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Indole-3-lactic Acid is a Weak Auxin Analogue but Not an Anti-auxin

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Abstract. Indole-3-lactic acid (ILA) is a naturally occurring indole derivative, preferably detected in soil bacteria and fungi and only in low amounts in plants. T-DNA gene 5 of *Agrobacterium tumefaciens* was found to be involved in the synthesis of ILA in transformed plant tissues, but the physiologic relevance for ILA production in plants is unclear. The related molecular structure of ILA to the natural auxin indole-3-acetic acid (IAA) makes ILA a good candidate for an auxin analogue. We examined the possible auxin activity of ILA on elongation, proliferation, and differentiation in *Pisum sativum* L. Results presented in this paper indicate that there are no or only weak effects of ILA toward the activity of auxins when used in the physiologic concentration range. Furthermore, no antagonistic effects of ILA were found. Biochemical analysis using the equilibrium dialysis binding system resulted in no high affinity ILA binding to an enriched protein fraction containing auxin-binding protein (ABP_{44}), whereas 1-naphthaleneacetic acid exhibited high affinity auxin binding.

Indoleacetic acid (IAA) and related compounds can induce different effects in plants. To understand better the effects of auxin structure and activity on morphogenesis and cell elongation, numerous studies have been carried out (Branca et al. 1991, Katekar 1979). The effects of auxin in cell elongation are well described (for review see Scott 1984), whereas their role in other morphogenetic responses (usually in combination with cytokinins) is still enigmatic.

The auxin analogue ILA (indole-3-lactic acid, Fig. 1) has been found in *Escherichia coli* (Körber et al. 1991) and in some fungi (Glombitza and Hartmann 1966, Perley and Stowe 1966). It also was reported to be a common tryptophan metabolite in many soil bacteria such as *Rhizobium* (Badenoch-Jones et al. 1983, Rigaud 1970) and *Agrobacterium* (Kaper and Veldstra 1958, Rovenska et al. 1988). In higher plants it was detected only in small amounts in tomato shoots (Gibson et al. 1972, Schneider and Wightman 1974).

Infection of susceptible plants by *Agrobacterium tumefaciens* can cause the formation of localized tumors, called crown galls (for review see Binns and Thomashow 1988, Zambryski et al. 1989). The tumorous growth induced by *Agrobacterium* was shown to be due to the integration of a part of the bacterial Ti-plasmid into the plant genome. The transferred DNA (T-DNA) contains growth-inducing genes (*iaaM, iaaH,* and *iptZ*) coding for enzymes that are involved in biosynthesis of auxin and cytokinin (for review see Morris 1986). Expression of these genes in transformed cells leads to higher levels of auxin and cytokinin, causing cell proliferation and tumor formation (Akiyoshi et al. 1983).

Other loci on T-DNA, such as gene 5 and 6b, are not required for tumorgenesis but have an influence on the phenotype of tumors (Zambryski et al. 1989). Gene 5 has been found to code for an enzyme that catalyzes the synthesis of ILA. ILA was suggested to modulate auxin responses in transformed plants by acting as an auxin antagonist and competing with some cellular auxin functions (Körber et al. 1991). This assumption is not in agreement with data published previously (Meudt and Bennett 1978, Thimann 1958). Since the effects of auxin on cell expansion, proliferation, or division depend on the type or state of maturation of the organ being studied (for examples see Evans 1985 and references therein) we used the following experimental systems to examine the role of ILA in the different

Abbreviations: IAA, indoleacetic acid; ILA, indole-3-lactic acid; T-DNA, transferred DNA; ABP, auxin-binding protein; NAA, naphthaleneacetic acid; MS, Murashige and Skoog; MES, 2-(*N*-morpholino)ethanesulfonic acid; BAP, 6-benzylaminopurine.

