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Uptake, accumulation and metabolism of auxins in tobacco leaf protoplasts

Alain Delbarre[1], Philippe Muller[1], Viviane Imhoff[1], Jean-Louis Morgat[2], Hélène Barbier-Brygoo[1]

[1] Institut des Sciences Végétales, CNRS, Bâtiment 22, Avenue de la Terrasse, F-91198 Gif-sur-Yvette, France
[2] Université Montpellier I, CRBA, Faculté de Pharmacie, 15 Avenue Charles Flahaut, F-34060 Montpellier, France

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Abstract. Uptake and metabolism of exogenous naphthalene-1-acetic acid (NAA) and indole-3-acetic acid (IAA) have been studied in tobacco (*Nicotiana tabacum* L. cv. Xanthi) mesophyll protoplasts. Both auxins entered protoplasts by diffusion under the action of the transmembrane pH gradient without any detectable participation of an influx carrier. Molecules were accumulated by an anion-trapping mechanism and most of them were metabolized within hours, essentially as glucose-ester and amino-acid conjugates. Protoplasts were equipped with a functional auxin-efflux carrier as evidenced by the inhibitory effect of naphthylphtalamic acid on IAA efflux. Basically, similar mechanisms of NAA and IAA uptake occurred in protoplasts. However, the two auxins differed in their levels of accumulation, due to different membrane-transport characteristics, and the nature of the metabolites produced. This shows the need to estimate the accumulation and the metabolism of auxins when analyzing their effects in a given cell system. The internal auxin concentration could be modulated by changing the transmembrane pH gradient, giving an interesting perspective for discriminating between the effects of intra- and extracellular auxin on physiological processes.

Key words: Auxin (accumulation, metabolism) – *Nicotiana* – Protoplast (leaf)

Introduction

Auxin controls various aspects of plant growth and development from the cellular level, such as cell elongation and cell division, to the organ level, such as morphogenesis. While several putative auxin receptors have been recognized in plant cells (Jones and Prasad 1992 and reference therein), pathways leading to the different responses are uncertain and localization of the initial site(s) of auxin reception at the cellular level is still a subject of debate.

Plant cells tend to concentrate natural and synthetic auxins, which are lipophilic weak acids, from the surroundings (Raven 1975; Goldsmith 1977; Rubery 1980, 1987). Accumulation mechanisms involve membrane diffusion of free molecules, driven by the transmembrane pH gradient and the electrical membrane potential, associated with carrier-mediated influx and efflux. The sequestration and distribution of auxins in cellular compartments are widely controlled by the intensity of cell metabolism, the nature and proportions of free and metabolized forms being dependent on the chemical structure, plant material and environmental conditions (Cohen and Bandurski 1982; Cohen 1983; Caboche et al. 1984; Vijayaraghavan and Pengelly 1986; Sandberg et al. 1990; Smulders et al. 1990; Sitbon et al. 1993). The capability of auxins to enter cells implies that they should be able to elicit cellular responses by interacting with internal as well as external receptors. Facing this situation, knowledge of the accumulation properties of cells with respect to auxins is a prerequisite when probing hormone effects and establishing dose-response comparisons for various molecules or different genotypes.

Owing to the lack of a rigid cell wall barrier, a good capacity for regeneration and easy obtainability from different plant tissues, protoplasts are valuable tools for investigating auxin effects at the cellular and molecular levels. This is evidenced by recent developments concerning studies of auxin and auxin-related drugs on plasma-membrane properties (Barbier-Brygoo et al. 1989, 1991; Venis et al. 1990, 1992; Marten et al. 1991; Rück et al. 1993) as well as gene regulation (Takahashi et al. 1989, 1990, 1991; Maurel et al. 1990, 1994; Capone et al. 1991; Boerjan et al. 1992; Takahashi and Nagata 1992a, 1992b; Walden et al. 1993). Electrophysiological and patch-clamp studies have shown the presence of receptors for naphthalene-1-acetic acid (NAA) and indole-3-acetic

Abbreviations: BA = benzoic acid; C_i/C_e = accumulation ratio of auxin; IAAasp = N-[3-indolylacetyl]-DL-aspartic acid; NAA = naphthalene-1-acetic acid; NAAasp = N-[1-naphthylacetyl]-L-aspartic acid; NPA = N-1-naphthylphthalamic acid

Correspondence to: A. Delbarre; FAX: 33 (1) 69823768

acid (IAA) at the outer face of the plasma membrane of protoplasts isolated from tobacco leaves (Barbier-Brygoo et al. 1989, 1991; Venis et al. 1990, 1992), *Vicia faba* guard cells (Marten et al. 1991), and maize coleoptiles (Rück et al. 1993). By contrast, nothing is known as yet concerning the location of receptors involved in auxin-regulated gene expression.

Accumulation of auxins in protoplasts is poorly documented. Although, in some cases, active auxin may be intracellular auxin, only the concentration supplied is generaly considered, regardless of uptake and metabolism. Within this context, the aim of the present study has been to investigate mechanisms by which exogenous NAA and IAA are absorbed and accumulated in tobacco mesophyll protoplasts. Both auxins enter protoplasts by diffusion across the plasma membrane and are accumulated inside by an anion-trapping mechanisms. There was no evidence supporting the existence of an auxin-influx carrier in protoplasts and only a low participation of an efflux carrier in the membrane transport of IAA. Metabolism plays a crucial role in the accumulation of both auxins, NAA being essentially converted to a glucose ester and IAA mainly conjugated with acidic amino acids. Results indicate that, though the mechanisms governing their accumulation are basically the same, NAA and IAA largely differ in uptake intensity and metabolism. Accumulation ratios are difficult to predict without information concerning pH value, volume and membrane permeabilities of the cell compartments. However, results also suggest that internal and external auxin concentrations can be independently modulated by changing the transmembrane pH gradient, giving a good opportunity for discriminating between intra- and extracellular auxin effects.

Materials and methods

Preparation of protoplasts from tobacco leaves. Protoplasts were prepared from *Nicotiana tabacum* L. cv. Xanthi, XHFD8 wild-type (Muller et al. 1985). Plants were grown at 22° C on a vermiculite support under 9 h daylight.

