PHYTOHORMONE INDUCED CHANGES IN THE NUCLEAR RNA POPULATION OF PLANT PROTOPLASTS

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1. Introduction

Although various regulatory effects exerted by phytohormones on RNA metabolism in plants have been demonstrated [1–4] the mechanism of the action of plant growth substances still remains obscure. The possibility that phytohormone may control transcriptional processes, inducing changes in the pattern of RNA synthesis has focused our attention. An isolated plant protoplast system has been chosen for experiment since it offers many advantages over other commonly used plant tissue systems. Application of isolated plant protoplasts allowed us to study phytohormone effect at the cellular level and to demonstrate directly that phytohormone induces significant changes in the population of the newly synthesized nuclear RNA.

2. Materials and methods

Twenty to 50 g batches of 4-day old apical parts of etiolated maize seedlings grown in the absence or presence of 10^{-7} M gibberellic acid (GA_3) were used for protoplast preparation. The modified procedure of enzymatic isolation of plant protoplasts was based on the methods previously described [5,6] and full details of our procedure will be published elsewhere [7]. Isolated protoplasts were suspended in a known volume of 0.3 M sorbitol and aliquots were taken for counting in a haemocytometer. A freshly prepared suspension of protoplasts ($1.6 - 3.5 \times 10^6$ per ml) in 0.3 M sorbitol containing 10 mM Na-phosphate, pH 6.5, and 5 mM $MgCl_2$ was directly incubated for desired periods of time at 30°C in a water bath (with gentle shaking and aeration) with 10 μCi/ml of [5,6–^{3}H]uridine (58 Ci/mmole), [G–^{3}H]adenosine (12.1 Ci/mmole), or [8–^{3}H]ATP (20.3 Ci/mmole) in the presence or absence of phytohormone. Incubation was terminated by addition of cold TCA upto 5% and after 30 min standing on ice the precipitated sample was collected on a glass fibre filter and the radioactivity monitored using a Packard Tricarb liquid scintillation spectrometer. Alternatively, when RNA was to be extracted, the protoplast suspension after incubation was rapidly cooled, and the remaining isotope diluted with 0.3 M sorbitol containing either 1 mM uridine, adenosine or AMP, respectively. Protoplasts were pelleted at 100 *g*, the supernatant syphoned off and discarded and the pellet used for RNA isolation. Two different procedures for RNA extraction were used. (1) Based on the conventional cold phenol method of Kirby [8] supplemented with additional re-extraction of the interphase at 65°C using a 1:1 phenol:chloroform mixture. This procedure was used for total cellular RNA isolation or for extraction of cytoplasmic and total nuclear RNA from previously separated cytoplamic and nuclear subfractions. (2) When total nuclear RNA was to be further fractionated into preribosomal and heterogeneous nuclear RNA (HnRNA) the hot phenol extraction method of Georgiev et al. [9] was used. This allowed one to obtain three major fractions of the total protoplast RNA: a) cytoplasmic RNA extracted at 0–4°C, b) preribosomal RNA released from 'phenol nuclei' at 45°C, and c) HnRNA extracted from RNA–DNA–protein complex of residual nuclei in the presence of 1% SDS at 85°C. All three stages of RNA extraction and its further purification were done in the presence of 0.5% ben-

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tonite. The final RNA preparation was submitted to DNAase and Proteinase K digestion and used either for 5–20% sucrose density gradient analysis or for oligodeoxythymidylate (oligo-dT) cellulose chromatography. Isolation of poly(A)-containing RNA species based on its affinity to oligo-dT-cellulose was done essentially according to Edmonds and Caramela [10]. Minor modifications consisted of the composition of the binding buffer (400 mM NaCl, 1 mM EDTA, 0.1% SDS and 10 mM Tris–HCl, pH 7.6) and in the additional introduction of a washing buffer (containing all other components of the binding buffer except for 100 mM NaCl).

3. Results and discussion

Protoplasts obtained enzymatically from etiolated maize seedlings retain their full morphological integrity and are very active in RNA synthesis showing linear [^{3}H]uridine incorporation over 0–90 min incubation (fig. 1). Photohormone treatment causes significant stimulation of RNA synthesis: about 2-fold when protoplasts were isolated from GA_3-grown plants (fig. 1a) and up to 30–50% stimulation when GA_3 was added to the protoplast suspension just at the beginning of incubation (fig. 1b).