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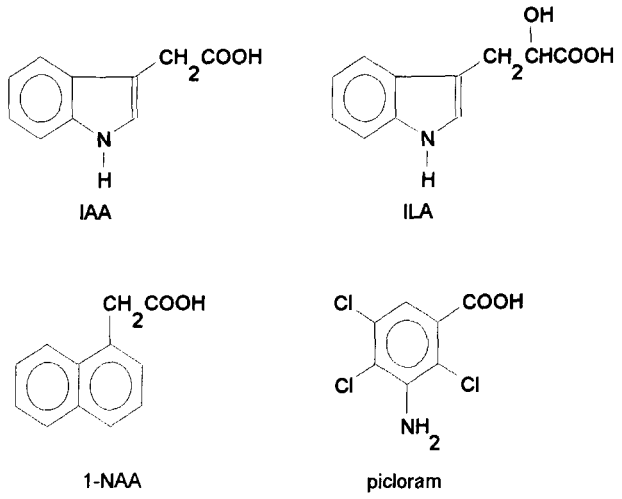

Fig. 1. Structures of IAA, ILA, 1-NAA, and picloram.

types of auxin response: elongation growth; callus induction on internodal segments (proliferation); root induction on multiple shoots and internodal segments; and, at a molecular level, binding to a cellular protein, the auxin-binding protein ABP_{44} (Reinard and Jacobsen 1995).

The promotion of cell elongation is one of the characteristic features of auxins studied extensively in the past. We examined the elongation of pea segments induced by active and inactive auxins. As a test system for the stimulation of cell proliferation we chose callus formation in tissue culture.

Auxins are also essential for specific differentiation processes in several cell types, which were examined by root formation in both pea epicotyl segments and multiple shoots. Koncz et al. (1991) and Körber et al. (1991) assumed an antagonistic effect of ILA on auxin action, caused by the competition of ILA and IAA/1-NAA for an auxin receptor or carrier. Therefore we also tested possible synergistic or antagonistic effects of ILA in all systems described above.

To examine the action of ILA on a specific ABP we assessed the action of ILA on the auxin binding to ABP_{44} (Reinard and Jacobsen 1995).

Materials and Methods

Plant Material

For elongation tests and binding assays seeds of *Pisum sativum* L. var. Birte (Norddeutsche Pflanzenzucht, H. G. Lemke KG, Hohenlieth, FRG) were soaked for 6 h in water and sown on moist vermiculite. The seedlings were grown for 5–7 days in the dark until the second internodal stage. For tissue culture seeds of uniform size and shape (cv. Madria and Solara) were surface sterilized for 30 s with 70% (v/v) ethanol followed by a 4-min treatment with 5% (v/v) sodium hypochlorite and three thorough washes with sterile distilled water. Thereafter, seeds were transferred to hormone-free medium containing 0.46 g^{-1} MS salts (Murashige and Skoog 1962), 1% (w/v) sucrose and solidified with 0.7% (w/v) agar. They were grown in the dark for 7 days at 22°C.

Chemicals

All chemicals were of analytical grade and were purchased from Sigma (Deisenhofen, FRG), Serva (Heidelberg, FRG), Applichem (Darmstadt, FRG) or Roth (Karlsruhe, FRG). Tritium-labeled 1-naphthaleneacetic acid 1-[^{3}H]NAA was obtained from Amersham (Braunschweig, FRG). Plant tissue culture media were obtained from Duchefa (Haarlem, The Netherlands).

Elongation Tests

Fifteen segments of 10-mm length were cut from the second internode of etiolated pea seedlings, starting 3 mm below the apical hook. The sections were incubated for 4 h in 20 mL of medium ± auxins with gentle continuous shaking in the dark at 22°C. Solutions were buffered with 5 mM MES and contained 1% MS salts and 1% sucrose. The pH was adjusted to 5.8. The following auxins and related analogues were tested in two different concentrations (10 μM and 1 mM): IAA, 1-NAA, picloram, 2-NAA, and ILA (Fig. 1). Hormone-free medium was used as a control.