Peeled leaf strips were spread on a Petri dish filled with 10 ml T_0 growth medium (Caboche 1980) containing 15 µM NAA and supplemented with (w/v) 0.1% cellulase "Onozuka" R-10 (Yakult, Tokyo, Japan), 0.02% macerozyme R-10 (Yakult), 0.05% driselase (Sigma, L'Isle d'Abeau Chesnes, France). After 16 h digestion at 25° C in the dark, the preparation was diluted with 2 vol. of potassium buffer [0.3 M KCl, 5 mM $CaCl_2$, 1 mM 2-(N-morpholino)ethanesulfonic acid (Mes), pH 5.7], cleared of fragments by filtration through a 56-µm gauze and centrifugated at 100 g for 5 min. The protoplast pellet was washed twice with 30 mL cold potassium buffer to rinse off the enzymes and to remove adsorbed auxin. Protoplasts were resuspended in T_0 medium depleted of auxin (T_{00} medium) at a density of 1×10^6 protoplasts · mL^{-1}, stored at 4° C, and used within 2–3 h following isolation.

Tritium labelling of NAA and naphthalene acetamide. Tritiated NAA ([^{3}H]NAA) was prepared from bromonaphthalene acetic acid by exchanging bromine with tritium. Naphthalene-1-acetic acid was reacted with one equivalent of bromine in acetic acid at 70° C for 2 h (Ogata et al. 1951) to give a 65% yield of bromo-4-naphthalene-1-acetic acid (needles in acetone-water; m.p.: 175–176). On reduction with tritium gas in the presence of 10% palladium on charcoal and sodium acetate in ethanol (Melhado et al. 1982), the brominated derivative quantitatively yielded [^{3}H]-4-naphthalene-1-acetic (1000 TBq · mol^{-1}) which was purified on a C_{18} µbondapack reversed-phase HPLC column (Waters; Millipore Corporation, Bedford, Mass., USA) using methanol-water-acetic acid (60:40:1; by vol) as eluent.

Tritiated naphthaleneacetamide([^{3}H]NAAam; 1000 TBq · mol^{-1}) was synthesized from bromonaphthalene acetamide using the same procedure, except that the HPLC purification was done in methanol-water (1:1; v/v). Bromo-4-naphthalene-1-acetamide was obtained from bromo-4-naphthalene-1-acetic acid. The acid was dissolved in freshly distilled thionyl chloride (15 mL · g^{-1}) and heated at 100° C for 2.5 h. Solvent was replaced with dry toluene and anhydrous ammonia was bubbled for 1.5 h into the ice-cold solution. The white precipitate of bromo-4-naphthalene-1-acetamide was crystallized in ethanol-water (m.p.: 196–198; yield: 67%).

Labelled molecules were stored at −20° C and were stable for several months.

Assays for auxin accumulation in protoplasts. Protoplasts in T_{00} medium (1×10^6 protoplasts · mL^{-1}) were incubated with 10–40 kBq · mL^{-1} (10–40 nM) [^{3}H]NAA or [^{3}H]IAA (Amersham France, Les Ulis, France; 999 TBq · mol^{-1}) and with appropriate quantities of unlabelled auxin when higher concentrations were needed. In some experiments, unlabelled auxin was replaced with benzoic acid (BA) or N-1-naphthylphthalamic acid (NPA; Interchim, Montluçon, France) in order to determine the effect of these competitors on accumulation. After the incubation, undamaged protoplasts were separated from medium by silicone-oil filtration (Werkheiser and Bartley 1957). Briefly, 200 µL of suspension was layered onto 100–200 µL AR200 phenylmethylsilicone oil (Wacker Chemie, München, Germany; viscosity: 1.9×10^4–2.1×10^4 · $m^2 \cdot s^{-1}$; density: 1.03–1.05) contained in a 400-µL microtube and immediately spun for 20 − 30 s in a tabletop centrifuge. After freezing in liquid nitrogen, tube tips were clipped and pellets were homogenized in 400 µL water. Radioactivity in the pellet and in 100 µL of supernatant was counted by liquid scintillation with quenching correction.

Volumes corresponding to protoplasts (V_{proto}) and to interstitial medium (V_{extra}; 8–20% of the pellet volume depending on the protoplast batches) in the pellet were estimated for each set of experiments. For that, protoplasts were incubated in the presence of 74 kBq · mL^{-1} tritiated water (Amersham France; 0.185 TBq · mL^{-1}) as a freely diffusive marker and 0.2 kBq · mL^{-1} [^{14}C]inuline carboxylic acid (Amersham France; 0.180 TBq · mol^{-1}) as a non-permeant marker. The values of $V_{extra} = P_i/M_i$ and $V_{proto} = P_w/M_w - P_i/M_i$ were calculated from P_w and M_w, the radioactivities measured for ^3H_2O in the pellet and per unit volume of incubation medium, and P_i and M_i, the corresponding values measured for inuline carboxylic acid. The mean diameter of the mesophyll protoplasts computed from V_{proto} (29.7 ± 0.8 µm, 34 independent accumulation experiments) was in reasonable agreement with that directly measured by using optical microscopy (32.8 ± 0.9 µm, 100 individual diameter measurements). The value of V_{proto} was not significantly modified when protoplasts were incubated for 3 h in the presence of up to 100 µM NAA.

Using P_{auxin} and M_{auxin}, the radioactivities in the pellet and per unit volume of incubation medium measured in the accumulation assays, the radioactivity actually accumulated in protoplasts was calculated by substracting the interstitial radioactivity ($M_{auxin} \cdot V_{extra}$) from P_{auxin}. Auxin bound to membranes was quantified after rapid vacuum filtration of 100–200 µL of protoplast suspension onto a nitrocellulose filter and washing off the unbound internal auxin with 5 × 5 mL of cold distilled water. Radioactivity released from filters dissolved in methoxyethanol was compared with radioactivity contained in the same number of intact protoplasts. Bound auxin represented less than 1% of the total content in protoplasts. It was checked that the incubation medium only contained unmetabolized hormone. Radioactivities per unit volume of protoplasts and of medium were converted to concentrations and the capacity of protoplasts to accumulate auxin was calculated as

the ratio of the internal to the external concentrations C_i/C_e. As the accumulated radioactivity corresponded to both free and metabolized auxin, we defined an apparent accumulation ratio calculated from the total radioactivity recovered in protoplasts and a net accumulation ratio, relative to the free intracellular auxin, calculated by substracting the contribution of labelled metabolites (see below).

