (**C**) or $4\times10^{-7}$M 1-NAA (**D**) and grown for 5 days in constant white light. (**E**) Dose–response curve for wild-type (Columbia ecotype) and *aux1-7* root elongation in the presence of varying concentrations of 1-NAA (see Materials and methods). Results are expressed as a percentage relative to the growth on hormone-free medium. Bars, 4 mm.

no discernible difference between *aux1* and wild-type could be detected when identical uptake experiments were performed using the membrane-diffusible auxin 1-NAA, the IAA-like amino acid tryptophan or the acid-trap control, benzoic acid (data not shown). The selectivity of the transport defect within *aux1* roots is therefore consistent with an auxin uptake carrier function for the permease-like protein, AUX1.

### *The diffusible auxin 1-NAA is able to rescue the aux1 agravitropic root phenotype*

Our results suggest that the *aux1* mutant has reduced carrier-mediated auxin uptake activity (Figures 1 and 2). AUX1 could therefore regulate gravitropism by mediating the uptake of auxin into elongating root cells. We rationalize that if AUX1 function is specifically associated with auxin uptake, the membrane permeable auxin, 1-NAA should bypass the *aux1* lesion within auxin uptake and

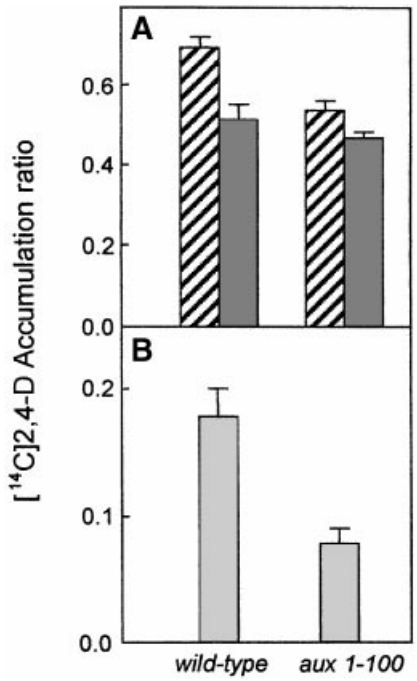


**Fig. 2.** The saturable uptake of the auxin 2,4-D is reduced in *aux1* mutant root cultures. (**A**) Root fragments from *Arabidopsis* wild-type and *aux1-100* mutant plants were incubated for 5 min with 150 nM [$^{14}$C]2,4-D. The [$^{14}$C]2,4-D accumulation ratio represents the ratio of the radioactivity retained per unit weight of root tissue to the radioactivity per unit volume of incubation medium (Materials and methods). Ratios are mean (± SE) of values obtained in 20 independent experiments, each in triplicate. (A) Total and diffusion-based accumulations were measured in the absence or presence of 50 μM unlabelled 2,4-D, respectively. (**B**) The saturable uptake of the auxin 2,4-D is reduced in *aux1* mutant root cultures. The saturable component of [$^{14}$C]2,4-D accumulation was calculated by subtracting diffusion-based from total accumulation.

