Regulation of poly(A) polymerase activity and poly(A)$^+$ RNA levels by auxin in pea epicotyls

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1. INTRODUCTION

The polyadenylation of hnRNA and mRNA in eukaryotes is catalysed by poly(A) polymerase [1]. The regulation of this enzyme is therefore likely to control the expression of various enzymes at the post-transcriptional level. We reported hormonal regulation of poly(A) polymerase activity by gibberellic acid in embryo-less half-seeds of wheat and wheat aleurone layers [2, 3]. The GA_3-induced poly(A) polymerase activity in wheat aleurone layers was dependent on de novo protein synthesis. However, fresh transcription was not necessary for the hormone-triggered enzyme activity. This indicated that GA_3 regulated the expression of conserved mRNA for poly(A) polymerase in wheat aleurone layers [3]. Gibberrellic acid is also known to increase the levels of total poly(A)$^+$RNA in barley aleurone layers [4]. Thus it was considered that the hormonal control of poly(A) polymerase could be responsible for a rise in total poly(A)$^+$ RNA in cereals [2].

A 10-fold enhancement of translatable cellulase mRNA was reported in auxin-treated pea epicotyls [5]. Since cellulase mRNA possessed poly(A) tail at the 3′-end, it was relevant to see whether the increase in specific poly(A)$^+$ RNA for cellulase was associated with the concomitant rise in poly(A) polymerase activity. We now report repression of poly(A) polymerase activity and also a decrease in total poly(A)$^+$ RNA levels in auxin-treated pea epicotyl apices. However, gibberellic acid exerted a positive control, since a 2-fold stimulation of poly(A) polymerase activity was witnessed in pea epicotyl internodes of seedlings grown in red light. Thus, it is inferred that auxin and GA_3 play a pivotal role in the regulation of poly(A) polymerase in pea epicotyls.

Abbreviations: ATP, adenosine triphosphate; DTT, dithiothreitol; 2,4-D,(2,4-Dichlorophenoxy) acetic acid (an analogue of the auxin type of plant hormone); GA_3, gibberellic acid; hnRNA, heterogeneous nuclear RNA; mRNA, messenger RNA; poly(A)$^-$ RNA, RNA without polyadenylated tail; poly(A)$^+$ RNA, RNA with polyadenylated tail

2. MATERIAL AND METHODS

2.1 *Source of enzymes*

Pea seeds (*Pisum sativum*, dwarf var. *HFP-4*) were soaked for 10 min in 0.02% $HgCl_2$ soln. and then washed thoroughly with sterile distilled water. The surface-sterilized seeds were imbibed in sterile water for 6 h at 25°C. The seeds were kept for germination on sterilized absorbant cotton pads in the dark at 25°C. The 6-day-old seedlings were sprayed with the following hormones: 2,4-D (0.1%), ethrel (2×10^{-3} M) and GA_3 (10^{-4} M) containing Tween 20 (0.1%) and NaCl (0.1 M). The pH of the hormone solutions was adjusted to 7.0. After spray application, the seedlings were grown in the dark for another 5 days. The epicotyl apices (1 cm long) of dark-grown seedlings were decapitated and used as a source of different enzymes. The method of spray application and the concentration of auxin (2,4-D, 0.1%) used in this study was essentially adopted from [5]. Our choice to assay poly(A) polymerase activity in pea epicotyls, after day 5 of hormone treatment, was also based on [5] for comparing our results with

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that of the cellulase–pea system under identical conditions. However, in other experiments, we also tested the effect of auxin on poly(A) polymerase activity at relatively low levels (2,4-D, 10^{-5} M, 10^{-4} M and 10^{-3} M). In addition, the effect of auxin on poly(A) polymerase activity was examined even after a shorter duration of treatment (24 h) instead of the usual 5 days. This approach would decrease the possibility of the secondary effects of auxin on enzyme activity.