Isolation and characterisation of auxin metabolites in protoplasts. Protoplast pellets obtained for a fivefold scaled-up assay were extracted twice with 500 µL 80% methanol at room temperature. Extracts were clarified by centrifugation, concentrated under a nitrogen stream, and chromatographed on silica gel thin-layer chromatography (TLC) plates using different solvent systems (A: chloroform-methanol-acetic acid (75:20:5; by vol.); B: chloroform-ethyl acetate-formic acid (5:4:1; by vol.); C: *iso*propyl alcohol-ammonium hydroxide-water (8:1:1; by vol.); Caboche et al. 1984; Smulders et al. 1990). Migration profiles were monitored by scanning radioactivity on TLC plates with a Trace Master Berthold 284 LB linear analyzer (Berthold/Frieske, Wildbad, Germany) and by counting radioactivity in 5-mm strips after elution with 80% methanol. Internal standards, naphthalene-1-acetic methyl ester, [^{3}H]naphthalene-1-acetamide, N-[1-naphthylacetyl]-L-aspartic acid (NAAasp) N-[1-naphthylacetyl]-L-glutamic acid in NAA-accumulation experiments, N-[3-indolylacetyl]-DL-aspartic acid in IAA-accumulation experiments, were included durint TLC.

Metabolites, separated from free auxin by TLC in solvent A, were further characterized by using alkaline hydrolysis and digestion with α- and β-glucosidases. Alkaline hydrolysis was run in 0.05 N KOH at 25° C (1 h) for identification of ester conjugates and in 7 N KOH at 100° C (1 h in sealed vessel) for identification of amide conjugates. For enzymic hydrolysis, metabolites were incubated for 1 h at 25° C with 0.1 unit α-glucosidase from yeast (Sigma) in 10 mM sodium phosphate buffer (pH 6.8), or 0.05 unit β-glucosidase from sweet almond (Sigma) in 10 mM sodium acetate buffer (pH 5.5). Usually, 1–2 µg equivalent auxin (>200 Bq) dissolved in 20 µL assay medium was needed for each analysis. After digestion, except in strong alkaline conditions when the reaction medium had to be acidified with phosphoric acid (pH 3) and extracted with ethyl acetate, samples were directly spotted onto TLC plates for product analysis in solvents A, B and D (butanol-acetic acid-water; 4:1:1, by vol.). Reducing-sugar residues were developed with benzidine-trichloroacetic reagent (Bacon and Edelman 1951).

The major NAA metabolite (metabolite A) was purified on a 500-µg scale for mass spectroscopy, starting from 10^8 protoplasts (100 mL suspension) incubated with 100 µM NAA (9.2 kBq · mL^{-1}) for 3 h. Metabolite A was isolated from methanolic extracts by two successive TLC separations in solvents A and B, and finally purified on a C_{18} µbondapack reversed-phase HPLC column using methanol-water-acetic acid (55:45:1, by vol.) as eluent. Mass spectra were recorded on an AEI MS9 mass spectrometer (AEI, Manchester, UK) with chemical ionization in *iso*butene or in ammonia.

Influx and efflux measurements. Influx rates of NAA and IAA were estimated from the quantity of [^{3}H]NAA (10 nM) or [^{3}H]IAA (40 nM) accumulated by protoplasts within the first minute of incubation. Inward membrane permeabilities were estimated from the ratio of the auxin quantity which entered one unit of membrane surface area (protoplast surface = 2.8×10^{-9} · m^2) per time unit to the external concentration.

To measure auxin efflux rates, protoplasts (2×10^6 protoplasts · mL^{-1} in T_{00}) were first loaded with 1 µM [^{3}H]NAA or [^{3}H]IAA for 15 min at room temperature. The protoplast suspension was then diluted with 9 vol. of auxin-free buffer and 500-µL aliquot fractions (10^5 protoplasts) were pipetted at intervals. Auxin concentrations were monitored both in protoplasts and medium. Metabolites were analyzed at the end of the loading period and after 30 min efflux. The effect of NPA on the efflux kinetics was assayed by adding 10 µM NPA to the protoplast suspension 5 min prior to dilution.

Results

Accumulation of NAA in protoplasts. Tobacco mesophyll protoplasts were able to concentrate [^{3}H]NAA several fold from the medium when incubated at pH 5.7 with micromolar NAA concentrations (Fig. 1). At 4° C, the apparent accumulation ratio was constant after 60 min incubation ($C_i/C_e = 8.6 \pm 1.2$, $n = 8$) and was stable for at least 2–3 h. Radioactivity accumulated at this temperature mainly corresponded to untransformed auxin, less than 5% of the absorbed radioactivity being incorporated into metabolites after 2 h incubation. By contrast, at 20° C, whereas the internal free-NAA concentration reached a constant value ($C_i/C_e = 11.2 \pm 0.9$, $n = 14$) with-

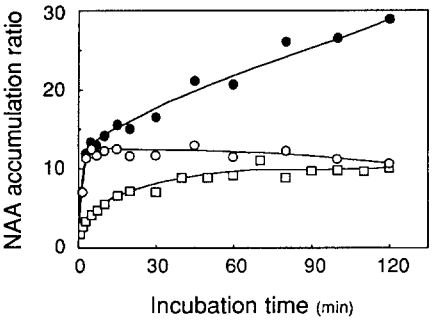

Fig. 1. Kinetics of NAA accumulation at 4 ° C (□) or 20° C (○, ●) in tobacco mesophyll protoplasts. Curves are representative of a series of experiments ($n = 11$ at 20° C and 5 at 4° C) in which 10^6 protoplasts · mL^{-1} were incubated in T_{00} buffer containing 1 µM (4° C) or 5 µM (20° C) [^{3}H]NAA at pH 5.7. Intra- and extracellular NAA concentrations were monitored as described in *Materials and methods*. Accumulation ratios were obtained by dividing the total NAA concentration (apparent accumulation ratio; ●) or the free-NAA concentration (net accumulation ratio; □, ○) in protoplasts by the external concentration. The curve showing the evolution with time of the apparent NAA accumulation ratio at 4° C was omitted for clarity because apparent and net accumulation ratios were very similar at this temperature

