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EARLY BIOCHEMICAL EVENTS DURING ADVENTITIOUS ROOT INITIATION IN THE HYPOCOTYL OF *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; French bean; auxin; root initiation; protein synthesis: RNA synthesis.

Abstract—Indole butyric acid (IBA) initiates roots in the hypocotyl tissue of *Phaseolus vulgaris* (French bean). The response is dependent on the concentration of IBA and the duration of exposure to the hormone. IBA enhances the rate of total protein synthesis in *ca* 30 min after exposure of the hypocotyl segments to the hormone. There is no detectable change in total or poly(A)-containing RNA synthesis in this period although significant increases are seen 2 hr after hormone pre-treatment. The early IBA-mediated increase in protein synthesis (30 min) is not sensitive to Actinomycin D but the antibiotic blocks the increase manifested 2 hr after hormone pre-treatment. Inhibition of early protein synthesis by cycloheximide depresses and delays root initiation. Cytosol prepared from IBA-treated hypocotyl tissue stimulates protein synthesis *in vitro* to a greater extent than that of the control.

INTRODUCTION

Adventitious root initiation in stems of dicotyledons is a phenomenon controlled and regulated by phyto-hormones, which bring about redifferentiation of pericyclic cells into root primordia. In the scheme for the sequence of events leading to root formation in the pea epicotyl, an increase in RNA synthesis and shift from monosomes to polysomes have been proposed [1]. These changes have been detected *ca* 2–3 hr after auxin treatment [2]. Thus, an early increase in *m*RNA synthesis is indicated, although polysome formation need not strictly depend on *m*RNA availability and could be the result of ribosome activation [3–5].

In view of these results, it was felt necessary to examine the rates of some of the macromolecular events at very early periods after auxin treatment. In the present study the rates of RNA and protein synthesis have been measured within 2 hr after indole butyric acid (IBA) treatment of the French bean hypocotyl tissue.

RESULTS

Some of the basic parameters influencing root forma-

Table 1. Effect of IBA concentration and duration of treatment on root production in the hypocotyl segment of *Phaseolus vulgaris*

IBA concentration (μg/ml)	No. of roots formed/segment (Average of 10 segments)*	Duration of IBA treatment (min)	No. of roots formed/segment (Average of 10 segments)*
0	1.2 ± 0.6	2	19.1 ± 1.0
2	45.2 ± 1.3	4	42.7 ± 2.5
4	58.3 ± 1.8	6	69.7 ± 6.5
6	71.1 ± 1.7	8	76.6 ± 4.0
8	86.9 ± 1.4	10	77.9 ± 1.7
10	82.8 ± 1.4	12	73.8 ± 2.6
12	77.6 ± 1.6	20	76.6 ± 1.5
20	75.8 ± 1.5	30	73.6 ± 3.3
30	77.9 ± 1.3		

Hypocotyl segments (10 segments of 8 cm length each) were placed erect in a flask containing 100 ml of half strength Hoagland's medium (basal medium). IBA was added to the basal medium in different flasks in increasing concentrations and the effect of IBA concentration on the number of roots formed was examined when the tissues were exposed to the hormone for 30 min and then placed in a fresh basal medium. The effect of the duration of the hormone treatment was examined at an IBA concentration of 10 μg/ml. The number of roots formed 72 hr after transfer of the treated hypocotyl to fresh basal medium was counted.
*Mean ± standard error.

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Table 2. Effect of cycloheximide and chloramphenicol treatment on root initiation by IBA in the hypocotyl tissue of *Phaseolus vulgaris*

Pre-treatment	No. of roots formed/segment (Average of 10 segments)*		
	72 hr	96 hr	120 hr
Basal medium	Nil	Nil	Nil
IBA	77.6 ± 1.3	79.8 ± 1.4	80.1 ± 5.3
IBA + cycloheximide	Nil	Nil	21.1 ± 2.4
IBA + chloramphenicol	72.2 ± 2.2	78.6 ± 1.9	74.1 ± 2.6