The efect of GA_3 was also tested under red light conditions, since there is destruction of endogenous levels of gibberellins in red light [6]. The dark grown seedlings (6-day-old) were sprayed with GA_3 (10^{-4} M). Thereafter, the GA_3-treated and untreated pea seedlings were transferred in a red light chamber at 25°C and allowed to grow for additional 5 days*. The upper epicotyl internodal regions of the control and GA_3-treated seedlings, were excised and processed for enzyme extraction.

2.2. *Enzyme preparation and assay of poly(A) polymerase*

The epicotyl apices and the epicotyl internodal tissue (3 g each) were homogenized in chilled Tris–HCl buffer (15 ml, 50 mM, pH 8.0) containing DTT (1 mM) in presence of polyvinyl polypyrrolidone (4%, w/v). The homogenate was spun at 20000 × *g* for 15 min at 4°C. The supernatant was subjected to ammonium sulphate precipitation (0–50% saturation). This fraction was desalted on a Sephadex G-25 column and will be referred to as 'G-25 fraction'. The procedure in [2,3] was followed for the assay of poly(A) polymerase activity, with the exception that β-mercaptoethanol (2 μmol) was substituted by DTT (2 μmol).

2.3. *Extraction and assay of peroxidase activity in pea epicotyl apices*

Peroxidase activity was assayed in dialysed crude extracts by the procedure in [8].

* In a standard procedure of dwarf pea bioassay for GA_3, the effect of hormone on epicotyl elongation is measured after a minimum duration of 5 days. GA_3-induced pea epicotyl elongation is a specific effect of this hormone, even after long duration of treatment [7]. Therefore, we adopted this procedure for studying the effect of GA_3 on poly(A) polymerase activity in the pea system

2.4. *Extraction and isolation poly(A)$^+$ RNA from pea epicotyls*

The pea epicotyl apices (1 g) were excised from control and 2,4-D-treated seedlings. The epicotyl segments were sliced into 5mm sections and transferred in conical flasks containing [^{14}C]uracil (80 μCi/flask; spec. act. 49.3 mCi/mmol), Tween 20 (0.1%) and chloramphenicol (50 μg/ml) in a 3 ml final vol. The tissue was incubated on an oscillatory shaker for 4 h at 25°C in the dark. Thereafter, the epicotyl apices were processed for the extraction and isolation of ^{14}C-labeled poly (A)$^+$ RNA as in [9]. The ^{14}C-labeled RNA (119 600 dpm) at 1 mg/ml was loaded on oligo(dT)-cellulose column (0.5 g dry wt) for the isolation of poly(A)$^+$ RNA in control and 2,4-D-treated pea epicotyls respectively. The radioactivity was measured in different fractions (0.7 ml each) by plating 0.1 ml sample on Whatman 3MM filter paper discs (24 mm).

2.5. *Isolation of ^{3}H-labeled poly(A) product by affinity chromatography*

The 'G-25 fraction' prepared from control and 2,4-D-treated pea epicotyls was employed for the routine assay of poly(A) polymerase activity. The assay mixture (× 3) was incubated at 35°C for 1 h. The reaction mixture was then diluted with 3 vol. Tris–HCl buffer (10 mM, pH 7.5) containing NaCl to make the final molarity of salt to 0.5 N. The diluted reaction product was heated at 70°C for 2 min, chilled instantly, and then loaded on an oligo(dT)-cellulose column. The ^{3}H-labeled poly(A)$^+$ RNA was eluted according to [9]. All the fractions obtained with elution buffer (Tris–HCl, 10 mM, pH 7.5) were pooled for measuring radioactivity of ^{3}H-labeled poly(A)$^+$ RNA.

2.6. *Protein and RNA estimation*

[Protein] was estimated according to [10] and [RNA] determined as in [11].