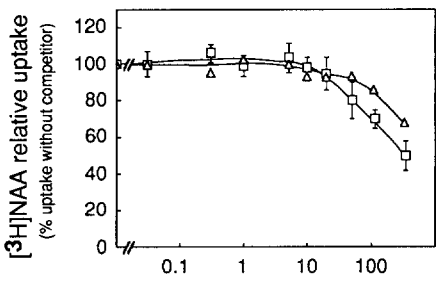

Fig. 2 Influence of the external NAA concentration (□) on initial [^{3}H]NAA uptake in tobacco mesophyll protoplasts at 20° C and pH 5.7. Accumulation of [^{3}H]NAA (10 nM) in protoplasts (10^6 · mL^{-1} in T_{00} buffer) was monitored during the first minute of incubation. Mean values (±SE; five experiments) are expressed as percent of tracer accumulation measured in the absence of unlabelled auxin (mean initial uptake = $74 \pm 9 \times 10^{-3}$ fmol · protoplast^{-1} · min^{-1}). The influence of increasing concentrations of benzoic acid (*BA*; △) on [^{3}H]NAA uptake was also investigated for comparison (one experiment)

in 2–3 min, protoplasts kept on accumulating auxin for hours due to extensive metabolism of the absorbed molecules (see below). Initial [^{3}H]NAA uptake did not change upon addition of unlabelled NAA up to 10 μM (Fig. 2), showing proportionality between influx and external concentration. This indicated than NAA molecules entered protoplasts mainly by diffusion (uptake rate $= 74 \pm 9 \times 10^{-3}$ fmol · min^{-1} · protoplast^{-1} at 1 μM external concentration, 20° C and pH 5.7, $n = 5$). There was no evidence for the participation of any saturable component. Uptake inhibition above 10 μM auxin proved to be non-specific because it also occurred when NAA was replaced by BA (Fig. 2), which has the same pK (4.2), and was accumulated to the same extent as NAA (data not shown).

Accumulation of NAA in protoplasts was strongly dependent on the pH gradient between the interior of the cells and the bulk solution (Fig. 3). Increasing the pH in the incubation medium from 4 to 7 decreased the NAA accumulation ratio by approximately 50-fold. The accumulation ratio was also reduced by 75% on addition of 100 μM nigericin, an ionophore expected to diminish cytosolic pH through an electroneutral H^+/K^+ exchange with the solution in our experimental conditions (external pH 5.5, low K^+). Data were compared with the theoretical absorption of a weak acid in a membrane-limited compartment under the influence of both the pH difference between the bulk phase and the compartment, and the transmembrane potential (Goldsmith 1977; Rubery 1980, 1987). The theoretical accumulation of NAA (pK = 4.2) was computed using Eq. 1 (adapted from Rubery 1980)

$$\frac{C_i}{C_e} = \frac{\{1+10^{(pH_i-pK)}\}\{P_r[1-Exp(-\frac{FE}{RT})]+\frac{FE}{RT}10^{(pH_e-pK)}\}}{\{1+10^{(pH_e-pK)}\}\{P_r[1-Exp(-\frac{FE}{RT})]+\frac{FE}{RT}10^{(pH_i-pK)}Exp(-\frac{FE}{RT})\}} \quad \text{(Eq. 1)}$$

where P_r is the ratio of the membrane permeabilities for the acid and for the anion, pH_i and pH_e the intra- and extracellular pH values, respectively, E the membrane potential, F the Faraday constant, R the gass constant and T the absolute temperature. The experimental accumulation curve was similar in shape to the calculated one but was shifted towards higher pH as observed in most plant cells (Goldsmith 1977). The possible origin of such an alkaline shift has been extensively commented on in several reports (Goldsmith 1977; Gutknecht and Walter 1980; Rubery 1980). Modifying the parameters, or nesting several compartments with different membrane permeabilities, pH or E values, changed the ordinate without moving the abscissa of the midpoint from $pH = pK_{NAA}$.

Formation of NAA conjugates in protoplasts. During the first 2 h of incubation at 20° C, protoplasts accumulated essentially two components which accounted for more than 80% of the accumulated radioactivity (Fig. 4), unmodified NAA ($R_f = 0.85$–0.87 in solvent A), identified by co-chromatography with an authentic sample, and

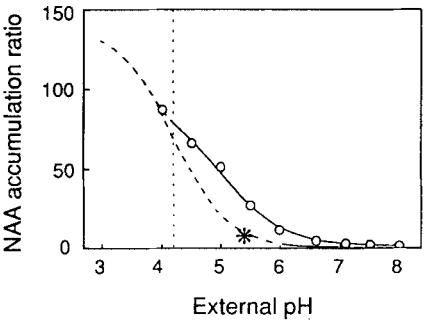

Fig. 3. Accumulation of free NAA in protoplasts as a function of pH in the bulk solution. Tobacco mesophyll protoplasts (10^6 · mL^{-1}) were suspended with 5 μM [^{3}H]NAA in T_{00} buffer containing 5 mM Mes and adjusted to the desired pH value by addition of 1 N HCl (pH < 4.6) or 1 N NaOH (pH > 4.6). Accumulated auxin was quantified after 60 min incubation at 20° C. The continuous curve (o), representative of two experiments, shows variations with external pH of the actual C_i/C_e, C_i referring to free intracellular NAA and C_e to the external concentration. *Asterisk* indicates the accumulation level of free auxin in the presence of 100 μM nigericin a pH 5.5. The theoretical NAA accumulation ratio at equilibrium (*dashed line*) was computed from Eq. 1 (see text) using $Pr = 10^3$ (Raven 1975), $pH_i = 7.5$ (Guern et al. 1991), $E = -11.4$ mV (Barbier-Brygoo et al. 1989) and $pK_{NAA} = 4.2$ (*vertical dotted line*). The curve was normalized to fit the experimental C_i/C_e at pH 4