In a second attempt, segments were incubated in media containing 10 μM 1-NAA, in addition to increasing concentrations of ILA (10 μM, 100 μM, 1 mM). After 4 h of incubation, segments were photographed under standardized conditions, and the final lengths of segments were measured from projected slides. Experiments were repeated three times. Elongation rates were calculated as the relative activity of hormones compared with elongation induced by 10 μM IAA.

Tissue Culture

Internodal segments of sterile grown 7-day-old seedlings (cv. Madria) were excised and transferred to basal MS medium containing 4.6 g^{-1} MS salts (enriched with 1% B_5 vitamins), 3% sucrose, 0.1% glycine, 5 mM MES and solidified with 0.35% Gelrite. The pH of the medium was adjusted to 5.8 before autoclaving. 1-NAA, picloram, and BAP were added prior to autoclaving while ILA was added filter sterilized after autoclaving.

Multiple shoot formation was obtained from nodal explants according to Griga et al. (1986) by placing nodal explants on MS medium in combination with 0.25 μM 1-NAA and 22.5 μM BAP. All cultures were maintained at 22°C in the dark.

Callus Induction

For proliferation tests, 30 internodal explants were cultivated on basal MS medium as described above. Callus formation was induced by adding 5 μM 1-NAA and 0.5 μM BAP. Increasing concentrations of ILA (0.5, 1, 5, 10, 25 μM) were added to the callus induction medium. As a control, segments were placed on hor-

mone-free medium. The same numbers of segments were placed on MS medium containing 5 μM ILA and 0.5 μM BAP.

Root Induction

Thirty internodal segments were placed on basal MS medium supplemented with 5 μM 1-NAA for root induction. ILA was added in increasing concentrations (0.5, 1, 5, 10, 25 μM). As a control, internodal segments were placed on hormone-free medium. Furthermore, segments were placed on medium complemented with 5 or 10 μM ILA.

In another set of experiments, the effect of ILA on the rooting of adventitious shoots was examined. Multiple shoots were cultured on medium added with either 5 μM 1-NAA or 5 μM ILA, respectively.

Binding Assay

All steps were carried out at 0–4°C. Five-day-old etiolated seedlings (cv. Birte) were homogenized (Bühler homogenizer HO 4) in an equal amount (w/v) of homogenization buffer (50 mM Tris-HCl, pH 7.8, containing 3% Polyclar-AT, 4 mM sodium diethyldithiocarbamate, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM 2-mercaptoethanol, and 1 μM leupeptin). The homogenate was filtered through nylon cloth (100 and 60 μm) and centrifuged for 2 h at 150,000 × g and 4°C. The supernatant was precipitated with 50% $(NH_4)_2SO_4$ and centrifuged at 12,000 × g for 15 min. The pellet was resuspended in binding buffer (citric acid/phosphate buffer, pH 5.5; filtered through nitrocellulose). Protein concentration was determined according to Bradford (1976). Binding assays were carried out using equilibrium dialysis (Reinard and Jacobsen 1989). For competition experiments 2×10^{-9} M [^{3}H]NAA (8.36 GBq/mM) was used in addition to various concentrations (3×10^{-8} to 6×10^{-7} M) of unlabeled 1-NAA or ILA. Kinetic data were calculated using a Scatchard fitting computer program (Munson and Robard 1980, Scatchard 1949).

Results

Effects of ILA on Stem Elongation

The elongation of internodal segments, induced by three different auxins (1-NAA, IAA, picloram) and the auxin analogue 2-NAA were compared with the effects of ILA. Stem elongation was found to be promoted significantly by 10 μM IAA compared with segments incubated in hormone-free buffer controls (Fig. 2). The effects of other hormones are expressed as relative activities compared with the elongation rate induced by 10 μM IAA.