3. RESULTS

3.1. *Effect of auxin on poly(A) polymerase and peroxidase activities*

The kinetic study of enzyme activity revealed a significant repression of poly(A) polymerase activity in auxin-treated (2,4-D, 0.1%) pea epicotyl apices over the control (fig. 1). The enzyme repres-

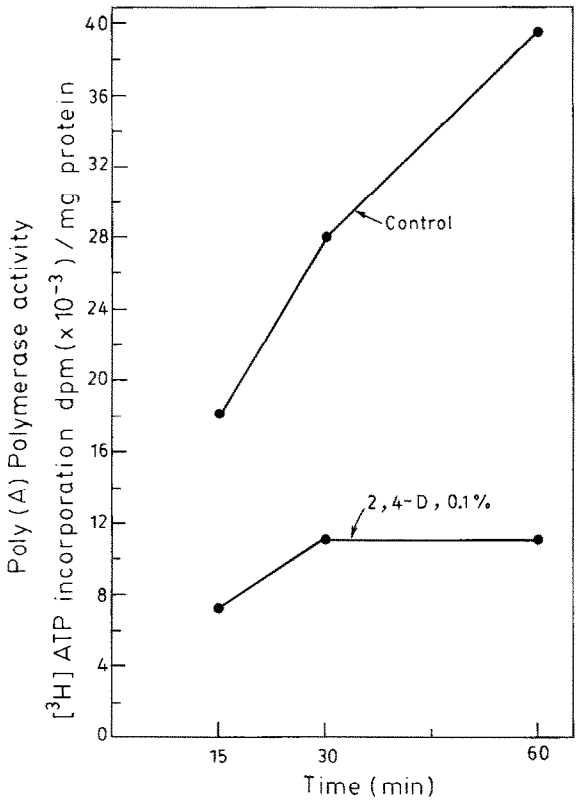

Fig. 1. [^{3}H]ATP incorporation into the poly(A) product as a function of time in control and 2,4-D-treated pea epicotyl apices. Poly(A) polymerase activity was assayed in 'G-25 fraction'.

sion, in response to auxin, was 49–64% (tables 1, 2). Since treatment of plant tissues with supraoptimal level of auxin is known to trigger ethylene production [12], it was possible that the repression of poly(A) polymerase in auxin-treated pea epicotyls was mediated by the endogenous levels of ethylene. This hypothesis was tested by assaying poly(A) polymerase activity in ethrel-treated pea epicotyls. Ethrel (2×10^{-3} M) treatment, failed to repress poly(A) polymerase activity in pea epicotyl apices (table 1). It thus appears that the repression of poly(A) polymerase activity by auxin may not be affected by the endogenous levels of ethylene. Both auxin and ethrel were, however, quite effective in inducing the swelling of the subapical zones of the epicotyl apices and also bringing about stunted growth of the seedlings. The auxin-mediated inhibition of poly(A) polymerase activity could not be ascribed to the general retarded growth of pea seedlings. This view is supported by the fact that auxin (2,4-D, 0.1%) treatment of pea epicotyls brought about a 10-fold stimulation of peroxidase activity over that of the controls. To a lesser extent, ethrel (2×10^{-3} M) mimicked the action of auxin, in that there was a 3-fold increase in peroxidase activity in ethrel-treated pea epicotyl apices (Fig. 2).

In the above experiments, the auxin treatment to the pea epicotyls was of 5-day duration. Thus, it could be argued that the repression of poly(A) polymerase activity by auxin, after a long duration of treatment, may represent some secondary effect. To obviate this objection, poly(A) polymerase activity was assayed after shorter duration of 2,4-D (0.1%) treatment. Pea epicotyls which were treated with 2,4-D (0.1%) for only 24 h showed a typical swelling of the subapical zone.

Table 1

Effect of 2,4-D and ethrel on poly(A) polymerase activity in pea epicotyl apices

Additions	Poly(A) polymerase activity ([^{3}H]ATP incorporation)			
	Expt 1		Expt 2	
	dmp/mg protein	Relative activity	dmp/mg protein	Relative activity
Control	23 152	1.00	22 448	1.00
2,4-D (0.1%)	11 948	0.51	9 648	0.42
Ethrel (2×10^{-3} M)	23 753	1.03	21 663	0.96

Epicotyl apices were excised from control, 2,4-D and ethrel-treated etiolated pea seedlings for the preparation of enzyme fraction. Poly(A) polymerase activity was assayed in 'G-25 fraction' by the incorporation of [^{3}H]ATP into the acid-precipitable polyadenylate product

