

The Site of Indole-3-acetic Acid Synthesis in Mesophyll Cells of *Spinacia oleracea*

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Using [^{14}C]tryptophan as a precursor, the intracellular localization of indole-3-acetic acid biosynthesis in spinach mesophyll cells was investigated. Chloroplasts as well as extraplastidic compartments were able to transform tryptophan into indole-3-acetic acid.

The cofactor requirement of plastidic and extraplastidic indole-3-acetic acid synthesis is shown.

Light and abscisic acid treatment inhibited indole-3-acetic acid production in all preparations.

Introduction

Phytohormones, e.g. auxin, regulate the development of plants. It is essential that also the intracellular concentration of these hormones are controlled. Important factors for such a control are intracellular hormone compartmentation, rates of hormone production and degradation and rates of inactivation by conjugation.

The mechanisms by which plants regulate the IAA level have not yet been defined in detail. It is known in which part of the plant auxins can be synthesized (e.g. Wightman [1, 2]). However, there is little information on intracellular sites of synthesis [3] and on intracellular compartmentation [4]. Earlier results indicated that up to about 45% of the total amount of free IAA in the laminae of spinach leaves are localized within illuminated chloroplasts and that darkened chloroplasts contain only 35% [4]. Since spinach leaves are capable of converting exogenously applied tryptophan to IAA [5], and since light seems to have an inhibitory effect on the production of IAA [6], the difference of the hormone distribution under light and dark conditions might be a factor in the regulation of IAA synthesis.

In the following we investigated the intracellular compartmentation of IAA biosynthesis.

Materials and Methods

Material

Spinacia oleracea was grown in a green house or under field conditions. The isolation of intact "type A" chloroplasts was carried out according to the method of Jensen and Bassham [7] with slight modifications. To decrease bacterial contamination of plant material the freshly harvested leaves were washed three times with distilled water, bathed in 1% (w/v) NaOCl solution for one minute and then washed ten times with sterilized water. All solutions, media and glass ware were autoclaved before use. The percentage of intact chloroplasts varied between 70–93% and their capacity for CO_2 assimilation between $65\text{--}129\ \mu\text{mol CO}_2 \times \text{mg}^{-1} \text{ chl} \times \text{h}^{-1}$. Carefully washed chloroplast preparations were contaminated with less than 0.5% of the extraplastidic cytosol.

Non-aqueously isolated chloroplasts and corresponding non-aqueous leaf fractions depleted of chloroplasts were prepared according to Heber and Willenbrink [8]. The cross-contamination of both fractions were measured as described earlier [4].

The isolation of intact protoplasts from spinach leaves and of chloroplasts and corresponding cytoplasmic fractions from these preparations followed procedures described recently [9, 10].

Radiochemicals: $\text{D}[\text{U-}^{14}\text{C}]\text{sorbitol}$, ^3HHO , [^{14}C]Trp (spec. act. $2.09\ \text{GBq} \times \text{mmol}^{-1}$) and [^{14}C]IAA (spec. act. $33.3\ \text{MBq} \times \text{mmol}^{-1}$) were purchased from Amersham Buchler (Braunschweig, Germany).

Abbreviations: ABA, abscisic acid; α -KG, α -ketoglutaric acid; Chl, chlorophyll; Chlpl, chloroplasts; HEPES, N-hydroxy-ethyl-piperazine-N'-2-ethanesulphonate; IAA, indole-3-acetic acid; [^{14}C]IAA, [$2\text{-}^{14}\text{C}$]indole-3-acetic acid; P-5'-P, pyridoxal-5'-phosphate; ThPP, thiamine pyrophosphate; Trp, tryptophan; [^{14}C]Trp, DL-[methylene- ^{14}C]tryptophan; TLC, thin layer chromatography.

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Methods

The uptake of [^{14}C]tryptophan into intact chloroplasts and protoplasts was measured using the silicone layer filtering centrifugation technique [13] as specified earlier [11, 12].