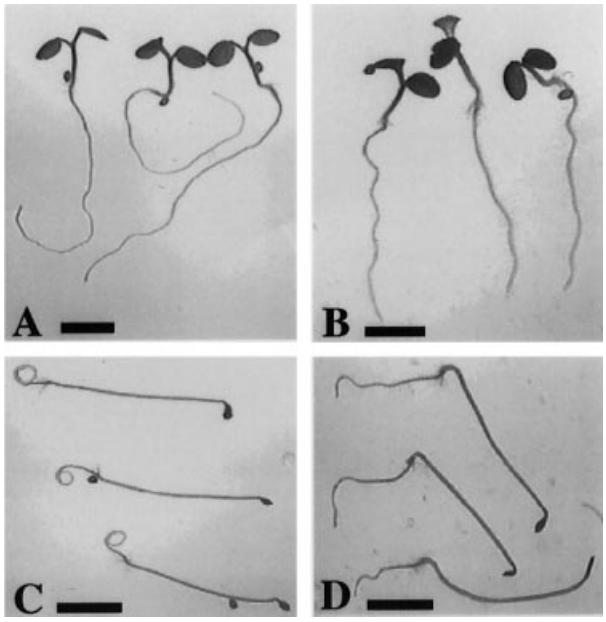


**Fig. 3.** The lipophilic auxin 1-NAA is able to restore the gravitropic root growth of the *aux1* mutant. Mutant *aux1-7* seedlings were germinated in the absence (**A** and **C**) or presence (**B** and **D**) of $10^{-7}$M 1-NAA. Seedlings were either grown vertically for 5 days in constant white light (A and B) or placed vertically in white light for 24 h followed by 48 h in the dark, then turned through 90° and grown for a further 24 h in the dark (C and D). Bars, 3 mm.

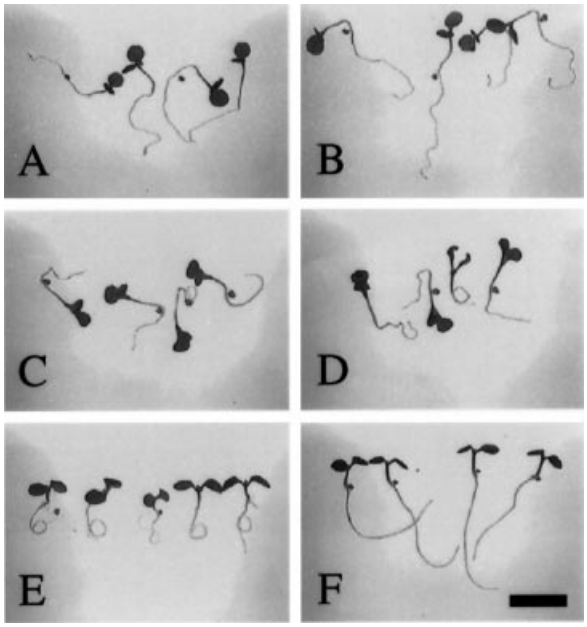


**Fig. 4.** (A and D) The agravitropic root growth phenotype of the auxin response mutants *axr2* and *axr3* cannot be rescued by 1-NAA. The *axr2-1* and *axr3-1* mutants were germinated on either hormone-free medium (**A** and **C**) or medium containing $10^{-7}$M 1-NAA (**B** and **D**) and grown vertically in constant white light for 5 days. (**E** and **F**) The agravitropic root growth phenotype of *aux1* can only be partially rescued by 2,4-D. The *aux1-7* mutant was germinated on either hormone-free medium (E) or medium containing $2\times10^{-7}$M 2,4-D (F) and grown vertically in constant white light for 5 days. Bar, 5 mm.

restore root gravitropism. We have tested whether 1-NAA could rescue the *aux1* agravitropic root phenotype by germinating mutant seedlings on medium containing levels of 1-NAA well below the $IC_{50}$ for root elongation (Figure 1E). On hormone-free medium, roots of *aux1* seedlings grew in a randomized manner (Figure 3A), whereas mutant roots germinated in the presence of $10^{-7}$ M 1-NAA grew vertically, exhibiting a positive gravitropic response (Figure 3B). Similarly, 1-NAA was also able to fully restore a root bending response within gravity-stimulated dark grown *aux1* seedlings (Figure 3C and D), confirming that 1-NAA rescued gravitropism (rather than phototropism) within mutant roots. The ability of 1-NAA to restore gravitropic root growth within *aux1* seedlings exhibited a dose-dependent relationship, in which the direction of *aux1* root growth became progressively more randomized at hormone concentrations $<10^{-7}$ M (data not shown).

In order to demonstrate the specificity of the effect of 1-NAA on the *aux1* mutant phenotype we have performed an identical experiment using the agravitropic auxin-response mutants, *axr2* (Wilson *et al.*, 1990) and *axr3* (Leyser *et al.*, 1996). Roots of both *axr2* and *axr3* seedlings appeared plagiotropic when grown in the absence (Figure 4A and C) or presence of 1-NAA (Figure 4B and D). Our inability to bypass the agravitropic defect within the *axr3* mutant, for example, is likely to reflect that the *AXR3* sequence represents a member of the *Aux*/*IAA* gene family (Abel *et al.*, 1995) which encodes nuclear-targeted proteins that are proposed to function as transcription factors (Rouse *et al.*, 1998).