Table 2

Inhibition of poly(A) polymerase activity in pea epicotyl apices after different durations of auxin treatment

Additions	Poly(A) polymerase activity ([^{3}H]ATP incorporation)	
	dpm/mg protein	Relative activity
1-Day treatment		
Control	41 656	1.00
2,4-D (0.1%)	19 404	0.47
5-Day treatment		
Control	47 816	1.00
2,4-D (0.1%)	17 504	0.36

Epicotyl apices were excised from etiolated pea seedlings which were treated with 2,4-D (0.1%) for a duration of 1 and 5 days. Enzyme fractions, prepared from control and hormone-treated epicotyl apices, were used for the assay of poly(A) polymerase activity

Enzyme fraction, prepared from this auxin-treated tissue, also showed significant repression of poly (A) polymerase activity (table 2). The degree of inhibition of poly(A) polymerase activity, witnessed in this case was nearly identical to that observed after 5 days of auxin treatment (tables 1,2). These results indicated that the repression of poly(A) polymerase activity was not a long-term effect of auxin in pea epicotyls. We also tested the effect of different concns of 2,4-D (10^{-3} M, 10^{-4} M, 10^{-5} M) on poly(A) polymerase activity in pea epicotyls. The data in fig. 3 showed a pronounced repression (~60% inhibition) of poly(A) polymerase activity at all [auxin]. Thus, it was apparent that a negative control of poly(A) polymerase activity can occur at a wide range of [auxin].

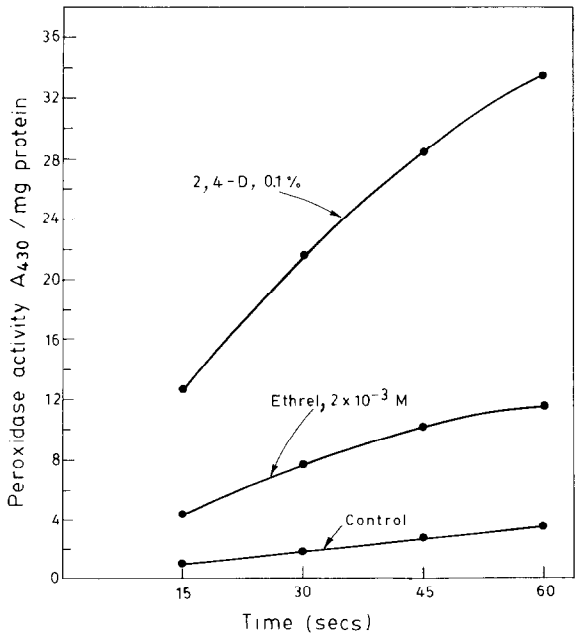

Fig. 2. Peroxidase activity as a function of time in control, 2,4-D-treated and ethrel-treated pea epicotyl apices. The enzyme activity was assayed in dialyzed crude extracts.