Ten μM 1-NAA or picloram stimulated elongation in the same ranges as 10 μM IAA (87% for 1-NAA, 108% for picloram). Higher concentrations (1 mM) of either IAA, 1-NAA, or picloram resulted in attenuated elongation responses (relative activities of 43% for IAA, 32% for 1-NAA, and 40% for picloram). ILA was found to be of much lower efficiency than the other compounds tested. It behaved similarly to the low active auxin analogue 2-NAA. At concentrations of 10 μM both substances had almost no influence on elongation (relative activities of 20% for 2-NAA, 4% for ILA). Elongation responses increased at higher concentrations (1 mM) for both, 2-NAA and ILA. Relative activities of approximately 65% (2-NAA) and 50% for ILA, respectively, were measured.

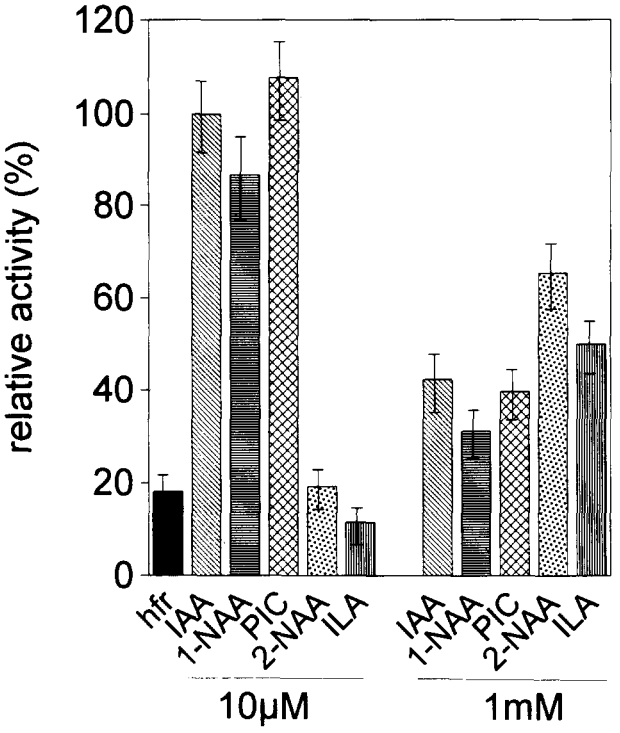

Fig. 2. Relative activities (%) of different auxins and the auxin analogue ILA on the elongation of pea stem segments (second internode, 10-mm sections, 4-h incubation) at two concentrations, 10 μM and 1 mM. The activity of hormones on elongation was compared with elongation induced by 10 μM IAA (= 100%). The following hormones and analogues were tested: IAA, 1-NAA, picloram, (PIC), 2-NAA, and ILA. Stem segments incubated in hormone-free buffer (hfr) were used as controls.

ILA was also investigated for its capability to act antagonistically on auxin-induced stem elongation. Therefore, competition experiments aiming to influence elongation growth (induced by 1-NAA) by adding increasing amounts of ILA were carried out. No significant changes were found in stem elongation by 10 μM 1-NAA and applied ILA up to 100 μM (Fig. 3).

Effects of ILA on Cell Proliferation

ILA was also studied for its auxin effects in inducing cell proliferation. Callus formation occurred in segments cultured on medium containing 0.5 μM