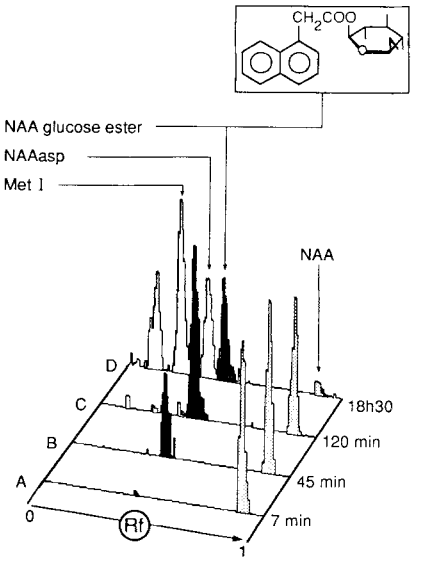

Fig. 4. Thin-layer chromatograms in solvent A of methanolic extracts of tobacco mesophyll protoplasts incubated for 7 min (*A*), 45 min (*B*), 120 min (*C*) and overnight (*D*) at 20° C and pH 5.7 in the presence of 5 μM [^{3}H]NAA (see experimental details in *Materials and methods*). The radioactivity on the silica plate was scanned with a linear analyzer. Peak intensities in each lane were normalized to the highest peak intensity in the same lane. Naphthalene-1-acetic acid ($R_f = 0.85$) and NAAasp ($R_f = 0.35$) were compared with synthetic standards. Metabolite A ($R_f = 0.42$) was identified as 1-(naphthyl-1-acetyl)-β-D-glucopyranose (formula in *inset*) by using mass spectroscopy, mild alkaline hydrolysis and β-glucosidase digestion (see *Results*). Unidentified "metabolite I" ($R_f = 0.23$) could correspond to metabolite I described without characterization by Smulders et al. (1990)

metabolite A ($R_f = 0.42$ in solvent A) characterized as an ester of glucose as described below. With longer incubations, at least three other radioactive products ($R_f = 0.11$, 0.23, 0.35) were synthesized (Fig. 4), the less polar of which was tentatively identified as NAAasp by comparison with a synthetic standard. The major compound at $R_f = 0.23$ probably corresponded to the so-called metabolite I described in tobacco explants by Smulders et al. (1990) and was not further characterized. After an overnight incubation, more than 95% of the accumulated radioactivity could be ascribed to metabolized NAA, a third of which being metabolite I, 20–25% NAAasp and 20–25% NAA glucose ester. This pattern was very similar to that observed at the end of a 16-h digestion period when protoplasts had been prepared in the presence of 15 μM [^{3}H]NAA (data not shown). Metabolite A was hydrolysed within minutes in 0.1 N KOH at 25° C to yield NAA and a reducing sugar (brown colour in the benzidine reagent test) chromatographically identical to glucose in solvents A, B and D. This suggested that unmodified NAA was coupled to glucose through an ester bond. The nature of the linkage was confirmed by the instability of metabolite A during chromatography in ammoniacal *iso*propanol (solvent C) giving naphthalene-1-acetamide in situ, identified by comparison with synthetic [^{3}H]NAAam, and the existence of a transesterification mechanism releasing NAA methyl ester instead of the free acid when the alkaline hydrolysis was performed in the presence of methanol. Mass spectra of metabolite A indicated that NAA was stoichiometrically coupled to glucose. Though the first ion was observed in most cases at $m/z = 331$ ($M + 1\text{-}H_2O$), the molecular ion, consistent with the molecular formula $C_{18}H_{20}O_7$ ($MW = 348$), was several times detected at $m/z = 349$ ($M + 1$) in *iso*butene and $m/z = 366$ ($M + 18$) in ammonia with a weak intensity. The intense peak at $m/z = 187$ was ascribed to the NAA moiety ($M_{NAA} = 186$). The other part of the molecule could be identified as a hexose from the observation of a weak ion at $m/z = 181$ ($M_{hexose} + 1$) and of several characteristic fragments ($m/z = 163$, 145, 127) resulting from successive dehydrations. Because, in addition, metabolite A was devoid of reducing properties and quantitatively hydrolyzed by β-glucosidase but not by α-glucosidase, it was finally identified as 1-(naphthyl-1-acetyl)-β-D-glucopyranose (see formula on Fig. 4).

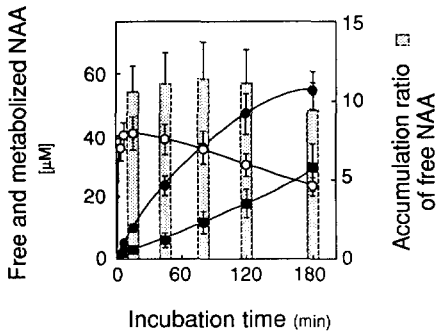

Fig. 5. Time course of accumulation of free NAA (○), NAA glucose ester (●) and pooled polar metabolites at $R_f \leq 0.35$ (■) by tobacco mesophyll protoplasts ($10^6 \cdot mL^{-1}$) incubated at 20° C and pH 5.7 in the presence of 5 μM [^{3}H]NAA. Results are means (±SE) of values determined in six independent experiments. Evolution with time of the accumulation ratio of free NAA (*dashed columns*) is given for comparison with data reported in other figures