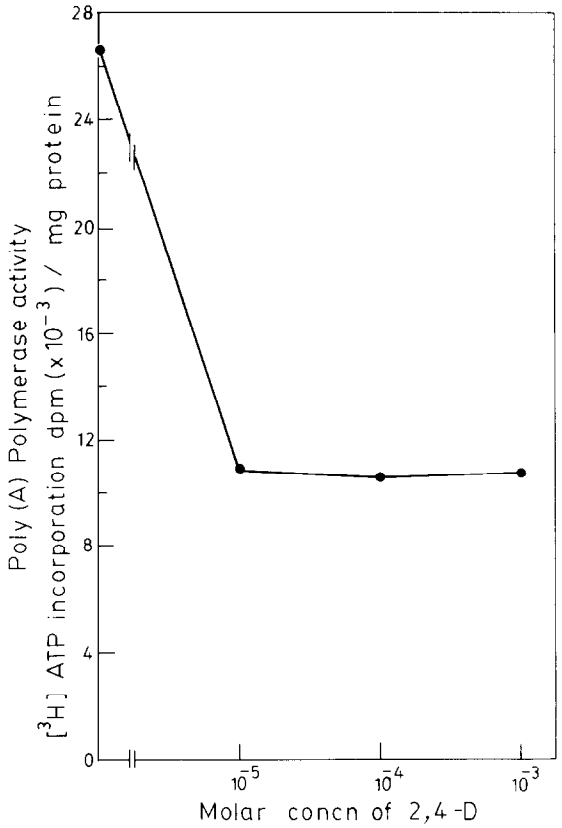

Fig. 3. Inhibition of poly(A) polymerase activity in 2,4-D-treated pea epicotyl apices. Poly(A) polymerase activity was assayed in 'G-25 fractions'. The repression of enzyme activity was identical at different [auxin].

3.2. *Effect of auxin on the levels of poly(A)$^+$ RNA*

Our next attempt was to determine whether the auxin-mediated repression of poly(A) polymerase activity in pea epicotyls also affected the total levels of poly(A)$^+$ RNA. This was examined by labeling the newly synthesized RNA by feeding precursor [^{14}C]uracil (4 h) to pea epicotyls, excised from control and 2,4-D-treated seedlings. The [^{14}C]RNA was extracted and purified from the tissue and was employed for the separation of poly (A)$^+$ RNA by affinity chromatography. Fractionation of labeled RNA on oligo(dT)-cellulose revealed a substantial decrease (70%) in total poly(A)$^+$ RNA in auxin-treated pea epicotyls (fig. 4). Likewise, a substantial decrease (56%) in the poly(A)$^+$ RNA was observed in auxin-treated epicotyl apices when the in vitro synthesized ^{3}H-labeled poly(A) product was fractionated on oligo(dT)-cellulose (table 3). We infer that the repression of

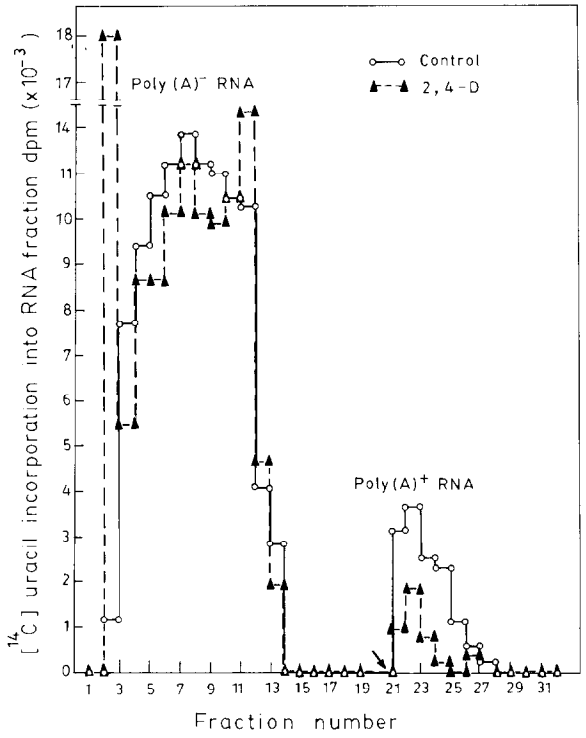

Fig. 4. Inhibitory effect of 2,4-D on the level of total poly(A)$^+$ RNA in pea epicotyl apices. The [^{14}C]RNA, isolated from control and 2,4-D-treated pea epicotyl apices, was fractionated on oligo(dT)-cellulose column for the separation of poly(A)$^-$ RNA and poly(A)$^+$ RNA.