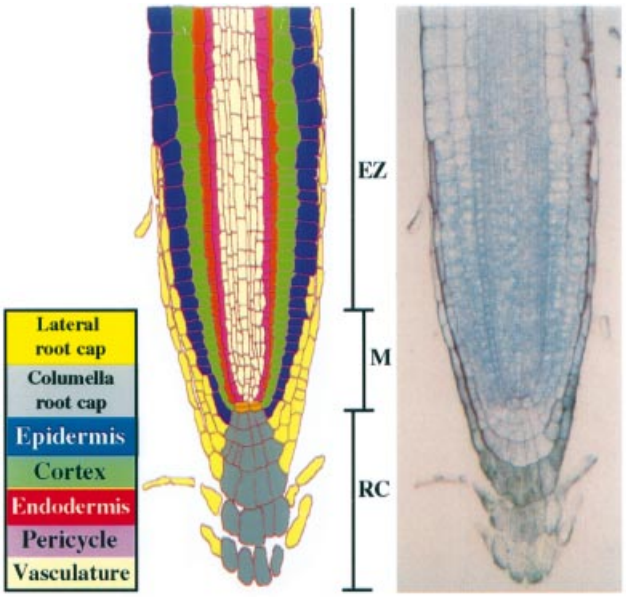


**Fig. 5.** (**A**) Schematic illustration of root apical tissues delineating root cap (RC), meristematic (M) and elongation zone (EZ). (**B**) The *AUX1* promoter drives GUS reporter gene expression within the majority of tissues within the primary root apex with the exception of the upper three tiers of columella cells within the root cap. Nine-day transgenic *Arabidopsis* seedlings were stained for GUS activity using conditions designed to minimize diffusion (see Materials and methods), embedded, then sectioned and viewed under bright field.

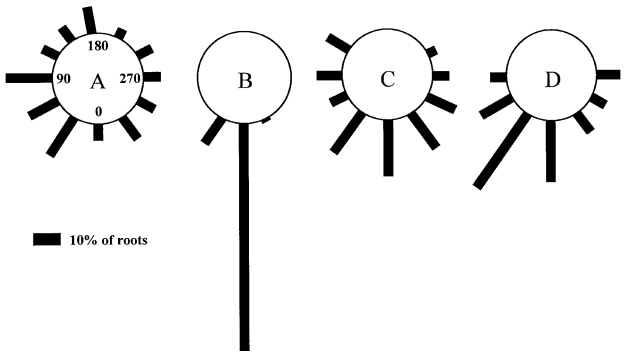


**Fig. 6.** NPA is able to block 1-NAA-mediated rescue of the *aux1* agravitropic mutant phenotype. Mutant seedlings were germinated on either hormone free medium (**A**), or medium containing $10^{-7}$M 1-NAA (**B**), $2\times10^{-7}$M NPA (**C**) or $2\times10^{-7}$M NPA and $10^{-7}$M 1-NAA (**D**) and grown for 5 days in constant white light. The orientation of root growth of 50 independent *aux1* seedlings was measured after 5 days. Each root was assigned to one of twelve 30° sectors. The length of each bar represents the percentage of seedlings showing direction of root growth within that sector. (A) Roots of untreated *aux1* roots remained plagiotropic; whereas (B) 1-NAA treatment completely restores gravitropism, (C) NPA has little effect on the plagiotropic phenotype of *aux1* roots, but (D) blocks the ability of 1-NAA to rescue the *aux1* gravitropic phenotype.

The synthetic auxin 2,4-D would not be expected to be as efficient in bypassing the *aux1* lesion due to the requirement for its carrier-mediated uptake (Figure 1A). We observed that roots of *aux1* seedlings germinated in the presence of $2\times10^{-7}$ M 2,4-D exhibited reduced root coiling (Figure 4F) in contrast to the seedlings grown on hormone-free medium (Figure 4E). However, the restoration of root gravitropism as obtained with 1-NAA (Figure 3B) was never observed using a similar range of 2,4-D concentrations below its $IC_{50}$ for root elongation (data not shown).