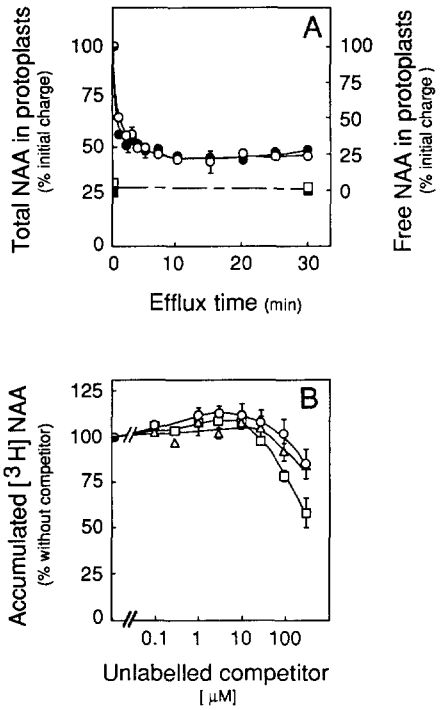

Fig. 6. A Kinetics of NAA efflux measured at 20° C in the absence (○, □) or in the presence of 10 μM NPA (●, ■). Protoplasts ($2 \times 10^6 \cdot mL^{-1}$ in T_{00} buffer, pH = 5.7) were incubated for 15 min with 1 μM [^{3}H]NAA then diluted tenfold with auxin-free buffer. N-1-naphthylphthalamic acid was added in samples 5 min prior to dilution when the effect of the inhibitor had to be assayed. Radioactivity accumulated in 10^5 protoplasts (○, ●) was measured at the end of the preincubation (zero time) and at the indicated times after dilution. Metabolites (□, ■) were quantified at zero time and after 30 min efflux. Internal free NAA was calculated by substracting the contribution of labelled metabolites from the total radioactivity recovered in protoplasts. Results (means ± SE of two experiments) are given as percent of total (*left ordinates*) and free NAA (*right ordinates*) accumulated in protoplasts at zero time. **B** Accumulation of free [^{3}H]NAA as a function of the external concentration of NAA (□), NPA (○) or BA (△) at 20° C. Protoplasts ($10^6 \cdot mL^{-1}$ in T_{00} buffer, pH 5.7) were incubated for 25 min in the presence of 10 nM [^{3}H]NAA and increasing concentrations of unlabelled auxin, NPA or BA. Values, expressed as percent of tracer accumulation measured in the absence of competitor, are means (±SE) of results obtained in two (BA effects), three (NPA effects) and four (NAA effects) independent experiments

As shown in Fig. 5, the concentration of NAA glucose ester became constant, at approx. 60 μM at 5 μM external NAA, after 3 h incubation at 20° C. By contrast, the more-polar metabolites were continuously produced throughout the incubation and, though they appeared four times more slowly than the ester, finally represented the major proportion of the accumulated NAA metabolites. For instance, protoplasts ($10^6 \cdot mL^{-1}$) incubated for 18 h with 5 μM NAA contained about 9 μM free NAA (0.18 ± 0.06 fmol · protoplast^{-1}), 30 μM NAA glucose ester (0.6 ± 0.2 fmol · protoplast^{-1}) and 90 μM polar metabolites (1.5 ± 0.4 fmol · protoplast^{-1}) at the end of

the incubation, having absorbed about 2.3 pmol auxin of the 5 pmol auxin supplied in the medium (four experiments).

Kinetics for NAA efflux from protoplasts. Half of the radioactivity accumulated in protoplasts during a 15-min preincubation with 1 μM [^{3}H]NAA was released to the medium within less than 2 min after a tenfold dilution (Fig. 6A). Afterwards, efflux declined drastically. The minimum efflux rate for NAA molecules was estimated to be 70×10^{-3} fmol · min^{-1} · protoplast^{-1} during the rapid phase. Since NAA metabolite levels remained unchanged in protoplasts during the 30-min efflux period, excreted molecules originated from unconjugated cellular pools (Fig. 6A). There was no evident participation of an auxin-efflux carrier in NAA efflux. Indeed, efflux kinetics were unmodified by addition of 10 μM NPA, an inhibitor of the auxin-efflux carrier in plant cells (Sussman and Goldsmith 1981; Fig. 6A). Besides, as shown by the constant trend of [^{3}H]NAA accumulation with increasing concentrations of NPA or unlabelled NAA (Fig. 6B), neither compound changed the balance between the processes governing the influx and efflux of the tracer. The diminution of the NAA internal concentration observed beyond 10 μM NPA or NAA might reflect a non-specific effect if one refers to the same decrease provoked by BA (Fig. 6B).

Influence of the digestion procedure on auxin accumulation. Protoplasts freshly isolated from leaf fragments digested overnight in T_0 growth medium containing 15 μM [^{3}H]NAA displayed a pattern of labelled metabolites very similar to that observed in protoplasts prepared in the presence of unlabelled NAA then incubated for 18.5 h in the presence of [^{3}H]NAA (Fig. 4, lane D). After washing, protoplasts were depleted of most unmetabolized NAA, but still contained large quantities of metabolites. However, residual free auxin and metabolites did not modify the protoplasts' capabilities to further accumulate and metabolize NAA. Indeed, in accumulation experiments, protoplasts isolated from leaf fragments digested in the presence or in the absence of auxin absorbed NAA with the same kinetics (four similar experiments). After 2 h incubation, NAA glucose ester represented 49 ± 10% and 49 ± 9% of the radioactivity accumulated in protoplasts prepared with and without auxin, and the accumulation ratios for free NAA were identical, 11.8 ± 1.3 and 11.8 ± 1.1 respectively.

Accumulation and metabolism of IAA in protoplasts. Basically, IAA accumulation in protoplasts proceeded from a mechanism similar to that described for NAA. Free IAA was accumulated under the action of the transmembrane pH gradient (data not shown) and sequestered in metabolized forms (Fig. 7). Influx velocity was proportional to the external concentration with no saturable component detectable up to 100 μM, indicating that IAA molecules entered protoplasts mostly by diffusion. However, it is worth to note (compare Figs. 5 and 7) that both, the accumulation ratio of free IAA ($C_i/C_e = 2.2 \pm 0.2$ at pH 5.7, $n = 7$), and its uptake rate

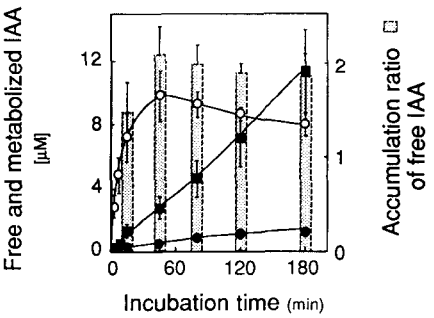

Fig. 7. Time course of accumulation of free IAA (○), IAA glucose ester (●) and pooled polar metabolites (including IAAasp) at $R_f \leq 0.25$ (■) in protoplasts (10^6 · mL^{-1}) incubated at 20° C and pH 5.7 in the presence of 5 μM [^{3}H]IAA. Results (±SE) are means of values determined in four independent experiments. Evolution with time of the accumulation ratio of free IAA (*dashed columns*) is given for comparison with data reported in other figures

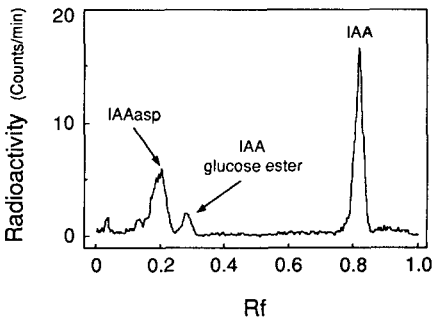

Fig. 8. Thin-layer chromatograms in solvent A of methanolic extracts of tobacco mesophyll protoplasts incubated for 3 h at 20° C and pH 5.7 in the presence of 5 μM [^{3}H]IAA (see experimental details in *Materials and methods*). Free IAA ($R_f = 0.83$) was compared with a synthetic standard. Indole-3-acetic acid glucose ester ($R_f = 0.30$) was identified by using mild alkaline hydrolysis and β-glucosidase digestion (see *Results*). The compound at $R_f = 0.20$ could be hydrolyzed in boiling 7 N KOH and was tentatively identified as IAAasp, as supported by its co-migration with synthetic IAAasp in solvents A, B, C

($8 \pm 1.5 \times 10^{-3}$ fmol · min^{-1} · protoplast^{-1} at 1 μM external concentration, 20° C and pH 5.7, $n = 2$) were five to ten times lower than the corresponding parameters measured with NAA.