Table 3

Relative binding of in vitro labeled ^{3}H-labeled poly(A) RNA product on oligo(dT)-cellulose column in control and 2,4-D-treated etiolated pea epicotyl apices

Additions	[^{3}H]poly(A)$^+$ RNA bound to oligo-(dT)-cellulose column (dpm)	Relative binding
Control	103 683	1.00
2,4-D (0.1%)	46 103	0.44

The 'G-25 fraction' (3 mg protein), prepared from control and 2,4-D-treated pea epicotyl apices, was employed for the assay of incorporation of [^{3}H]ATP (1 h incubation) into the ^{3}H-labeled poly(A) RNA product. The ^{3}H-labeled reaction product was diluted with binding buffer (Tris–HCl, 10 mM, pH 7.5 + 0.5 N NaCl) and loaded on the oligo(dT)-cellulose column. After washing the column with binding buffer (20 ml), the bound poly(A)$^+$ RNA was eluted with 8 ml of a low ionic strength buffer (Tris–HCl, 10 mM, pH 7.5)

Table 4

Stimulation of poly(A) polymerase activity by gibberellic acid in pea epicotyls

Additions	Poly(A) polymerase activity ([^{3}H]ATP incorporation)	
	dpm/mg protein	Relative activity
Control	5 620	1.00
GA_3 (10^{-4} M)	10 932	1.95

The dark-grown pea seedlings (6-day-old) were sprayed with GA_3 (10^{-4} M). After the hormone treatment, the seedlings were maintained for 5 days in continuous red light at 25°C. The untreated seedlings were also placed in red light and served as controls. The 'G-25 fraction' was prepared from the internodal tissue of pea epicotyls and was used for the assay of poly(A) polymerase activity

poly(A) polymerase by auxin was responsible for the decreased levels of total poly(A)$^+$ RNA in vivo.

3.3. *Effect of GA_3 on poly(A) polymerase activity*

Spray application of GA_3 (10^{-4} M) to etiolated pea seedlings (6-day-old) failed to alter the poly(A)

polymerase activity in epicotyl apices, when the seedlings were grown in the dark (5 days) subsequent to hormone treatment (not shown). However, if the dark-grown seedlings (6-day-old) were sprayed with GA_3 and then transferred to red light (5 days), there was a dramatic elongation of internodal regions over that of untreated controls. This physiological response by GA_3 was also associated with ~2-fold stimulation of poly(A) polymerase activity in pea epicotyl internodes (table 4).

4. DISCUSSION

This study reveals that auxin repressed the activity of poly(A) polymerase with a concomitant reduction in the level of total poly(A)$^+$ RNA in pea epicotyls. The hormone-elicited decrease in total poly(A)$^+$ RNA was possibly an outcome of the repression of this processing enzyme. Despite the prevalent decreased levels of poly(A) polymerase in auxin-treated pea epicotyls, a substantial increase (10-fold) in the translatable poly(A)$^+$ RNA, specific for cellulase enzyme, is reported in this tissue [5]. This preferential increase in specific poly(A)$^+$ RNA for cellulase enzyme is possibly achieved by the site-specific action of auxin on cellulase gene.

The repression of poly(A) polymerase together with the induction of cellulase and peroxidase in auxin-treated pea epicotyls indicated a pleiotropic effect of the hormone on enzyme regulation. However, there was no appreciable difference in the incorporation of [^{3}H]leucine into the total protein fraction in control and 2,4-D-treated pea epicotyls (unpublished).

In barley aleurone layers, GA_3 is known to increase the total poly(A)$^+$ RNA content [4]. Subsequently, we reported stimulation (2–2.5-fold) of poly(A) polymerase activity by GA_3 in wheat aleurone layers [3]. Thus a positive correlation was envisaged between poly(A) polymerase activity and total poly(A)$^+$ RNA levels in cereals [2]. We now propose that the regulation of poly(A) polymerase activity by GA_3 in wheat aleurone layers and by auxin in pea epicotyls, in turn controls the overall levels of total poly(A)$^+$ RNA.

While auxin significantly repressed poly(A) polymerase activity in pea epicotyls, GA_3 brought about a 2-fold increase in the activity of this processing enzyme. Thus, it was evident that a common hormone (GA_3) controlled the stimulation of poly(A) polymerase activity both in wheat aleurone layers [3] and in pea epicotyls. Briefly then, the levels of poly(A) polymerase, in pea epicotyls, are under a dual control of GA_3 and auxin.

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