### *AUX1 is expressed within tissues associated with gravitropic signal transduction*

The coordinated redistribution of auxin within the root apex has been proposed to regulate root curvature following a gravitropic stimulus (Evans, 1991). We have previously observed that the *AUX1* mRNA is localized to the primary root apex using whole-mount *in situ* hybridization (Bennett *et al.*, 1996). We have performed a more detailed examination of the *AUX1* expression pattern within the *Arabidopsis* primary root in order to pinpoint the specific tissues within which AUX1 functions. A 2.2 kbp *AUX1* promoter fragment was fused to the *uidA* (GUS) reporter gene (Jefferson *et al.*, 1987) and transformed into wild-type *Arabidopsis* (May *et al.*, 1998). All transgenic lines selected expressed the GUS reporter gene in an identical pattern within *Arabidopsis* root and shoot meristematic tissues. The spatial expression of the GUS reporter within the primary root apex for one of these transgenic lines, termed LL4, was characterized in greater detail.

A longitudinal section through a GUS-stained LL4 root highlights the tissue organization of the *Arabidopsis* primary root apex (Figure 5). The collection of cuboid-shaped cells at the apex compose the columella tissue which senses changes in root orientation using starch filled plastids termed statoliths (Blancaflor *et al.*, 1998). The root meristem, lying immediately behind the columella tissues, represents the zone of root cell division. The files of cells originating from the meristem demarcate the individual tissues which make up the mature root (Dolan *et al.*, 1993). Each cell progressively enlarges longitudinally as it passes through the zone of elongation. GUS staining can be detected within every tissue of the LL4 root which is distal to its meristem, extending back to the distal and central elongation zones (Figure 5). Closer scrutiny reveals that the meristematic initials, their elongating daughter cells and the lateral root cap stain for GUS activity. In contrast, little or no GUS activity could be detected within the upper three tiers of the gravity-sensing columella cells within the root cap. *AUX1* expression is therefore most closely associated with root tissues which transduce and respond to, rather than initially perceive, the gravitropic stimulus.

### *1-NAA requires auxin efflux carrier activity to rescue aux1 root gravitropism*

The spatial expression of AUX1 within the epidermal and cortical tissues of the root apex (Figure 5) overlaps with the pattern recently described for the putative auxin efflux carrier component, AtPIN2 (Muller *et al.*, 1998). As a substrate for the auxin efflux carrier (Delbarre *et al.*, 1996), the ability of 1-NAA to rescue the *aux1* agravitropic phenotype (Figure 3B and D; Figure 6B) may reflect its capacity to enter root cells by diffusion and be remobilized via the auxin efflux carrier. In order to investigate the requirement for auxin carrier activity to redistribute 1-NAA within root apical tissues following gravistimulation, we have examined whether the efflux carrier inhibitor, NPA, is able to disrupt the ability of 1-NAA to rescue the *aux1* agravitropic phenotype. NPA treatment clearly reverses the ability of 1-NAA to rescue *aux1* gravitropism

(Figure 6D), whereas the addition of NPA alone has little effect on the *aux1* agravitropic phenotype (Figure 6C) though the roots tend to be less tightly coiled. Our observations suggest that the ability of 1-NAA to rescue the *aux1* agravitropic phenotype reflects its polar movement within root apical tissues through its capacity to enter cells by diffusion and then act as a substrate for auxin efflux carrier activity.

## Discussion

### *The permease-like AUX1 polypeptide represents a component of the auxin influx carrier machinery*

We report several independent lines of evidence which conclude that the *AUX1* gene encodes a component of the auxin influx carrier. Auxin accumulation experiments demonstrate that *aux1* roots have a significantly reduced capacity to mediate the uptake of the auxin influx carrier substrate, 2,4-D (Figure 2), yet retain a wild-type level of uptake for the membrane permeable auxin 1-NAA. Mutations within AUX1 selectively impair the action of auxins that require carrier-mediated uptake (Figure 1). The selective response of *aux1* towards IAA and 2,4-D versus 1-NAA contrasts with that described for other auxin signalling mutants such as *axr2* which indiscriminately perturbs responses towards all three hormones (Wilson *et al.*, 1990). Evans *et al.* (1994) have observed previously that elongating *aux1* roots exhibited a significant delay in their response to inhibitory levels of IAA compared to *axr2* and wild-type *Arabidopsis*, prompting the authors to suggest that the *aux1* mutant was defective for auxin uptake.