Indole-3-acetic acid was rapidly converted to several metabolites (Fig. 8; $R_f = 0.04, 0.15, 0.20, 0.30$ in solvent A) which accounted for half of the accumulated hormone, approx. 17 μM at 15 μM external IAA, after 2 h of incubation (Fig. 7). IAA glucose ester ($R_f = 0.30$), identified from its reactivity to hydrolysis with dilute KOH (0.1 N, 25° C) and with β-glucosidase, was a minor conjugate only representing 10% of the total metabolites (Figs. 7, 8). The spot at $R_f = 0.20$ co-migrated with synthetic IAA aspartyl amide in solvents A, B, C and was more than half-hydrolyzed to IAA in boiling 7 N KOH. This spot was then assumed to contain IAAasp which is a common IAA metabolite in plants (Cohen and Bandurski 1982; Cohen 1983; Vijayaraghavan and Pengelly 1986; Sitbon et al. 1993).

Following a ten-fold dilution, protoplasts released, in 30 min, approx. 50% of the free IAA accumulated during

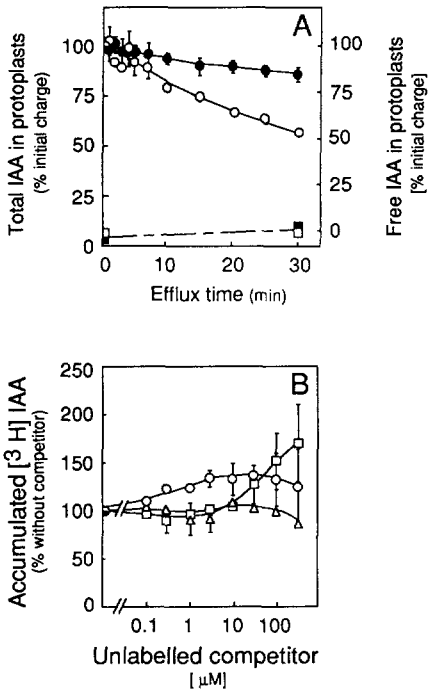

Fig. 9. A Kinetics of IAA efflux measured at 20° C in the absence (○, □) and in the presence of 10 μM NPA (●, ■). Experimental procedures and presentation of results are as given in Fig. 6A for NAA efflux. Radioactivity accumulated in 10^5 protoplasts (○, ●) and that corresponding to metabolites (□, ■) are shown as means (±SE) of two experiments. **B** Accumulation of free [^{3}H]IAA as a function of the external concentration of IAA (□), NPA (○) or BA (△) at 20° C. The [^{3}H]IAA concentration was 40 nM in the incubation medium. Experimental procedures are as given in Fig. 6B for NAA accumulation. Values, expressed as percent of tracer accumulation measured in the absence of competitor, are means (±SE) of results obtained in two (NPA and BA effects) and three (IAA effects) independent experiments

a 15-min preincubation in the presence of a 1-μM external concentration (Fig. 9A). Auxin was excreted at an almost constant velocity of 2×10^{-4} fmol · min^{-1} · protoplast^{-1}. Because the efflux rate was reduced by three- to fowfold in the presence of 10 μM NPA (Fig. 9A) and the accumulation of [^{3}H]IAA was raised by increasing concentrations of either NPA or IAA (Fig. 9B), while BA used as the control had only negligible effect, it was concluded that IAA efflux in protoplasts was partly catalyzed by a carrier.

Discussion

Accumulation of free auxin in protoplasts. One purpose of this work was to determine whether auxin accumulation in protoplasts could be predicted from simple considerations. Accumulation of free NAA and IAA in protoplasts fitted a diffusive model by anion trapping, the general mechanisms of which are well documented (Goldsmith 1977; Rubery 1980, 1987). Yet, using this model to predict auxin accumulation in multicompartmented protoplasts was not possible without a precise knowledge of the parameters controlling repartition of the molecule between compartments (pH, membrane potential, membrane permeabilities, volume). Auxin-absorption curves were shifted to higher pH and flattened compared with those computed by using Eq. 1, possibly due to unstirred-layer effects (Gutknecht and Walter 1980). In spite of relatively close pK values, accumulation of IAA (pK = 4.7) in protoplasts was severalfold lower than that of NAA (pK = 4.2). Such a difference in accumulation could result from differences between membrane permeabilities for protonated and dissociated species, and/or unequal participation of carriers in their membrane transport. Finally, measured internal auxin concentrations were averaged using the protoplast volume whereas molecules were partitioned between small basic compartments strongly accumulating auxin anions (e.g. cytosol, chloroplasts) and large acidic compartments containing low auxin concentration (e.g. vacuole).

Permeability of the plasma membrane to neutral NAAH and IAAH, species assumed to enter protoplasts, was calculated by correcting values measured at pH 5.7 in 1-min accumulation experiments, respectively 4×10^{-7} and 0.4×10^{-7} m · s^{-1}, for dissociation (NAAH/NAA$^-$ = 0.03 and IAAH/IAA$^-$ = 0.1). Permeabilities at 20° C were 12×10^{-6} m · s^{-1} for NAAH and 0.4×10^{-6} m · s^{-1} for IAAH, in reasonable agreement with those reported for other plant cell membranes (Raven 1975; Rubery 1980; Gimmler et al. 1981) and artificial lipoid-bilayer systems (Bean et al. 1968; Gutknecht and Walter 1980) ($P_{IAAH} = 1 \times 10^{-6}$ to 40×10^{-6} m · s^{-1}). The membrane permeability coefficient for a solute is proportional to its partition coefficient between membrane material and aqueous surroundings (Nobel 1974, pp. 26–32). We measured partition coefficients between water (pH 2) and decane of 0.25 for NAAH and 0.005 for IAAH, showing that the larger membrane permeability of NAAH mainly resulted from a stronger hydrophobicity.