IAA synthesis in leaves of spinach. Intact spinach rosettes were transferred from the green house to an environmental chamber (14 h $5\text{ W} \times \text{m}^{-2}$ at 293 K and 10 h darkness at 288 K). 7.5×10^{-5} Bq [^{14}C]Trp in small drops were applied to 3-mm cuts in the midrib of young leaves during the light period or in darkness. This solution which contained also $0.1\text{ mg} \times \text{ml}^{-1}$ chloramphenicol to suppress the growth of contaminating bacteria was taken up within about 30 min. During the following reaction time the wounds were moistened with distilled water containing chloramphenicol ($0.1\text{ mg} \times \text{ml}^{-1}$). After 4 h the leaves were immersed in liquid nitrogen and immediately homogenized.

IAA synthesis in chloroplasts, protoplasts and cytoplasmic fractions. [^{14}C]Trp ($8 \times 10^3 - 2.5 \times 10^4$ Bq) was added to 200 μl aliquots of suspensions containing aqueously isolated intact chloroplasts (corresponding to 50–200 μg Chl), intact chloroplasts isolated from protoplasts (corresponding to about 500 μg Chl.), extraplastidic fractions of protoplast preparations, 20–50 mg of non-aqueously isolated chloroplasts (corresponding to 0.5–1 mg Chl) or 5–100 mg of the extraplastidic non-aqueously isolated leaf fractions.

The assay medium for intact chloroplasts was the "C" – medium [7] with 0.33 M sorbitol as osmoticum, whereas the assay medium for protoplasts contained 0.5 M sorbitol [9]. In experiments with non-aqueously isolated preparations the basic reaction media consisted of 60 mM HEPES/KOH buffer (pH 7.6) and 1 mM MgCl_2 . Reaction mixtures contained $0.1\text{ mg} \times \text{ml}^{-1}$ chloramphenicol or $0.3\text{ mg} \times \text{ml}^{-1}$ of both Penicillin G and Ampicillin. Where indicated, cofactors were added: pyridoxal-5'-phosphate (0.1 mM), α -ketoglutaric acid (5 mM), thiamine pyrophosphate (0.1 mM), NAD (0.1 mM) and NADH (0.1 mM).

Reactions were stopped by adding methanol to aliquots of the samples (final concentration 80% v/v). Prior to extraction and analysis of radioactive labelled IAA, small quantities (ca. 10 mg per assay) of unlabelled IAA was dissolved in the reaction mixture in order to minimize losses of labelled [^{14}C]IAA by oxidation. To correct for possible contributions of

epiphytic bacteria to the measured synthesis of IAA in spite of the present antibiotics, the different plant fractions were heated to 363 K for 5–8 min to destroy spinach enzymes. The amount of [^{14}C]IAA synthesized in these control assays were only insignificantly higher than the [^{14}C]IAA present in blanks supplied with radioactive Trp solutions in the absence of plant material (0.1–0.2%). The IAA synthesis in the unboiled suspension was corrected for these values.

Extraction and analysis of IAA. Each sample, stored under N_2 at 260 K, was extracted three times with methanol (80%) containing $100\text{ mg} \times \text{l}^{-1}$ of the antioxidant 2,6-di-*tert*-butyl-4-methyl-phenol (BHT). The combined extracts of each assay were evaporated in a rotary-film evaporator under reduced pressure at 310 K. The residue was dissolved in a small volume of methanol and chromatographed on silica-gel 60 F-254 TLC-plates (Merck, Germany) with the solvent system chloroform/methanol/95% glacial acetic acid (75 : 20 : 5, v/v/v) [14].

After autoradiography, radioactive material was scraped off the plates and counted in a liquid scintillation counter (BF 8000, Berthold, Wildbad, Germany). All samples were corrected for quenching.

To ascertain that the radioactivity of the IAA zone actually originated from auxin, the following different tests were performed:

- (1) TLC in solvent medium chloroform/96% ethanol (65 : 35, v/v) [15].
- (2) TLC using *n*-propanol/methylacetate/7N ammonia (45 : 35 : 20, v/v/v) as mobile system [14].
- (3) UV fluorescence quenching on the applied stationary phase, silica gel plates with fluorescence indicator.
- (4) The red colour of the polymeric IAA oxidation product [16], slowly appearing when IAA is exposed to oxygen.
- (5) Detection with the sensitive chromogenic Van Urk – Salkowski reagent [17].

Results and Discussion

The uptake of Trp into intact protoplasts and chloroplasts

The synthesis of ^{14}C -labelled IAA in plant cells or organelles from ^{14}C -labelled Trp requires uptake of the precursor and the internal transfer to the site of IAA synthesis.