The *AUX1* gene encodes a highly hydrophobic polypeptide containing 10–12 transmembrane spanning domains whose primary sequence exhibits homology with a family of plant and fungal amino acid transport proteins (Bennett *et al.*, 1996; Fischer *et al.*, 1998). However, *aux1* is unlikely to represent an amino acid permease mutation since wild-type and *aux1* roots exhibit identical rates of uptake for the indole amino acid tryptophan. AUX1 lacks sequence homology with the recently described family of auxin efflux carrier proteins which appear related to a family of bacterial transporters (Chen *et al.*, 1998; Galweiler *et al.*, 1998; Luschnig *et al.*, 1998; Muller *et al.*, 1998; Utsuno *et al.*, 1998). Auxin influx and efflux carrier proteins therefore appear to have originated from separate families of transporter sequences.

We note that the modified phytohormone response signatures of *aux1* and *Atpin2* mutants parallel the known substrate specificities of the auxin influx and efflux carriers, respectively (Delbarre *et al.*, 1996). The increased resistance of *aux1* roots towards exogenous IAA and 2,4-D contrasts with the elevated sensitivity of *Atpin2* roots towards 1-NAA (Chen *et al.*, 1998; Muller *et al.*, 1998). Mutations within *AUX1* and *AtPIN2* also confer elevated resistance towards the plant hormone ethylene and its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) (Pickett *et al.*, 1990; Chen *et al.*, 1998; Luschnig *et al.*, 1998; Muller *et al.*, 1998). Epistasis experiments indicate that *AUX1* and *AtPIN2* (*EIR1*) act downstream of the ethylene receptor *ETR1* and its signal transduction component, *EIN2* (Roman *et al.*, 1995). Ethylene has been demonstrated to influence polar auxin transport within etiolated pea epicotyls by down-regulating auxin efflux carrier activity (Suttle, 1988). Such observations suggest that ethylene mediates many of its growth regulatory effects by acting as a global regulator of the auxin transport machinery.

### *Root gravitropism requires auxin influx carrier activity*

Auxin transport plays an important role during root gravitropism. Earlier workers have demonstrated that roots treated with inhibitors which block auxin efflux carrier activity disrupt gravitropism (reviewed by Lomax *et al.*, 1995). Results presented in this study highlight the importance of auxin influx carrier activity for root gravitropism.

We have demonstrated that roots of the *aux1* mutant have impaired carrier-mediated auxin uptake activity (Figures 1 and 2). Artificially elevating the rate of phytohormone uptake into plant cells by growing mutant seedlings in the presence of the membrane-diffusible auxin 1-NAA restores root gravitropism (Figure 3). In contrast, the less diffusible auxin 2,4-D only partially restores root gravitopism (Figure 4). We conclude that the reduced rate of auxin uptake into root apical cells represents the physiological basis for the agravitropic root phenotype of the *aux1* mutant. Moreover, the response of *aux1* roots towards 1-NAA (Figures 1 and 3) suggests that the auxin signalling machinery within the *aux1* root is essentially intact and that the biochemical defect is limited to the auxin uptake machinery. This contrasts with the agravitropic phenotype of the mutant *axr3* which cannot be bypassed by 1-NAA (Figure 4), in agreement with its genetic lesion disrupting a later step within the auxin signalling pathway (Rouse *et al.*, 1998).