The hypocotyl segments of 8 cm length were treated with IBA (30 μg/ml). Cycloheximide (10 μg/ml) or chloramphenicol (40 μg/ml) was added to some flasks. The segments were exposed to IBA ± additives for 30 min, washed thoroughly and then transferred to a fresh basal medium. The number of roots formed were counted at different time intervals.
*Mean ± standard error.

tion in the present system were first determined. The results presented in Table 1 indicate that the number of roots produced by the IBA is proportional to the concentration up to 8–10 μg/ml. Higher concentrations, up to 30 μg/ml, though not inhibitory, do not significantly increase root formation. It is possible to induce root formation not only with 2 cm length segments (placed either upright or upside down), but also in segments, which are vertically split into four strips (data not given).

Several cells of the pericycle, situated near and between small phloem patches and lying opposite the split exarch xylem elements, start dividing within 24 hr of auxin treatment. Many of these cells also have large nuclei with conspicuous nucleoli. During the next 24 hr the divided cells of this region appear grouped. At the end of 72 hr after auxin treatment, the cluster of divided cells are distinctly organized as root initials in four rows reflecting the tetrarch nature of the vasculature below. An invariant 4 row pattern of adventitious root production is maintained from the base up to the cotyledonary node.

In order to understand the biochemical processes which are activated on exposure of the hypocotyl tissue to IBA, the effects of cycloheximide and chloramphenicol on root initiation were examined. The results presented in Table 2 indicate that if cycloheximide is present during the first 30 min of IBA treatment, the initiation of root initials is considerably delayed and the number of roots ultimately formed is also decreased. Under similar conditions, chloramphenicol has no effect on root initiation induced by the hormone. The results indicate the possibility that the hormone may have an early effect on cytoplasmic protein synthesis which may determine the subsequent sequence of events.

Thus, it was of interest to examine the effect of IBA on early protein synthesis. It was also pertinent to examine how long the inhibitory effect of cycloheximide on protein synthesis persisted. For this purpose, the hypocotyl tissue was treated with IBA or IBA + cycloheximide for 30 min, washed thoroughly and placed in a fresh basal medium. The rate of protein synthesis was estimated immediately as well as at different time intervals after transfer to the fresh medium using [^{14}C]-chlorella hydrolysate. The results presented in Table 3 indicate that exposure of the hypocotyl tissue to IBA for 30 min results in a significant increase in the rate of total protein synthesis. The inhibitory effect of cycloheximide is significant up to a period of 30 min after transfer to the fresh medium. However, 2 hr after the transfer, there is no significant inhibition of protein synthesis.

The effect of IBA on early RNA synthesis was next examined. For this purpose, the hypocotyl tissue was exposed to IBA for 30 min, thoroughly washed and transferred to the fresh basal medium. RNA synthesis was measured immediately, as well as 2 hr after transfer to the fresh medium using ^{32}P as well as uridine-[^{3}H]. Total RNA as well as poly(A)-containing RNA synthesis were measured in these experiments. IBA-pre-treatment had no effect on total RNA or poly(A)-containing RNA synthesis when measured immediately after transfer to the fresh basal medium. However, after 2 hr there is a striking increase in the rates of total RNA as well as poly(A)-containing RNA synthesis (Tables 4 and 5).

Table 3. Rate of protein synthesis after exposure of the hypocotyl tissue of *Phaseolus vulgaris* to IBA and cycloheximide

Pre-treatment	Immediately after transfer to the fresh basal medium			30 min after transfer to the fresh basal medium			2 hr after transfer to the fresh basal medium		
	cpm/μg amino acid pool (A)	cpm/μg protein (B)	(B/A)	cpm/μg amino acid pool (A)	cpm/μg protein (B)	(B/A)	cpm/μg amino acid pool (A)	cpm/μg protein (B)	(B/A)
Basal medium	26.8	39.38	1.46	24.8	36.95	1.49	28.0	42.84	1.53
Basal medium + IBA	18.0	59.2	3.28	22.6	66.30	2.96	21.6	59.4	2.75
Basal medium + IBA + cycloheximide	20.2	23.1	1.15	23.0	38.3	1.66	17.0	52.0	3.05

Hypocotyl tissue (1 g, 4 cm length) was placed in 5 ml of basal medium with or without IBA (30 μg/ml). To some flasks containing the IBA-medium, cycloheximide (10 μg/ml) was added. The segments were exposed for 30 min washed thoroughly and transferred to a fresh basal medium. Protein synthesis was followed at different time intervals after transfer to the fresh basal medium using [^{14}C]-chlorella hydrolysate (10 μCi/5 ml). The labelling period was 30 min.

