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Short Communication

Differential Effects of 1-Naphthaleneacetic Acid, Indole-3-Acetic Acid and 2,4-Dichlorophenoxyacetic Acid on the Gravitropic Response of Roots in an Auxin-Resistant Mutant of Arabidopsis, *aux1*

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The agravitropic nature of root growth of an auxin-resistant mutant of Arabidopsis, *aux1*, was restored when the synthetic auxin 1-naphthaleneacetic acid (NAA) was added to the growth medium; *aux1* roots were not resistant to NAA. Neither indole-3-acetic acid nor 2,4-dichlorophenoxyacetic acid had the same effects as NAA. These differential effects of the three auxins on *aux1* defects suggest that *AUX1* may encode the auxin influx carrier according to the model proposed by Delbarre et al. [(1996) *Planta* 198: 532].

Key words: *Arabidopsis thaliana* — *AUX1* — Auxin influx carrier — Auxin-resistant mutant — Gravitropism of root — Growth of root.

aux1 is an auxin-resistant mutant of Arabidopsis that can grow in the presence of natural and synthetic auxins, IAA and 2,4-D (Maher and Martindale 1980, Mirza et al. 1984). It is also resistant to ethylene (Pickett et al. 1990, Roman et al. 1995) and inhibitors of the auxin efflux carrier (Fujita and Syono 1996). Morphological defects of *aux1* are confined to roots; in particular the gravitropic response of *aux1* roots is severely impaired (Maher and Martindale 1980, Mirza et al. 1984, Mirza 1987, Okada and Shimura 1992, Roman et al. 1995, Timpte et al. 1995). Recently the *AUX1* gene has been cloned by T-DNA tagging, and shown to encode a protein similar to amino-acid permeases. The structural similarity of IAA to tryptophan suggests that AUX1 protein could be an auxin influx carrier (Bennett et al. 1996). In fact, it has been suggested that *aux1* is defective in auxin uptake since the latent period for inhibition of root growth by auxin was longer for roots of *aux1* than for other auxin-resistant mutants (Evans et al. 1994).

If the *aux1* phenotype results from lower intracellular concentrations of auxin due to the lack of a functional auxin influx carrier, exogenously added auxin may rescue defects in *aux1*. We therefore determined the effects of IAA, NAA and 2,4-D on the gravitropic responses and growth of *aux1* roots. To our surprise, we found that only NAA corrected the aberrant gravitropic response to the normal level, and that *aux1* was not resistant to NAA at all. Discussion of these results with reference to differential uptake of auxins by tobacco suspension-cultured cells reported by Delbarre et al. (1996) strongly suggests that the *AUX1* product is the auxin influx carrier.

Seeds of *aux1*, derived from *Arabidopsis thaliana* Columbia ecotype, were surface-sterilized, as described by Watahiki et al. (1995), and were plated on nutrient agar that contained half-strength MS salts (Murashige and Skoog 1962), 1%(w/v) sucrose, half-strength B5 vitamin (Gamborg et al. 1968), and 1%(w/v) agar. They were kept at 4°C for two d, and then at 22°C for 24 h under continuous white light (76 W m^{-2}) to induce germination. They were further grown at 22°C in darkness for 4 d on vertically-held plates. Photographs of the seedlings were taken from the back of the plate through the agar medium. The root length and the root-tip angle from the vertical were determined for each seedling using image-analyzing software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, U.S.A.). The angle was measured from the vertical counterclockwise.