### *Root gravitropism requires local auxin transport mediated by auxin influx and efflux carrier components AUX1 and AtPIN2*

Microautoradiographic studies have highlighted the presence of two separate auxin transport streams within roots (Tsurumi and Ohwaki, 1978). The polar auxin transport stream mediates basipetal, long distance movement of IAA from its apical site(s) of synthesis, whereas during local auxin transport within the root apex IAA is redistributed acropetally amongst the rapidly dividing and growing cells of the meristematic and elongation zones, respectively (Figure 7). Components from both auxin transport streams have recently been identified (Bennett *et al.*, 1996; Chen *et al.*, 1998; Galweiler *et al.*, 1998; Luschnig *et al.*, 1998; Muller *et al.*, 1998; Utsuno *et al.*, 1998). These studies have concluded that AUX1 and AtPIN2 are expressed within root apical tissues mediating local auxin transport (Figure 5; Muller *et al.*, 1998), whereas AtPIN1 is localized within vascular tissues associated with polar auxin transport (Galweiler *et al.*, 1998). Until these recent studies, the relative importance of local versus polar auxin transport in root gravitropism had been unclear since phytotropins such as NPA block both auxin transport pathways. Molecular genetic studies have now demonstrated that mutations within *AUX1* and *AtPIN2* (but not *AtPIN1*) cause agravitropic root phenotypes (this study; Chen *et al.*, 1998; Galweiler *et al.*, 1998; Luschnig *et al.*, 1998; Muller *et al.*, 1998; Utsuno *et al.*, 1998), indicating that local auxin transport is of primary importance to root gravitropism.

We have presented evidence that auxin influx and

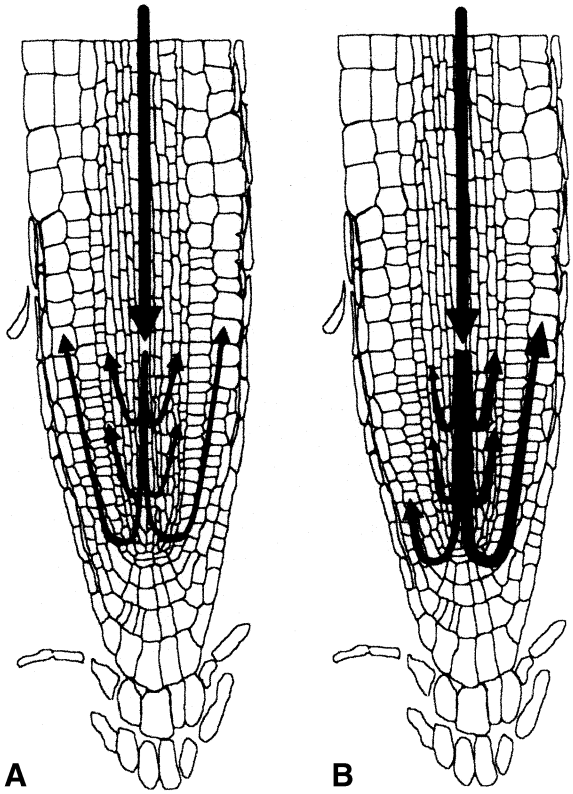


**Fig. 7.** Contrasting models for the role of auxin transport during root gravitropism featuring either facilitative (**A**) or regulatory (**B**) modes of action for auxin. The (upper) single, large arrow signifies polar auxin transport whereas the (lower) bifurcated arrow represents local auxin transport mediating hormone redistribution within root apical tissues.

efflux carriers act in unison to coordinate the localized redistribution of IAA within root apical cells (Figure 6). Our model is supported by the observation that auxin influx and efflux carrier components AUX1 and AtPIN2 are expressed in an overlapping pattern within root apical tissues (Figure 5; Muller *et al.*, 1998). The AUX1 transcript is expressed within all root apical tissues from the root cap to the distal/central elongation zone with the exception of the upper three tiers of the columella tissues (Figure 5). The AtPIN2 protein has been immunolocalized within epidermal and cortical cells of the distal/central elongation zone (Muller *et al.*, 1998) which represents a subset of the tissues expressing the GUS transgene under the control of the *AUX1* promoter (Figure 5). Significantly, the AtPIN2 protein has been immunolocalized to the basal end of elongating root cells (Muller *et al.*, 1998), prompting suggestions that its asymmetric subcellular distribution underpins the localized redistribution of auxin within the root apex (Figure 7). We are currently attempting to determine whether AUX1 is also required to be localized asymmetrically within root apical cells, in an apical orientation, to channel auxin redistribution.