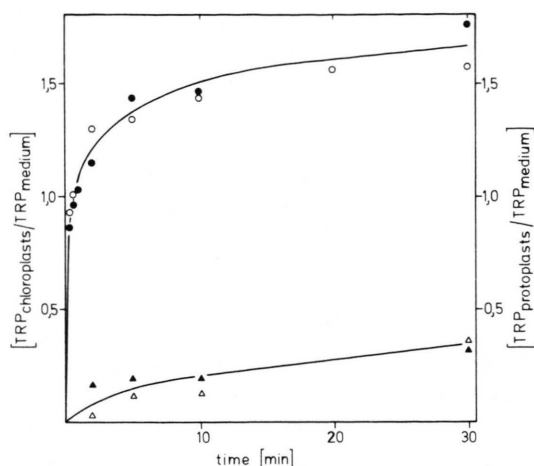


Fig. 1. Time course of $[^{14}\text{C}]\text{Trp}$ uptake into intact chloroplasts ($\circ$, $\bullet$) and intact protoplasts ($\triangle$, $\blacktriangle$) of spinach at 293 K. Open symbols indicate the accumulation ratio of this amino acid during illumination ($24 \text{ W} \times \text{m}^{-2}$), filled symbols show the accumulation ratio in the dark. Chloroplast were incubated with $1.6 \times 10^{-5} \text{ mol} \times \text{l}^{-1}$ (pH 7.0).

Fig. 1 demonstrates 1) that slow uptake of $[^{14}\text{C}]\text{Trp}$ through the plasmalemma into the cytosol takes place and 2) that the chloroplast envelope is much more permeable to Trp than the plasmalemma. Within about 10 min equilibrium between internal and external Trp concentration was reached in chloroplasts. At equilibrium the internal Trp concentration was higher by a factor of 1.5 than the external concentration. The uptake of $[^{14}\text{C}]\text{Trp}$ into protoplasts was much slower. In both cases light dark difference in uptake were not observed. An almost linear relationship does exist between external Trp concentration and the rate of Trp uptake (not shown). The permeability coefficient P_s of the chloroplast envelope was $2.13 \times 10^{-8} \text{ m} \times \text{s}^{-1}$ and that of the plasmalemma $6.85 \times 10^{-6} \text{ m} \times \text{s}^{-1}$. These data fit very well into the "Collander plot" – diagram described recently for the membranes of spinach mesophyll cells [12]. The result suggest that the Trp uptake is due to simple diffusion. The slowly increasing accumulation ratio of Trp within the chloroplasts after prolonged incubation times could be the consequence of the removal of metabolized molecules from the pool of free Trp.

IAA synthesis by whole, living spinach leaves

Table I demonstrates the ability of spinach leaves to produce IAA when fed with its precursor Trp

Table I. IAA synthesis from $[^{14}\text{C}]\text{Trp}$ ($7.5 \times 10^5 \text{ Bq}$) in young leaves under illumination ($5 \text{ W} \times \text{m}^{-2}$) or in the dark. Incubation time 4 h at 293 K.

	IAA synthesis	
	$\text{mol} \times \text{mg}^{-1} \text{ chl}$	$\text{mol} \times \text{g}^{-1} \text{ dry weight}$
Illuminated leaves	4.3×10^{-10}	6.3×10^{-9}
Darkened leaves	7.0×10^{-10}	10.0×10^{-9}

(compare Wildman *et al.* [5]). Light inhibited auxin biosynthesis. The reason for this fact is not understood [6].

However, since $[^{14}\text{C}]\text{IAA}$ applied to non-aqueously isolated cytoplasmic fractions of spinach mesophyll cells was not significantly degraded or metabolized (not shown) after 4 h, the difference of the amount of IAA measured in light and dark reflects light inhibition of the activity of enzymes catalyzing synthesis rather than light-stimulated IAA degradation.

In the following experiments all incubations were carried out in the dark.