Sucrose density gradient fractionation of the total protoplast RNA preparation showed that GA_3 treatment stimulated principally the synthesis of the heterodisperse RNA fraction sedimenting between 16 S and 4 S and to a lesser extent the synthesis of both ribosomal RNAS (fig. 2). Further separation of the total RNA into cytoplasmic and nuclear subfractions showed the main effect of phytohormone to be on the nuclear RNA (table 1). More than a 2-fold increase of the specific activity of the nuclear RNA caused by GA_3 treatment, accompanied by only a 37% rise in the cytoplasmic RNA compared to the control values, indicated that after a 90 min labelling period most of the RNA synthesized in response to the hormone was still present in the nucleus and only a relatively small portion of it was transported to the cytoplasm.

To obtain more decisive evidence whether or not significant hormonal stimulation of the synthesis of the heterodisperse RNA fraction (see fig. 2) reflected increased transcription of messenger RNA molecules, total protoplast RNA was fractionated by the hot phenol extraction method and the content of poly-(A)-containing molecules was determined in the respective RNA fractions. Data presented in table 2 illustrate the radioactivity distribution among three RNA fractions obtained from a control and a GA_3-treated protoplast sample. Increase of the specific activity of the cytoplasmic fraction by 34% was accompanied by a 54% stimulation of synthesis of pre-

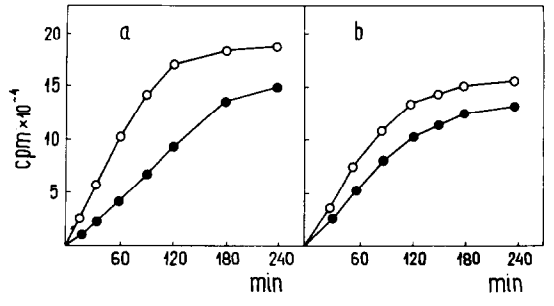

Fig. 1. Time course of [^{3}H]uridine incorporation into RNA of protoplasts: (a) protoplasts were isolated from control (•) or GA_3-treated plants (○); (b) protoplasts isolated from control plants (•) and GA_3 (10^{-7} M) was present in the medium only during the incubation (○). 10 ml of protoplast suspension (1.6 × 10^6/ml) was incubated with 10 μCi/ml of [^{3}H]uridine. At the time indicated 0.3 ml aliquots (in triplicate) were withdrawn and treated with trichloroacetic acid.

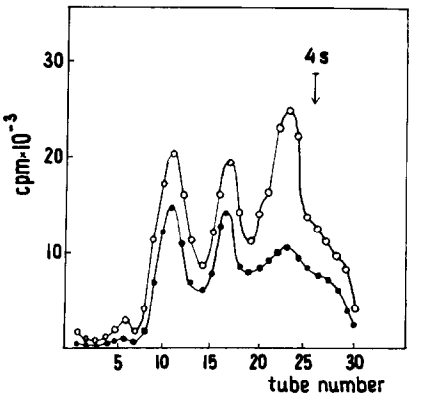

Fig. 2. Radioactivity profiles of the total protoplast RNA after sucrose density gradient centrifugation. Protoplasts were isolated either from control (•) or GA_3-treated plants (○). 5 ml of protoplasts suspension (3.5 × 10^6/ml) was incubated 90 min with 10 μCi of [^{3}H]adenosine.

Table 1
Specific activity of cytoplasmic and nuclear RNA fractions isolated from control and GA_3-treated maize protoplasts

Treatment	Cytoplasmic RNA		Nuclear RNA	
	cpm X 10^{-3}/OD	% of control	cpm X 10^{-3}/OD	% of control
(Control H_2O)	4.9	100	35.3	100
GA_3 (10^{-7} M)	6.6	137	81.7	231

For experimental details see legend under fig. 2.