### *Auxin: gravitropic regulator or permissive signal?*

The Cholodny–Went hypothesis proposes that the auxin transport machinery regulates gravitropism by creating an asymmetric gradient of auxin within root apical tissues (Evans, 1991). Our results indicate that the influx carrier is unlikely to create a differential auxin distribution during gravitropism. The restoration of gravitropism using the membrane-diffusible auxin 1-NAA suggests instead that the influx carrier is simply required to overcome the biophysical limitations of membrane diffusion imposed on the endogenous auxin, IAA (Figure 3). Our observations therefore infer that the influx carrier acts to facilitate, rather than regulate, the uptake of auxin during a gravitropic root bending response. Nevertheless, we and others have demonstrated that the efflux carrier is also required during a gravitropic response (Figure 6; Chen *et al.*, 1998; Galweiler *et al.*, 1998; Luschnig *et al.*, 1998; Muller *et al.*, 1998; Utsuno *et al.*, 1998). However, we are currently unable to ascertain whether the efflux carrier acts by facilitating symmetrical auxin movement (Figure 7A), or undertakes a more complex role, to create a gradient of auxin following a gravitropic stimulus (Figure 7B). If the facilitator model operated (Figure 7A), the efflux carrier would simply act in unison with the auxin influx carrier to facilitate the symmetrical redistribution of auxin within wild-type root apical tissues. Within this model, no hormone gradient would be formed and auxin would act instead as a permissive signal. Alternatively, if auxin performed the regulatory function attributed to the phytohormone within the Cholodny–Went hypothesis (Figure 7B), it would have to pass through the auxin efflux carrier to impose the formation of a gradient.

Several researchers have questioned the original evidence supporting auxin redistribution-based models for root gravitropism, arguing that auxin levels were frequently measured following the completion of a gravitropic response and that logarithmic (rather than arithmetic) differences between upper and lower surfaces were necessary to account for the dramatic changes in organ elongation (Trewavas, 1992). However, several studies have demonstrated that auxin redistribution occurs prior to a gravitropic response (Harrison and Pickard, 1989; Parker and Briggs, 1990; Young *et al.*, 1990; Iono, 1991) and that arithmetic changes in auxin distribution are sufficient to account for gravitropic curvature (Migliaccio and Rayle, 1989). Nevertheless, an exclusively auxin transport-based model cannot fully account for the complex pattern of elongation which occurs on the upper and lower side of a gravity stimulated organ (Ishikawa *et al.*, 1991). For example, a transport-based model could not explain how tissues on the upper and lower surfaces reverse their relative growth rates prior to the root reaching the vertical. In response to these observations, Evans (1991) has proposed a model which better describes the dynamic changes in elongation taking place within gravitropically stimulated root tissues, combining asymmetric changes in auxin transport rates with alterations in tissue sensitivity. Changes in auxin sensitivity are proposed to arise as a result of adaptation by root cells following the initial gravity-induced redistribution of IAA by the auxin transport machinery. Whilst seeming complex, the identification of gravitropic regulators such as AUX1, PIN2 and AXR3 provides an ideal starting point with which to dissect such a challenging biological question.

## Materials and methods

### *Assay for auxin sensitivity of root growth*

Wild-type (Columbia ecotype) and *aux1-7* (Pickett *et al.*, 1990; Bennett *et al.*, 1996) seed was surface sterilized (Forsthoefel *et al.*, 1992) and

sown onto MS agar [4.3 g/L MS salts (Sigma), 1% sucrose and 1% bactoagar, pH to 6.0 with 1 M KOH] containing either $10^{-7}$ M 2,4-D, $2\times10^{-7}$ M IAA or $4\times10^{-7}$ M 1-NAA. The seed was vernalized in the dark at 4°C for 48 h and then germinated vertically under constant light conditions at 22°C for 5 days. The dose–response curve of wild-type and *aux1-7* seedlings to 1-NAA was determined as follows. Seed were surface sterilized and sown on MS agar, vernalized in the dark at 4°C for 48 h and then germinated vertically under constant light conditions at 22°C for 3 days. Seedlings (15 per concentration) were individually transferred to MS agar plates containing various concentrations of 1-NAA together with a hormone-free control. The seedlings were grown vertically for a further 3 days under the same growth conditions as previously described and the increase in root length after transfer measured. The percentage inhibition was determined by comparison to the growth of the hormone-free control.