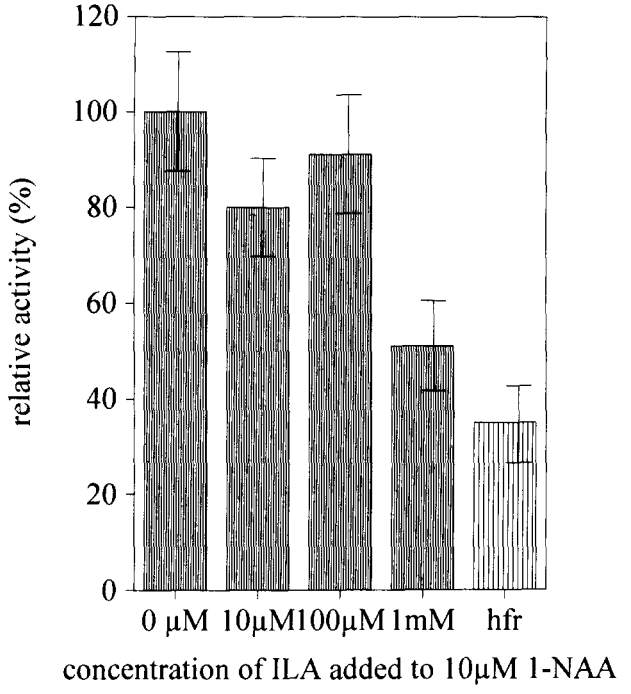

Fig. 3. Effect of increasing concentrations of ILA (10 µM, 100 µM, 1 mM) on 1-NAA-induced pea stem elongation. The activity of 10 µM 1-NAA on elongation of segments (= 100%) was only inhibited significantly by adding 1 mM ILA. hfr, hormone-free buffer.

BAP supplemented with 5 µM 1-NAA and 0.5 µM picloram, but not on that with ILA (Fig. 4, a–c).

ILA at 5 µM was not able to induce effects similar to 1-NAA or picloram. A small callus formation was observed at the cut surface, possibly due to the polar transport of endogenous IAA. This was also observed in the hormone-free control, which is shown in Fig. 5g and may be due to internal IAA produced by the explant itself.

Additionally, ILA was assayed for its ability to compete with auxin effects on callus induction. Therefore, ILA was added to the induction medium at different concentrations. Our experiments showed that increasing concentrations of ILA (0.5–25 µM) apparently have no influence on callus formation, compared with controls (Fig. 5, a–h). Callus formation was neither inhibited nor stimulated in this range of ILA concentrations.

Effects of ILA on Cell Differentiation

The ability of ILA to influence cell differentiation was tested by inducing roots on epicotyl segments or multiple shoots. Adventitious shoots formed roots when cultured on media containing 5 µM

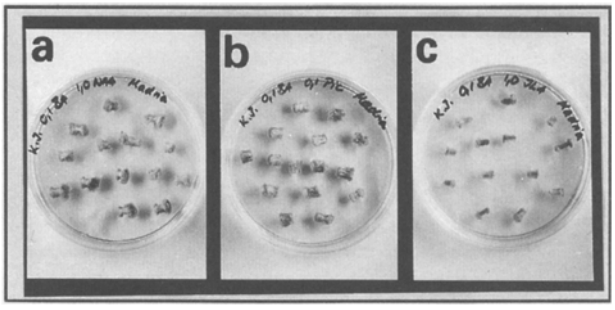

Fig. 4. Effects of auxins and ILA on callus induction. Shown are internodal explants of 7-day-old pea seedlings, 20 days after cultivating on MS medium added with **(a)** 5 µM 1-NAA, **(b)** 0.5 µM picloram, **(c)** 5 µM ILA. Callus formation occurred in segments cultured on 5 µM 1-NAA and, even more rapidly, on 0.5 µM picloram. No callus formation could be induced by adding 5 µM ILA to the medium.