Protoplasts excreted NAA 350 times more rapidly than IAA, suggesting that the outward permeability of membranes was 70 times higher for NAA than for IAA, considering that accumulation of IAA was 5 times lower. Efflux kinetics for a solute distributed between several compartments cannot be solved, except in particular cases, in terms of membrane permeabilities characteristic of each compartment (see analysis in Walker and Pitman 1976). However, the quantity of solute excreted by the system per unit time at a given instant is proportional to both the solute concentration in the outer compartment, namely the cytosol, at this instant and the permeability of the membrane limiting this compartment. Accordingly, the importance of the NAA efflux rate in protoplasts, comparable in size to its influx rate, might indicate that there was no major limiting barrier to the NAA exchanges between compartments containing hormone and the medium. On the contrary, the slow efflux rate measured for IAA might indicate that either the plasma membrane was poorly permeant to hormone, essentially the anion form, or IAA was sequestered in some internal compartment, or both.

Short uptake experiments failed to demonstrate the existence of a carrier operating in the influx mode for

both auxins in protoplasts. Inhibition of [^{3}H]NAA uptake by high NAA or BA concentrations was likely due to an acid-load effect on intracellular pH owing to the large amounts of NAA or BA entering the protoplasts during the first seconds of incubation. An acid-load effect probably also accounted for the decrease in NAA accumulation observed beyond a competitor concentration of 10 μM.

By contrast, tobacco protoplasts were equipped with a functional auxin-efflux carrier, as evidenced by the inhibitory effect of NPA on IAA efflux and the stimulatory effect of high IAA concentrations on its own accumulation. The presence of the carrier was not detected with NAA, probably because the membrane transport of this auxin involved a larger diffusive component, making carrier participation negligible. The observation of a functional auxin-efflux carrier in mesophyll tobacco protoplasts is in apparent contradiction with data reported by Libbenga et al. (1985) showing that freshly prepared protoplasts lack NPA-binding sites. In these experiments, however, NPA-binding sites were possibly destroyed during leaf digestion, since the authors used more enzymes (× 20) though for shorter periods (2–4 h). Another explanation is that the sites which bind NPA with high affinity and were analyzed by Libbenga et al. (1985) are not implicated in the membrane transport of auxin, as recently postulated by Michalke et al. (1992).

Auxin metabolism in tobacco mesophyll protoplasts. Auxin metabolism was quite similar in mesophyll protoplasts and in other tobacco-derived systems (Caboche et al. 1984; Vijayaraghavan and Pengelly 1986; Smulders et al. 1990; Nilsson et al. 1993; Sitbon et al. 1993). Naphthalene-1-acetic acid was essentially converted into glucose ester upon short incubations but, after several hours, more slowly synthetized metabolites such as aspartyl amide conjugate became predominant. By contrast, exogenous IAA accumulated as an amide conjugate, probably IAAasp, from the very beginning of the incubation, and glucose ester was only a minor metabolite. It is more important to note, however, that metabolism strongly amplified auxin accumulation in protoplasts, as would occur if an anion-trapping mechanism only were present. We also observed that protoplasts, incubated at a density of $10^6 \cdot mL^{-1}$ in the presence of 5 μM NAA, consumed overnight half the auxin exogenously supplied, which suggests that metabolism, in some conditions, should be able to provoke an auxin shortage in the incubation medium within 1–2 d of culture.

Stable amide derivatives of auxin could constitute an irreversible output from the free-auxin pool (Vijayaraghavan and Pengelly 1986; Smulders et al. 1990; Sitbon et al. 1993). By contrast, auxin conjugates such as glucose esters, because they are readily hydrolyzed in vivo (Vijayaraghavan and Pengelly 1986; Smulders et al. 1990; Sitbon et al. 1993; Delbarre et al. 1994), are increasingly considered to be implicated in the regulation of hormone levels in cells (Cohen and Bandurski 1982; Smulders et al. 1990; Palme and Schell 1993). In relation to that, a comparison between the rates of NAA uptake (0.3 fmol · protoplast^{-1} · min^{-1} at 5 μM external concentration), formation of NAA glucose ester (0.008 fmol · min^{-1} · protoplast^{-1} at 5 μM external NAA) and ester hydrolysis in vivo (0.003 fmol · min^{-1} · protoplast^{-1} at 50 μM internal ester) (Delbarre et al. 1994) indicates that NAA esterification could contribute to long-term auxin homeostasis by sequestering or releasing hormone when the available amount increases or decreases.

In conclusion, the present work shows that NAA and IAA are concentrated in protoplasts above their level in the incubation medium and are accumulated mostly in metabolized forms. However, both auxins behave quite differently. In fact, intracellular auxin concentrations cannot be easily predicted because auxin distribution between protoplasts and the external medium is widely dependent on membrane permeability, carrier efficiency and metabolism, all properties linked to the chemical structure of the auxin assayed. This clearly emphasizes the need to estimate accumulation and metabolism of auxins when analyzing their effects in a given cell system. Our data also indicate that it may be difficult to prepare auxin-free protoplasts when necessary, since active hormone can be released from labile conjugates for a long time following protoplast isolation, a problem reinforced by the fact that some proportion of the internal free auxin, being contained in compartments in slow exchange with the external medium, will be hardly eliminated from protoplasts. Finally, particular attention must be paid to metabolism intensity when monitoring hormone effects over long culture periods because protoplasts are able to deplete the incubation medium of auxin and consequently may be rapidly deprived of hormone. The time required to reach an auxin shortage in the culture medium depends on both the protoplast density and the nature of the auxin assayed. Nevertheless, since the accumulation of free auxin molecules is closely related to the size of the pH gradient across the plasma membrane, the internal auxin concentration can be easily modified over a large range just by increasing or decreasing the external pH. This would be an interesting way of manipulating independently the extra- and intracellular concentrations of auxin with the aim of discriminating their effects on physiological processes.

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