Table 4. Effect of IBA on RNA synthesis in the hypocotyl tissue of *Phaseolus vulgaris*

Pre-treatment	Immediately after transfer to fresh basal medium			2 hr after transfer to fresh basal medium		
	Nycleotide pool (cpm/A_{260} Unit)	Total RNA	Poly(A)-RNA (cpm/30 A_{260} units of RNA)	Nucleotide pool (cpm/A_{260} Unit)	Total RNA	Poly(A)-RNA (cpm/30 A_{260} units of RNA)
Basal medium	22500	2420	678	18500	2510	692
Basal medium + IBA	20600	2330	626	19600	3520	1200

The experimental details are as given in Table 3. RNA synthesis was followed at different time intervals after transfer to the fresh basal medium using ^{32}P (200 μCi/10 ml/10 g tissue). The labelling period was 30 min.

In view of these results, the Actinomycin D sensitivity of the IBA-mediated early increase in the rate of protein synthesis was assessed. The results (Table 6) indicate that Actinomycin D treatment of the hypocotyl tissue along with IBA, does not abolish the increased rate of protein synthesis detected immediately after transfer to the fresh basal medium. However, Actinomycin D prevents the increased rate of protein synthesis detected 2 hr after transfer to the fresh basal medium. In all these experiments, striking changes in the sp. radioact. of the free amino acid pool were not detected. In separate experiments, Actinomycin D was found to inhibit RNA synthesis by over 90% at both the time periods examined.

Finally, an attempt was made to determine whether the enhanced rate of protein synthesis detected *in vivo* in hypocotyls treated with IBA for 30 min could also be demonstrated *in vitro*. For this purpose ribosomes and post-ribosomal supernatant (S 150) were prepared from IBA-treated and control hypocotyls. Protein synthesis was measured *in vitro* using the ribosomes and S 150 fraction as described in the experimental section. A linear reaction rate could be demonstrated at least for 30 min. The results (Table 7) indicate that the rate of protein synthesis was maximal with the combination of ribosomes and S 150 fraction prepared from the hypocotyl tissue treated with IBA for 30 min. Addition of the S 150 fraction from IBA-treated hypocotyl to control ribosomes also results in a significant stimulation of protein synthesis *in vitro*.

DISCUSSION

The absolute requirement of apical buds for root initiation has been suggested [6, 7]. In the present study, the stem tissue obtained after excision of cotyledons, leaves, apical and axillary buds followed by thorough washing of the hypocotyls has been found not to initiate any roots in the basal medium. Exposure of the stem tissue to the hormone for a very short period is sufficient to induce maximum root formation. These results suggest that the root induction is primarily due to exogenously applied hormone and that perhaps the hormone commits the cells to undergo redifferentiation into root initials in a very short period of time.

It has also been suggested that the induction of root on stem cuttings by the application of hormone is unusual in showing no apparent optimum or plateau in the dose response curve, with the higher concentration of auxin eliciting a greater number of roots [1]. In the present system employed, the IBA-induced root formation has been found to be dependent on hormone concentration and time of exposure.