Since roots of wild-type Columbia (Okada and Shimura 1992, Liscum and Briggs 1996) and *aux1* mutants (Mirza 1987) exhibit negative phototropism, we grew seedlings in darkness to avoid the effects of light. The plants were grown on a medium containing 1% sucrose because growth of Arabidopsis roots in darkness is slow in the absence of sugars (Kurata and Yamamoto 1997). After growth for 4 d in the dark in the presence of various concentrations of auxin, the length and direction of growth of roots were determined for the wild type and *aux1*. A series of experiments with NAA is shown in Fig. 1, in which the root length is plotted against the root-tip angle from the vertical for each seedling. The mean and the standard deviation (SD) of the root-tip angles can give a measure of root gravitropism of a given genotype (Liscum and Hangarter 1993, Golan et al. 1996). Thus, dose-dependent effects of NAA, IAA and 2,4-D on the two parameters of root gravitropism are shown in Fig. 2. Their effects on root growth are also shown in Fig. 3.

Abbreviations: NAA, 1-naphthaleneacetic acid; SD, standard deviation.

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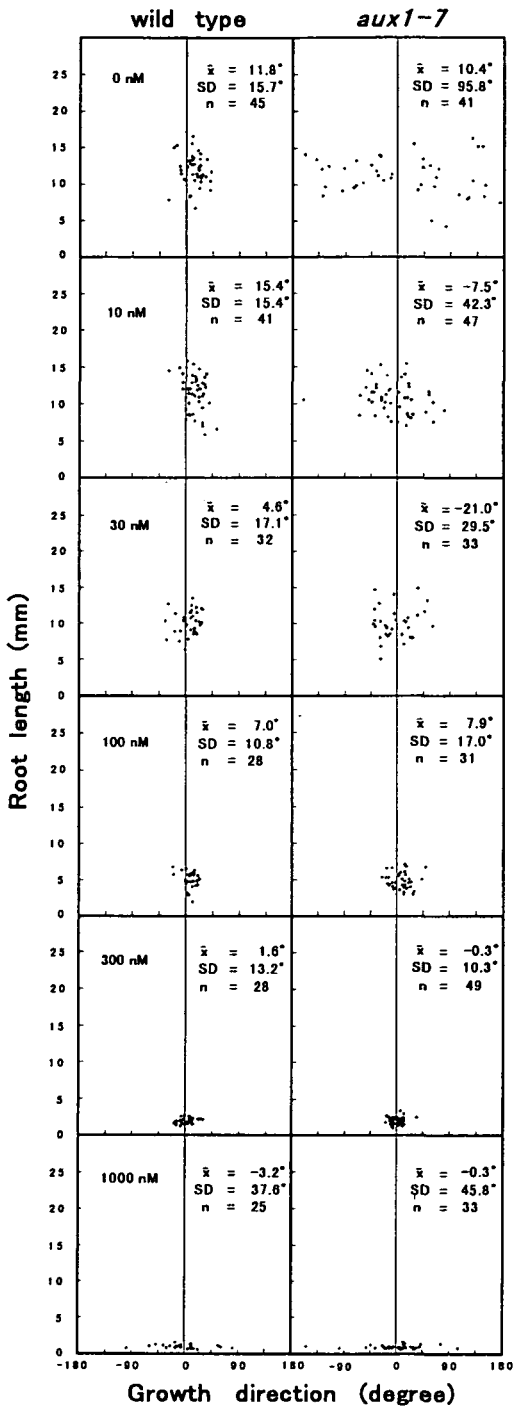

Fig. 1 Effects of NAA on root growth and the direction of growth of root tips of wild-type (left panels) and *aux1-7* (right panels) seedlings. After induction of germination, seedlings were grown in the dark for 4 d at 22°C. The results from each seedling were plotted according to root length and growth direction of root tip. The direction of gravity is shown as 0°, and the angle was measured counterclockwise.