IAA synthesis in isolated intact protoplasts and mesophyll cell fractions

In comparison with the IAA formation by intact leaves (Table I), protoplasts showed a lower rate of synthesis (Table II). Chloroplasts from such proto-

Table II. IAA synthesis in protoplasts, in chloroplasts isolated from protoplasts and in the corresponding extraplastidic fraction of the protoplast preparation. The samples were incubated for 8 h (at 293 K in the dark, pH 7.0) in a medium which contained 0.1 mM each of P-5'-P, ThPP, NAD and NADH, 5 mM α -KG and 0.1 $\text{mg} \times \text{ml}^{-1}$ chloramphenicol.

Preparation	$[^{14}\text{C}]\text{Trp}$ concentration in the medium ($\text{mol} \times \text{l}^{-1}$)	IAA synthesis $\text{mol} \times \text{mg}^{-1} \text{ chl}$
intact protoplasts	1.6×10^{-5}	3.3×10^{-10}
protoplasts fractionated into		
1) intact chloroplasts	0.9×10^{-5}	3.4×10^{-10}
2) chloroplast free residue	0.9×10^{-5}	2.0×10^{-10a}

^a This value was based on the chlorophyll content of the protoplasts yielding the cytoplasm [12].

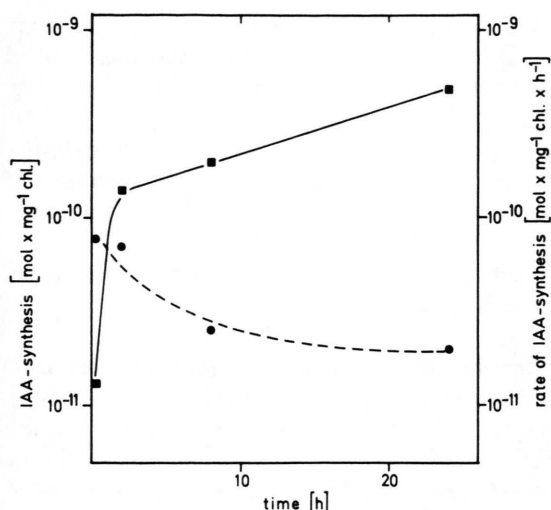


Fig. 2. Time dependent synthesis of IAA by intact chloroplasts isolated under sterile conditions from spinach leaves [■]. [^{14}C]Trp was applied at an external concentration of $6.5 \times 10^{-5} \text{ mol} \times \text{l}^{-1}$ (pH 7.6; darkness; all co-factors added). Rate of synthesis (● — — ●).

plasts exhibited a higher IAA synthetic rate over 8 h than the corresponding extraplastidic fraction. If the rates of synthesis in both compartments are added, more than the total capacity of the protoplasts for IAA synthesis from external Trp is obtained. This is because uptake across the plasmalemma is the limiting step of IAA synthesis in protoplasts. The fact that chloroplasts synthesize auxin in the presence of chloramphenicol ($0.1 \text{ mg} \times \text{ml}^{-1}$), which inhibits plastidial protein syn-

thesis indicates that the enzymes necessary for IAA synthesis are present in these organelles. This is confirmed by the observation that protein synthesis in chloroplasts is reduced in the dark [21], but the synthesis of IAA from Trp is stimulated (Table I).

Fig. 2 shows IAA synthesis of type "A" chloroplasts as a function of time. The rate is highest in the first two hours but then decreases continuously.

In Table III the co-factor requirement of IAA synthesis from Trp in chloroplasts was investigated. Sterile isolated and almost completely purified spinach chloroplasts are capable of transforming Trp to IAA, even if not supplied with cofactors whose presence are thought to be essential for this pathway [6, 19, 20]. Each assay was supplied with the following cofactors:

- 1) 0.1 mM P-5'-P known as a prosthetic group of transaminases;
- 2) 5 mM- α -KG which can be shuttled into chloroplasts by the dicarboxylate translocator [18], as a cosubstrate of aminotransferases;
- 3) 0.1 mM ThPP with coenzyme function in (de)carboxylation reactions;
- 4) 0.1 mM of both NAD and NADH, because variable results were obtained applying pyridine nucleotides of only one species [19].

These substances or biochemically related analogues must be present within the plastids, because IAA is synthesized also when these cofactors were omitted from the medium. Nevertheless in all investigations the addition of cofactors resulted in a

Table III. The effect of cofactors and ABA on the IAA synthesis from [^{14}C]Trp by aqueously isolated chloroplasts and non-aqueously prepared cytoplasm. Incubation time 8 h; pH 7.6; $3.5 \times 10^{-5} \text{ mol} \times \text{l}^{-1}$ [^{14}C]Trp; $0.3 \text{ mg} \times \text{ml}^{-1}$ both Penicillin G and Ampicillin.