One of the early effects of IBA detected in the present study is an increase in the rate of total protein synthesis. An increase in total RNA as well as poly(A)-containing RNA synthesis can be demonstrated only 2 hr after transfer of IBA-pretreated hypocotyls to fresh basal medium, which is in agreement with the data available in other systems [3]. The early increase in protein synthesis is not sensitive to Actinomycin D, whereas the increase detected after 2 hr is sensitive to the antibiotic. The results suggest the possibility that the hormone may primarily act at the level of translation or post-transcriptional steps. The subsequent increase in protein synthesis could depend on the synthesis of fresh messenger RNA. The early increase in protein synthesis is probably vital to root initiation, since its abolition by cycloheximide results in a delay and decrease in root formation. The enhanced rate of early protein synthesis could also be demonstrated *in vitro* using the ribosomes and S 150 fraction prepared from hypocotyls treated with IBA for 30 min. The significant stimulatory activity of the S 150 fraction prepared from IBA-treated hypocotyls on protein synthesis by control

Table 5. Effect of IBA on RNA synthesis in the hypocotyl tissue of *Phaseolus vulgaris*

Pre-treatment	Immediately after transfer to fresh basal medium		2 hr after transfer to fresh basal medium	
	Total RNA (cpm/A_{260} unit)	Poly(A)-RNA (cpm/50 A_{260} units of total RNA)	Total RNA (cpm/A_{260} unit)	Poly(A)-RNA (cpm/50 A_{260} units of total RNA)
Basal medium	143	35	153	42
Basal medium + IBA	159	39	309	96

The experimental details are as given in Table 4 except that RNA synthesis was followed using uridine-[^3H] (500 μCi/5 ml/5 g tissue).

Table 6. Effect of Actinomycin on IBA-mediated increase in the rate of protein synthesis in the hypocotyl tissue of *Phaseolus vulgaris*

Pre-treatment		Immediately after transfer to fresh basal medium			2 hr after transfer to to fresh basal medium		
		Amino acid pool (cpm/μg amino acids) (A)	Protein (cpm/μg protein) (B)	(B/A)	Amino acid pool (cpm/μg amino acids) (A)	Protein (cpm/μg protein) (B)	(B/A)
Basal medium	−Act.D	20.8	21.3	1.02	26.2	26.9	1.03
	+Act.D	22.7	19.7	0.87	23.2	24.5	1.06
Basal medium + IBA	−Act.D	19.8	34.3	1.73	21.4	43.1	2.01
	+Act.D	18.5	36.4	1.97	24.2	21.3	0.88

Hypocotyl tissue 4 cm length, was treated with Actinomycin D (100 μg/10 ml/1 g) for 15 min before the addition of IBA (30 μg/ml). After 30 min of IBA addition, the tissue was washed thoroughly and placed in a fresh basal medium. Protein synthesis was followed at different time intervals after transfer to the fresh basal medium using [^{14}C]-chlorella hydrolysate (10 μCi/5 ml). The labelling period was 30 min.

Table 7. Effect of IBA-pre-treatment of the hypocotyl tissue on protein synthesis *in vitro*

Treatment	Leucine-[^{3}H] incorporated (cpm/A_{260} unit)	
	Expt I	Expt II
Control polysomes + control S 150	1030	1110
IBA-polysomes + IBA-S 150	2950	3630
Control polysomes + IBA-S 150	2172	2380
IBA polysomes + control S 150	1240	1450

The incubation conditions are given in text. The incubation period was 30 min. The results obtained in two independent experiments are given.

ribosomes indicates that some soluble factors induced by—or in combination with—IBA are at least partially responsible for the early increases in protein synthesis.

EXPERIMENTAL

Morphological studies. Phaseolus vulgaris L. var. Burpees stringless seedlings were raised in sand in a dark chamber. When the hypocotyls attained a height of 20–25 cm, they were cut 15 cm below the cotyledons and washed for 30 min in running H_2O. Hypocotyl segments of 4 or 8 cm length were kept in a suitable vol. of half strength Hoagland's nutrient soln [8] with or without IBA. In all the long term expts,a 12 hr day period was maintained . The medium was changed every day. To reduce bacterial contamination, 30 μg/ml of Ampicillin (Hindustan Antibiotic Limited) was used. The number of root initials were counted at different periods of time.

For anatomical studies serial hand sections were made, stained with 2% (W/V) safranine and observations were made under the microscope.