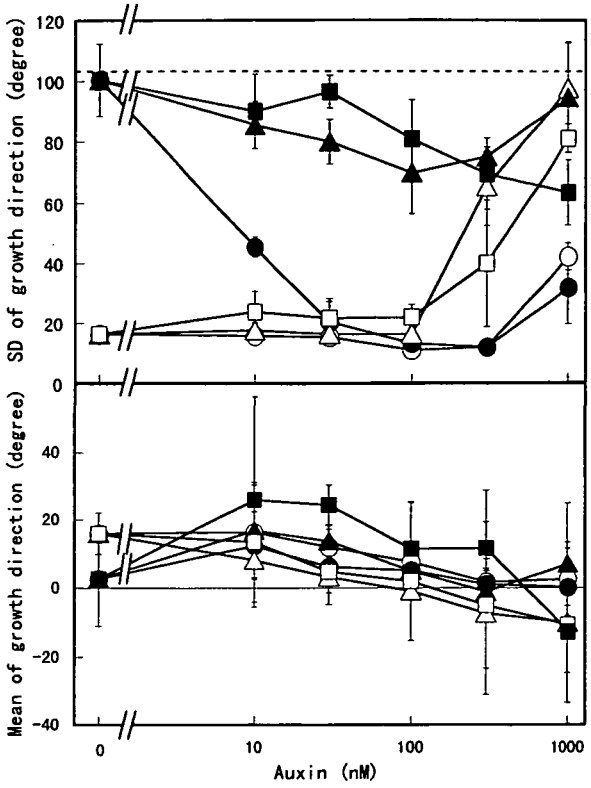

Fig. 2 Effects of NAA (○, ●), 2,4-D (△, ▲) and IAA (□, ■) on the mean (bottom) and the standard deviation (SD) (top) of the growth direction of root tips in wild-type (open symbol) and *aux1-7* (closed symbol) seedlings grown in the dark for 4 d at 22°C. Error bars show the SD of at least 3 independent experiments, in which 28–50 seedlings were used. The broken line in the top panel shows the level of SD in perfect agravitropic roots (103.9°).

Wild-type roots showed strong positive gravitropism: the mean root-tip angle only slightly deviated from 0°, and the SD was as small as 16.3±2.6° (n=13) (Fig. 1, top and Fig. 2). In *aux1-7* roots, no gravitropism was observed because the SD of the root-tip angles was 100.2±12.1° (n=9) (Fig. 1, top and Fig. 2), which agreed well with 103.9° ($=180°/\sqrt{3}$), the theoretical SD value if there is no preferential distribution in direction of growth (see Appendix). This is consistent with previous reports on the agravitropic nature of *aux1* roots (Maher and Martindale 1980, Mirza et al. 1984, Mirza 1987, Okada and Shimura 1992, Roman et al. 1995, Timpte et al. 1995), although no quantitative analysis has been carried out on the *aux1* root gravitropism.

When *aux1-7* seedlings were germinated and grown on a culture medium containing 10 nM NAA, the SD of the root-tip angle was smaller than that observed in the absence of NAA (Fig. 1, 2); the growth rate of the roots was not changed significantly by this concentration of NAA (Fig. 1, 3). In wild-type roots, neither growth rate nor gravitropism was affected by 10 nM NAA. Addition of

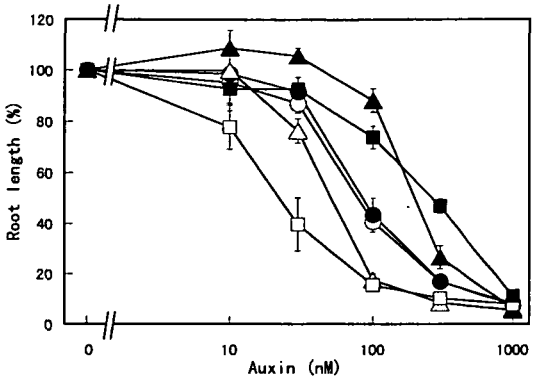

Fig. 3 Effects of NAA (○, ●), 2,4-D (△, ▲) or IAA (□, ■) on root growth of wild-type (open symbol) and *aux1-7* (closed symbol) seedlings grown in the dark for 4 d at 22°C. Root growth is expressed relative to the mean root length of the same genotype on medium without auxin. Each point represents the mean and standard error of the mean of at least 3 independent experiments, in which 28–50 seedlings were used.