	1	2	3	4	5	6	7	8	9	
cofactors: α -KG	—	+	+	+	+	+	+	+	+	concentration
P-5'-P	—	—	+	+	+	+	+	+	+	$5 \text{ mmol} \times \text{l}^{-1}$
ThPP	—	—	—	+	+	+	+	+	+	$0.1 \text{ mmol} \times \text{l}^{-1}$
NAD	—	—	—	—	+	—	+	+	+	$0.1 \text{ mmol} \times \text{l}^{-1}$
NADH	—	—	—	—	—	+	+	+	+	$0.1 \text{ mmol} \times \text{l}^{-1}$
ABA	—	—	—	—	—	—	—	10^{-7}	10^{-6}	$\text{mol} \times \text{l}^{-1}$
preparation: chloroplasts										synthesis
(isolated from young leaves)	10.0	11.3	13.1	13.9	13.8	23.1	20.9	—	11.1	$10^{-11} \text{ mol} \times \text{mg}^{-1} \text{ chl}$
chloroplasts (isolated from old leaves)	2.8	1.6	5.5	5.0	3.0	2.8	3.3	—	1.7	$10^{-11} \text{ mol} \times \text{mg}^{-1} \text{ chl}$
cytoplasmic fraction										
(isolated from young leaves)	1.3	—	8.5	7.7	7.8	4.1	8.9	8.7	3.3	$10^{-9} \text{ mol} \times \text{g}^{-1} \text{ d.w.}$
	14.0	—	92.0	83.4	83.9	44.9	96.8	93.2	54.0	$10^{-11} \text{ mol} \times \text{mg}^{-1} \text{ chl}$

higher IAA formation than without these substances. IAA synthesis was more stimulated by co-factors given to cytoplasmic preparations than to chloroplasts. At best a 7 fold stimulation was observed. This indicated a limiting step at the level of the chloroplast envelope.

The synthesis carried out in the cytoplasm was 3–4 times lower than that of the chloroplast (complete medium, column 7). In experiments using non-aqueously isolated chloroplasts and corresponding cytoplasmic fraction depleted of chloroplasts the contribution of chloroplasts to total IAA synthesis reached a maximum of 57% (not shown). Chloroplasts isolated from young leaves (maximal leaf area 5 cm²) produce more IAA from [¹⁴C]Trp than those isolated from older leaves. This fact seems to be a consequence of an enhanced synthesis rather than of an inhibited degradation, because we could not ascertain a faster metabolism of IAA in the latter systems. This could be one of the reasons that younger leaves contain higher levels of IAA *in vivo* [2, 4, 22].

The phytohormone ABA inhibits auxin production with an optimum at 2×10^{-6} to 5×10^{-6} mol ABA $\times$ l⁻¹. These findings agree with the report of Anker [23] who investigated the auxin production of the physiological tip of *Avena* coleoptiles.

Concluding remarks

The experimental results demonstrate 4 major facts:

- 1) Sterile isolated chloroplasts from *Spinacia oleracea* leaves show an autonomous capability of IAA synthesis from [¹⁴C]Trp, which penetrates the chloroplast envelope.
- 2) IAA production also takes place in the cytoplasm.
- 3) In both compartments application of certain co-factors is not prerequisite of synthesis, but stimulates auxin formation.
- 4) Light and ABA in naturally occurring concentrations [11] inhibit IAA production.

Regarding the former observations [11] that ABA is accumulated in the chloroplasts in the light *in vitro* and *in vivo*, the ABA distribution between the compartments could be a mechanism for the regulation of the *de novo* synthesis of auxin in spinach mesophyll cells. Obviously ABA only decreases the enzyme activity concerned in the biosynthetic pathway but does not accelerate the metabolism of IAA because no increased amounts of degradation products could be detected. The inhibitory effect of ABA on IAA synthesis demonstrate the necessity to always consider possible mutually antagonistic hormonal interactions when studying their biosynthesis, and also during developmental studies on the plant.

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