Protein synthesis in vivo. Protein synthesis in the hypocotyl tissue was followed by placing 1 g of suitably treated and washed segment in 5 ml medium containing 10 μCi of [^{14}C]-chlorella hydrolysate. The different treatments given are described in the tables. After 30 min of labelling, the tissues were thoroughly washed to remove adhering radioactivity and a tissue homogenate was prepared by grinding in NaPi buffer (0.1 M pH 7) in the cold. $HClO_4$ (0.3 N in final concn) was added to the homogenate and the ppt. recovered by centrifugation after standing a few hr in the cold. Protein was extracted from the ppt. with N NaOH, the extract neutralized and re-precipitated with TCA (10% w/v ,in final concn). The ppt. was subsequently washed with hot TCA, EtOH–Et_2O (3:1) and finally with Et_2O. The final prepn. was dissolved in HCO_2H and aliquots were counted on Whatman No. 3 filter paper discs. Aliquots were also used after neutralization for the measurement of protein content by the method of ref. [9]. The sp. radioact. of the amino acid pool was estimated in the $HClO_4$ supernatant. The supernatant was neutralized and aliquots were used for radioactivity measurement and total amino acid was estimated using ninhydrin by the procedure of ref. [10].

Protein synthesis in vitro. For this purpose the polysomal fraction was isolated from the hypocotyl tissue. The isolation procedures and *in vitro* amino acid incorporation were similar to those described in ref. [11]. Briefly, the tissue was homogenized in 5 vols. of buffer A containing Tris–HCl, pH 7.5, 50 mM; $MgCl_2$, 5 mM; KCl, 15 mM; DTT, 0.6 mM and sucrose, 300 mM. The homogenate was centrifuged at 20 000 *g* for 15 min and 2 ml of the supernatant were layered over 3 ml of 1.5 M sucrose and centrifuged at 150 000 *g* for 2 hr. The pellet was suspended in buffer A. Another batch of homogenate was centrifuged at 20 000 *g* for 15 min and the supernatant centrifuged at 150 000 *g* for 2 hr. The final supernatant was passed through a G-25 column and was used as the source of soluble enzymes (S 150 fraction). The *in vitro* protein synthesizing incubation mixture contained in 0.1 ml total vol.: HEPES-buffer, pH 7.5, 20 mM; $MgCl_2$, 5 mM; KCl, 50 mM; ATP, 0.4 mM; GTP, 0.12 mM; phosphoenol pyruvate, 5 mM; pyruvate kinase, 2 enzyme units; amino acid mixture without leucine, 25 μM each amino aicd, polyribosome, 100 μg RNA; S 150 fraction, 100 μg protein; 1 μCi of leucine [^{3}H] (7 Ci/nmol). The mixture was incubated at 37° for 30 min. Aliquots (20 μl) were applied to filter paper discs and the discs, after air-drying, were successively washed with hot and cold 5% TCA, EtOH–Et_2O (2:1) and finally Et_2O. The discs were then counted for radioactivity.

RNA synthesis was measured using either carrier-free ^{32}P-orthophosphate (200 μCi/10 ml/10 g tissue) or uridine-[^{3}H] (500 μCi/5 ml/5 g tissue). The labelling period was 30 min. After thorough washing, RNA was isolated from the tissue homogenate using the PhOH–$CHCl_3$ extraction procedure [12]. Poly(A)-containing RNA was isolated from a known amount of total RNA using poly(U)-Sepharose columns [13]. For measurement of nucleotide pool sp. radioact. in expts using ^{32}P, the tissue homogenate was treated with $HClO_4$ (0.3 N final concn). After standing for a few hr in the cold, it was centrifuged and the free nucleotides in the supernatant were adsorbed using charcoal. The charcoal pellet was washed repeatedly with H_2O and finally the nucleotides were eluted using 50% EtOH–0.3 N NH_4OH [14]. Aliquots of the eluate were used for radioactivity and A_{260} measurements. Radioactivity of the filter paper discs was measured in a liquid scintillation counter with 10 ml of toluene containing 0.5% PPO (w/v) with an efficiency of 6% for ^{3}H and 35% for ^{14}C.

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