higher concentrations of NAA inhibited root growth of wild type and *aux1-7* in a similar manner; the SD of growth direction of *aux1-7* roots decreased further to the wild-type level, while the gravitropism of wild-type roots was not affected at all. The presence of 1,000 nM NAA increased the SD of *aux1-7* and wild-type roots to a similar extent. In both genotypes there was no apparent correlation between the growth rate and the gravitropic response (Fig. 1). These results indicate that the agravitropic nature of *aux1-7* roots is corrected by the addition of NAA up to 300 nM, but that 1,000 nM NAA disturbs root gravitropism. They also indicate that root growth is inhibited by NAA in a similar way in wild type and *aux1-7*.

Next, we checked the effects of IAA and 2,4-D on root gravitropism (Fig. 2). 2,4-D and IAA also decreased the SD of the root-tip angle of *aux1-7*, although they were much less effective than NAA. The addition of higher concentrations of 2,4-D and IAA increased the SD of the wild-type angle. 2,4-D was the most effective of the three auxins in disrupting root gravitropism; NAA was the least effective. Wild-type roots completely lost gravitropism at 1,000 nM 2,4-D.

Growth of wild-type and *aux1-7* roots was inhibited differentially by the three auxins (Fig. 3). Growth of wild-type roots was inhibited most effectively by IAA. NAA was least effective and no difference was observed between the sensitivity of wild-type and *aux1-7* roots to NAA. The effectiveness of 2,4-D in the inhibition of wild-type root growth was between that of IAA and NAA, and the resistance of *aux1-7* root growth to 2,4-D and IAA was similar.

In the present study, we showed that exogenously added NAA rescued the agravitropic trait of *aux1-7* roots much more effectively than did IAA and 2,4-D (Fig. 2). *aux1* has been isolated as an auxin-resistant mutant that can grow in the presence of 2,4-D (Maher and Martindale 1980). Although *aux1-7* showed growth resistance to IAA and 2,4-D (Fig. 3; Maher and Martindale 1980), it was not resistant to NAA (Fig. 3). Delbarre et al. (1996) have recently shown that in suspension-cultured tobacco cells the relative contributions of diffusion and carrier-mediated influx and efflux to the membrane transport of auxin are different for IAA, NAA and 2,4-D. NAA enters cells by passive diffusion, while uptake of IAA and 2,4-D is mostly by the influx carrier. The differential effects of the three auxins on gravitropism and growth of *aux1-7* roots can be explained if we assume that *AUX1* encodes an influx carrier of auxin, and that Delbarre et al.'s model also applies to Arabidopsis root cells. IAA and 2,4-D cannot enter *aux1* root cells efficiently because of the lack of the auxin influx carrier, resulting in growth resistance to IAA and 2,4-D. In contrast, NAA can enter the *aux1* cells by passive diffusion through the plasma membrane. The infiltrated NAA substitutes for endogenous auxin in root cells and thus root gravitropism is restored in *aux1*. Since NAA can enter *aux1* cells as freely as wild-type cells, growth of *aux1* roots is not resistant to NAA. The fact that NAA rescues an *aux1-7* defect in root gravitropism and that *aux1-7* roots are not resistant to NAA strongly supports the supposition that *AUX1* encodes an auxin influx carrier, and that Delbarre et al.'s model is valid in Arabidopsis cells. It is also suggested that the *AUX1* product does not contribute significantly to the lateral transport of auxin, which has been postulated as a key step of tropic responses in the Cholodny-Went hypothesis (Went and Thimann 1937), because *aux1-7* roots can express normal gravitropism if NAA is supplied to the root cells through diffusion.