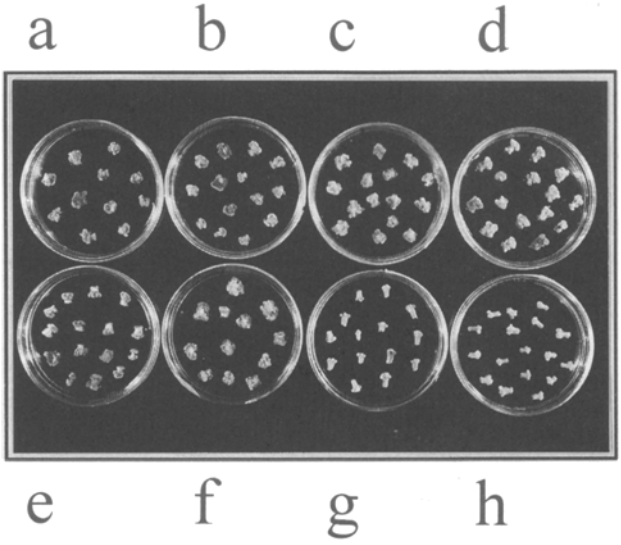

Fig. 5. Effects of different ILA concentrations on callus induction. Shown are internodal explants cultured for 4 weeks on callus-inducing media: **(a)** 5 µM 1-NAA and 0.5 µM BAP, and callus-inducing media added with increasing amounts of ILA: **(b)** 0.5 µM, **(c)** 1 µM, **(d)** 5 µM, **(e)** 10 µM; **(f)** 25 µM. No effects on callus induction, even at higher concentrations of ILA, could be found. **(g)**, segments cultured on hormone-free MS medium. **(h)**, segments cultured on MS medium containing 5 µM ILA and 0.5 µM BAP.

1-NAA but not on media with 5 µM ILA (Fig. 6, a and b). Furthermore, the shoots grown on ILA-containing medium failed to develop.

Rooting of internodal segments could be induced on MS medium containing 5 µM 1-NAA (Fig. 6c) but not on media with 5 or 10 µM ILA (Figs. 6d and 7, g and h). Additionally, ILA was assayed for its ability to compete with auxin effects on root formation using epicotyle segments. Increasing concen-

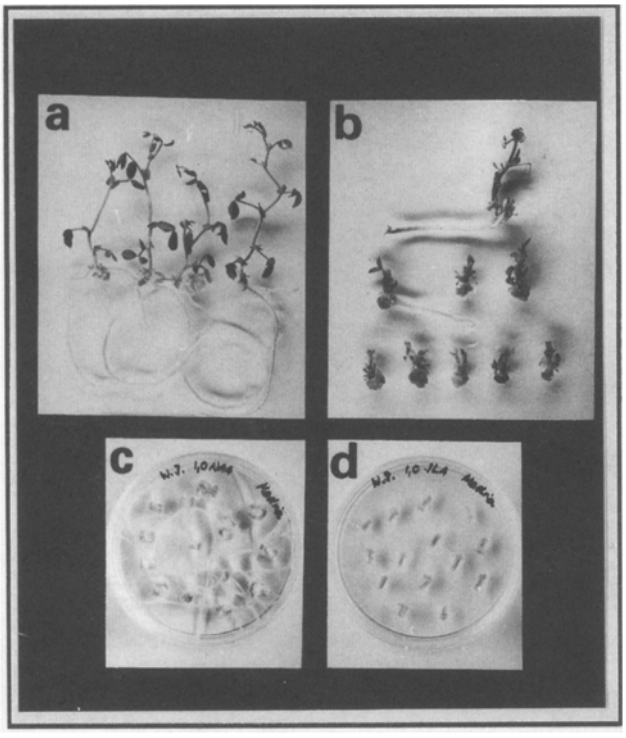

Fig. 6. Effects of ILA on rooting ability of multiple shoots (**a, b**) and internodal segments (**c, d**). Segments were cultivated on MS medium, and either 5 μM 1-NAA or 5 μM ILA. Root formation was found at shoots and segments cultivated on 5 μM 1-NAA (**a, c**) but not on segments cultured on 5 μM ILA (**b, d**).