Table 2
Specific activity of cytoplasmic, preribosomal and heterogeneous nuclear RNA isolated from maize protoplasts, control and treated with GA_3

Treatment	Cytoplasmic RNA (0–4°C)		Preribosomal RNA (45°C)		Heterogeneous nuclear RNA (85°C)	
	cpm X 10^{-3}/OD	% of control	cpm X 10^{-3}/OD	% of control	cpm X 10^{-3}/OD	% of control
Control (H_2O)	6.9	100	38.6	100	118.3	100
GA_3 (10^{-7} M)	9.3	134	59.6	154	284.2	240

Protoplasts isolated from control or GA_3-grown plants were incubated for 120 min in the presence of [^{3}H]ATP (10 μCi/ml). For other experimental details see legend under fig. 2.

Table 3
Effect of GA_3-treatment on the binding of particular RNA fractions to oligo-dT-cellulose

Treatment	Cytoplasmic RNA			Preribosomal RNA			Heterogeneous nuclear RNA		
	cpm X 10^{-3} given	cpm bound	bound fraction % of total	cpm X 10^{-3} given	cpm bound	bound fraction % of total	cpm X 10^{-3} given	cpm bound	bound fraction % of total
Control (H_2O)	50	885	1.77	25	< 50	< 0.2	31	1980	6.39
GA_3 (10^{-7} M)	50	1110	2.22	25	< 50	< 0.2	34	3390	9.97

For details of experimental conditions see legens under table 2 and fig. 3.

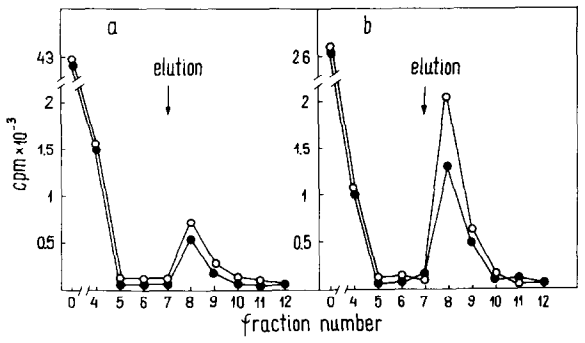

Fig. 3. Fractionation of: (a) cytoplasmic and (b) heterogeneous nuclear RNA by oligo-dT-cellulose chromatography. RNA sample dissolved in 1 ml of the binding buffer (50 000 cpm each, in the case of cytoplasmic control (•) or GA_3-treated sample (○) and 31 000 cpm or 34 000 cpm in the case of HnRNA, control (•) or GA_3-treated sample (○), respectively) was passed three times through the column (75 mg of oligo-dT-cellulose). Unbound RNA was washed out with three 1 ml portions of the binding buffer followed by two portions of the washing buffer. Elution of the bound RNA was achieved with five 1 ml portions of the elution buffer.

ribosomal RNA and by more than a two-fold increment in the HnRNA fraction. This result might indicate that GA_3 caused in the first place an acceleration of the synthesis of messenger type RNA molecules. To check this assumption particular RNA fractions were submitted to oligo-dT-cellulose chromatography in order to evaluate the content of poly-(A)-containing RNA species. The results presented at fig. 3 and table 3 clearly demonstrate that both cytoplasmic RNA and HnRNA fractions derived from GA_3-treated protoplasts showed an increased proportion of poly(A)-containing RNA molecules.

Polyadenylate sequences covalently bound to mammalian messenger RNA have been known for some time [10,11]. More recently an adenylating enzyme has been found in maize [12] and the presence of poly(A) segments in the RNA of cultured rice tissue has been also reported [13]. It has been suggested that addition of the poly(A) segment takes place after completion of RNA transcription and is very important for transfer of mRNA from the nucleus to the cytoplasm [14]. Thus poly(A) sequences may serve as natural chemical markers for examining the precursor–product relationship between heterogeneous nuclear RNA and cytoplasmic messenger. From our studies it can be concluded that poly(A)-containing RNA species synthesized in a 2 hr period of protoplast incubation are largely in the nucleus and a relatively small amount of AMP-rich RNA is recovered from the cytoplasm (table 3). It should be stressed, however, that degradation of RNA during isolation and purification may have decreased our estimates. On the other hand, the higher percentage of poly(A)-containing RNA molecules both in cytoplasmic RNA and in HnRNA of protoplasts trested with GA_3 gives strong support to the proposition that phytohormone induced significant changes in the pattern of transcription and that the increased proportion of messenger RNA species was synthesized in response to GA_3 treatment.

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