### *Auxin uptake assay*

Sterile seeds (three per culture) were placed in a 250 ml flask containing 100 ml of Gamborg's B5 medium containing 20 g/L sucrose. The flasks were wrapped in foil leaving a 1 cm gap near the top to allow a small amount of light to enter and placed on a rotating shaker (20–30 r.p.m.) at a temperature of 22°C. Root material from 3- to 5-week-old plants grown in liquid culture was collected, rinsed with 2×100 mL of uptake buffer (10 mM sucrose, 0.5 mM calcium sulfate and 20 mM 2-(*N*-morpholino)ethane sulfonic acid (Mes), pH to 5.7 with KOH), then transferred into 100 mL of fresh uptake buffer. Roots were cut into pieces of ~5 mm length. The fragments (0.3–0.4 g wet weight) were placed in 25 mL vials and made up to 4 g with uptake buffer. The uptake reaction was initiated by adding 200 μL of a solution containing [$^{14}$C]2,4-D (2.07 TBq/mol from Amersham-France, Les Ulis, France; 150 nM final concentration) plus or minus unlabelled 2,4-D (50 mM final concentration). After 5 min of incubation, the root fragments were filtered onto GFC glass fibre filters (Whatman International, Maidstone, UK) under suction to rapidly remove the incubation medium and rinsed with 2× 5 mL ice-cold water, then weighed. The radioactivity, extracted from tissue with 1 mL ethanol, was counted by liquid scintillation with quenching correction. The auxin accumulation ratio was defined as the ratio of the radioactivity retained per unit weight of tissue (Bq/mg/FW) to the radioactivity per unit volume of incubation medium (Bq/μL).

### *Assay for root gravitropism*

Seed was surface sterilized, vernalized in the dark at 4°C for 48 h and then germinated vertically in constant white light at 22°C for 24 h. The plates were then placed in the dark at 22°C maintaining the same orientation and allowed to grow vertically for a further 2 days. The plates were then turned through 90° and grown for a further day under the same growth conditions before scoring for reorientation of root growth.

### *Plasmid constructions*

All cloning procedures used standard techniques (Sambrook *et al.*, 1989). The *AUX1* promoter (2.6 kb) including the first exon and intron (0.9 kb) prior to the translation start site was cloned as a *Hin*dIII–*Bam*HI fragment into the corresponding sites of the pBI121 GUS expression vector (Jefferson *et al.*, 1987). Recombinant binary vectors were transformed into *Agrobacterium tumifaciens* strain C58 using the electroporation protocol (Wen-jun and Forde 1989). *Arabidopsis* plants were transformed using the vacuum infiltration procedure (Bechtold *et al.*, 1993). Seed from the vacuum infiltrated plants was sterilized and sown on MS medium containing kanamycin (50 μg/mL) and grown under constant light at 22°C for 21 days. Resistant plants were transferred to a 1:1 mixture of compost and vermiculite under long-day conditions (16 h light) at 22°C. Plants homozygous for the transgene were selected in the $F_2$ generation and analysed further.

### *Histochemical staining and sectioning of transgenic Arabidopsis roots*

Glucuronidase (GUS) activity was assayed in a buffer containing 50 mM sodium phosphate (pH 7.0), 10% (v/v) methanol and 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (Melford Laboratories, Ipswich, UK). Samples were incubated in 1 mL of GUS buffer at 37°C in the dark for 3 h. GUS stained roots were placed in fixation solution (4% glutaraldehyde, 4% formaldehyde, 50 mM sodium phosphate buffer pH 7.2) for 3 h at 20°C. A serial ethanol dehydration was then performed [30, 50, 70, 90, 95% (twice)] at room temperature for 1 h at each step. Samples were embedded in Technovit 7100 resin (Heraeus Kulzer) according to the manufacturer's instructions. Sections (4 μm) were cut, dried onto glass slides and stained for 8 min in an aqueous 0.05% ruthenium red solution. The samples were mounted in DePeX (BDH, Poole, UK) prior to photography.

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