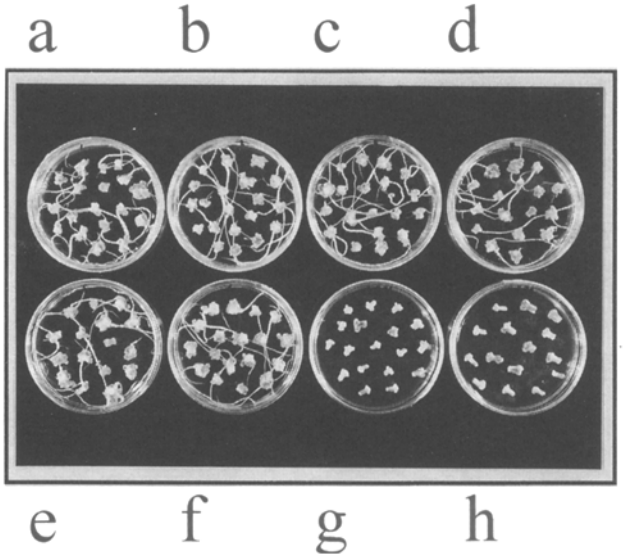

Fig. 7. Effects of different ILA concentrations on root induction. Shown are internodal explants cultured 4 weeks on root-inducing medium containing 5 μM 1-NAA (**a**). Rooting could not be affected by adding increasing amounts of ILA: 0.5 μM (**b**), 1 μM (**c**), 5 μM (**d**), 10 μM (**e**), 25 μM (**f**). (**g**), segments cultured on MS medium containing 5 μM ILA. (**h**), segments cultured on MS medium containing 10 μM ILA.

trations of ILA were added to media containing 5 μM 1-NAA.

ILA did not influence rooting caused by 0.5 μM 1-NAA (Fig. 7a) when tested in concentrations of 0.5–25 μM (Fig. 7, b–f). No root formation occurred on 1-NAA-free medium with 5 μM or 10 μM ILA (Fig. 7, g and h).

Binding Assays

ILA and 1-NAA were assayed for their capability to compete for the binding to protein fractions containing soluble ABP_{44}. Equilibrium dialysis binding tests resulted in Scatchard plots as indicated in Fig. 8a (for 1-NAA as competing ligand) and Fib. 8b (for ILA as competing ligand). Binding parameters for experiments using 1-NAA as competing ligand were calculated as follows:

$$\text{dissociation constant } (K_D) = 1.6 \times 10^{-7}\ \text{M}$$

$$\text{binding sites } (R_T) = 7 \times 10^{-9}\ \text{M/mg of protein.}$$

In contrast, competition of [^{3}H]NAA by ILA resulted in no high affinity binding.

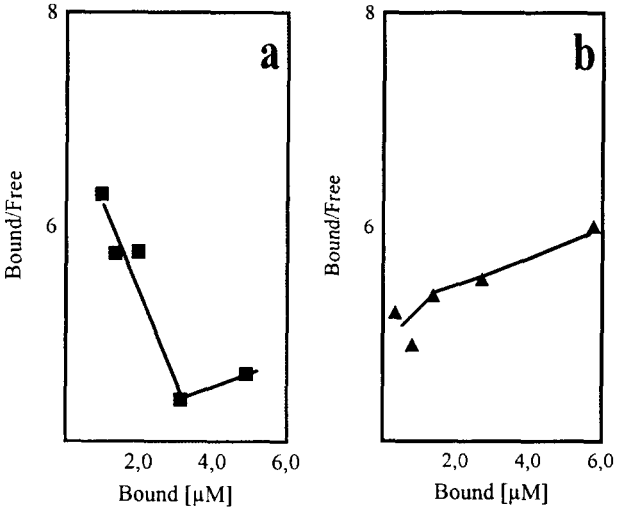

Fig. 8. Scatchard plot analysis of binding data from 1-NAA (**a**) and ILA (**b**) used in equilibrium dialysis binding assays as competitors of [^{3}H]NAA (2×10^{-9} M; 8.36 GBq/mM). The ability of 1-NAA and ILA to compete for high affinity [^{3}H]NAA binding to a protein fraction containing ABP_{44} was tested over a concentration range of 3×10^{-8} to 6×10^{-7} M. Data were calculated according to Scatchard (1949). While 1-NAA replaced [^{3}H]NAA binding ($K_D = 1.6 \times 10^{-7}$ M (**a**), no competition was found using ILA as unlabeled ligand (**b**).

Discussion

Although ILA has been detected in plants earlier (Ehmann 1976: grape vine, Schneider and Wightman 1974: tomato) its physiologic role, if any, has remained obscure. Among the large number of compounds tested for auxin activity (for review see Katekar 1979), in only few of these was ILA examined (Meudt and Bennett 1978, Thimann 1958). Our data are in good agreement with reports published previously. ILA showed no auxin activity in proliferation or differentiation processes (Figs. 4 and 6) and only a very weak activity in elongation assays (Fig. 2).