Higher concentrations of either auxin perturb gravitropism of wild-type roots. 2,4-D was most effective and NAA was least effective (Fig. 2). According to the model of Delbarre et al., efflux of 2,4-D is accomplished through simple diffusion; efflux of IAA or NAA is mediated by both diffusion and the auxin efflux carrier, and the relative contribution of diffusion to the total efflux of IAA (19%) is larger than that of NAA (6.4%). The relative effectiveness of the three auxins in the disturbance of root gravitropism shown in this study (2,4-D > IAA > NAA) is in accord with the extent of the relative contribution of diffusion to the total efflux of the auxins. This suggests that it is the isotropic outward diffusion of auxin that interferes with the root gravitropism, and that the auxin efflux carrier plays a central role in root gravitropism. The unequal auxin distribution induced by gravistimulation, which is postulated in the Cholodny-Went hypothesis, may be brought about by the auxin efflux carrier. Although an auxin influx carrier is very likely to be encoded by *AUX1* as described above, the gene for the auxin efflux carrier remains obscure. Genes, such as *PIN1* (Okada et al. 1991) and *MONOPTEROS* (Przemeck

et al. 1996), have been proposed as candidates for it.

aux1-7 is a missense mutation, which results in a substitution of Gly-459 to Asp in the *AUX1* polypeptide consisting of 485 amino acid residues (Bennett et al. 1996), suggesting that *aux1-7* is not a null mutant. Therefore, it is rather surprising that *aux1-7* completely loses root gravitropism (Fig. 1, 2). Gly-459 might be important for the function of the *AUX1* protein. We also checked root gravitropism of another allele of *aux1*, *aux1-21* (Roman et al. 1995). *aux1-21* is caused by deletion of one base (Bennett et al. 1996) and its mRNA is barely detectable (M. J. Bennett, personal communication), suggesting that it is likely to be a null mutant. *aux1-21* roots also were completely agravitropic in the absence of auxin (SD=95.1±3.2° (n=3)), and the effects of the three auxins on the root gravitropism were very similar to those observed in *aux1-7* (data not shown). These results show that the differential effects of the auxins observed in the present study are not allele-dependent.

In this study, the mean direction of growth of wild-type (ecotype Columbia) roots deviated from the vertical by 10 to 15°. The addition of increasing concentrations of either auxin seemed to decrease the deviation in parallel with an inhibition of root growth (Fig. 1, 2). Rutherford and Masson (1996) reported that in Arabidopsis the direction of root growth was dependent on the ecotype: The roots of ecotype Columbia grew straight down in both the light and dark, but root growth of the Wassilewskija and Landsberg *erecta* ecotypes tended to deviate away from the vertical by about 20° under 16-h day/8-h night cycles. We are currently unable to explain the difference between their results and ours. The growth rate of roots was similar in *aux1* and wild type in this study (Fig. 1). This is in contrast to previous reports (Mirza et al. 1984, Roman et al. 1995, Timpte et al. 1995), in which *aux1* roots grew faster than did those of the wild type. Again, we are unable to explain the causes of the difference.

Appendix

Determination of the SD of growth direction when roots of a very large number of seedlings grow in a random manner—The direction of gravity is set as 0°. When the growth directions of $(2n+1)$ roots are distributed evenly between $-180°$ and 180°, the mean of the angles is 0°. Let s be the SD,

$$s^2=\frac{1}{2n}\sum_{i=-n}^{n}\left(180°\times\frac{i}{n}\right)^2$$
$$=\frac{1}{n}\sum_{i=1}^{n}\left(180°\times\frac{i}{n}\right)^2$$
$$=\frac{(180°)^2}{n^3}\sum_{i=1}^{n}i^2$$
$$=\frac{(180°)^2}{n^3}\times\frac{n(n+1)(2n+1)}{6}$$
$$=\frac{(180°)^2}{3}\times\left(1+\frac{1}{n}\right)\times\left(1+\frac{1}{2n}\right)$$

Thus, as the sample size, $2n+1$, increases, s^2 tends to $(180°)^2/3$. In this ideal condition, the SD of the growth angle, s, should be $180°/\sqrt{3}$.

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