The discovery that ILA is produced by the *Agrobacterium* T-DNA gene 5 product and the influence of gene 5 on tumor growth in transformed tissue (Joos et al. 1983) led to the assumption that ILA is involved in modulating the auxin responses in plants by acting as an auxin antagonist ("anti-auxin," Körber et al. 1991). Körber et al. (1991) found that expression of gene 5 in transgenic tobacco plants, driven by the mainly constitutive 35 S promoter, resulted in the synthesis of ILA up to concentrations, that are as high as free IAA levels. This accumulation of ILA did not appear to influence normal plant growth and differentiation. On the other hand, tobacco seedlings, transformed with gene 5, were able to overcome inhibitory effects of exogenous applied auxins. From these and other data Körber et al. (1991) concluded that the anti-auxin ILA antagonizes auxin stimulation of gene expression in tumors and normal plant cells by competition for binding to cellular proteins. According the definition of Söding (1961) anti-auxins are substances that decrease the effects of auxins, for example, IAA, by competitive inhibition. Our own results show that ILA was not able to inhibit the effect of the synthetic auxin 1-NAA either in elongation or in proliferation or differentiation processes (Figs. 2 and 4–7). Only when ILA was applied in unphysiologically high concentrations (1 mM) were minor effects on 1-NAA-induced elongation found (Fig. 3). There are several reports on the effect of ILA on binding to ABPs, mostly by using photoaffinity labeling with azido-IAA. Competition of azido-IAA labeling was found for pm24 (Körber et al. 1991) and pm23 (Feldwisch et al. 1992) when using 1 μM azido-IAA and 1 mM ILA. The same pattern was also found for the so-called p23, of which labeling was competed by IAA indole-3-butyric acid, ILA, and tryptophan, and which was assumed to be unspecifically labeled (Feldwisch et al. 1995). Another putative ABP, which was characterized as a β-glucosidase by Campos et al. (1992), was found not to bind ILA.

For the up to now best characterized ABP-1 (Jones 1994 and references therein) ILA was found to compete IAA binding (Körber et al. 1991) when added in 1,000-fold excess (1 mM) in photoaffinity labeling experiments. On the other hand, data derived from tobacco protoplasts are not in agreement with this finding. Barbier-Brygoo et al. (1989) developed an assay system for monitoring early auxin effects at the cell level, based on auxin-induced modulations of the transmembrane potential differences of tobacco mesophyll protoplasts. This response was found to be modulated by the external addition of ABP-1 (Venis et al. 1992). Based on this system, Barbier-Brygoo et al. (1992) found no influence of ILA on the membrane potential nor an interference with the 1-NAA-induced responses. Our in vivo data of ILA affecting stem elongation (Figs. 2 and 3), proliferation (Figs. 4 and 5), and differentiation (Figs. 6 and 7) are in agreement to the data published by Barbier-Brygoo et al. (1992).

Additionally, we tested ABP_{44} (Reinard and Jacobsen 1995) for its ability to bind ILA. Results obtained from binding studies showed no competitive effect of ILA on auxin binding to ABP_{44} (tested over a concentration range of 3×10^{-8} M to 6×10^{-7} M).

In summary, our results indicate that there is no antagonistic effect of ILA when examined at concentrations physiologically effective for the naturally occurring auxin IAA. Therefore it can be excluded that ILA can compete with auxin action, at least in the cases studied in the present contribution. Our findings show that only 100-fold excess of ILA is able to interfere weakly with auxin effects in stem elongation, but not on the other morphogenetic effects described here.

The data presented in this report do not agree with the findings of Körber et al. (1991) who consider ILA an anti-auxin. Our data confirm ILA as an inactive auxin analogue, comparable to indole-3-butyric acid or 2-NAA.

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