EXPRESSION OF CONSERVED MESSAGE OF POLY(A) POLYMERASE THROUGH HORMONAL CONTROL IN WHEAT ALEURONE LAYERS

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1. Introduction

Poly(A) polymerase has been reported in both animal and plant cells [1]. Increased polyadenylation of a messenger fraction has been reported for fertilized sea urchin eggs [2] and GA_3-treated barley aleurone layers [3]. However, the real significance of polyadenylation is somewhat cryptic [4,5]. The hormonal control of poly(A) polymerase was first reported in [6]. Essentially, we observed a 2–3-fold stimulation of poly(A) polymerase activity in GA_3-treated embryoless half seeds of wheat. We found that this increased enzyme activity is not due to any structural modification of a preformed enzyme. Inhibitor studies indicated the necessity of de novo protein synthesis for the GA_3-triggered enzyme activity [6]. However, this approach did not tell us whether the hormone regulates the enzyme activity at the transcriptional, post-transcriptional or translational level. Here, we provide evidence for the translation of a GA_3-stimulated poly(A) polymerase from conserved message in wheat aleurone layers. The hormone-stimulated enzyme activity was completely inhibited by abscisic acid (ABA). Clearly, the hormones control poly(A) polymerase activity by operating at the post-transcriptional level. We propose that GA_3 activates the conserved message of poly(A) polymerase, while ABA inhibits this process.

Abbreviations: ABA, abscisic acid; Act D, actinomycin D; ATP, adenosine triphosphate; dpm, disintegrations per minute; GA_3, gibberellic acid; mRNA, messenger RNA; poly(A), polyadenylic acid

2. Materials and methods

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

The surface sterilized embryo-less half-seeds of wheat (*Triticum aestivum* var. Sonalika), prewashed for 5 h in distilled water (20°C), were imbibed at 25°C in the dark for 48 h, in the presence or absence of GA_3 (10 μM) under aseptic conditions. Chloramphenicol (50 μg/ml) was routinely added during imbibing wheat half-seeds. The effect of the following chemicals was tested on poly(A) polymerase in GA_3-treated half-seeds of wheat: ABA (100 μM), cycloheximide (20 μg/ml), actinomycin D (Act D; 100 μg/ml), cordycepin (500 μM), 6 amino acid analogues (1 mM each of L-canavanine, D,L-ethionine, D,L-7-azatryptophan, L-thioproline, 3,5-diiodotyrosine, D,L-*o*-fluorophenylalanine) and 6 amino acids (2 mM each of L-arginine, L-methionine, L-tryptophan, L-proline, L-tyrosine, L-phenylalanine). The effect of ABA (100 μM) and cycloheximide (20 μg/ml) was also tested on poly(A) polymerase activity in wheat half-seeds which were not treated with GA_3 (10 μM). After imbibing half-seeds (5 g) for 48 h, the aleurone layers were manually isolated and the starchy endosperm discarded. The excised wheat aleurone layers were immediately frozen in liquid nitrogen and stored overnight at −15°C. The thawed aleurone tissue was homogenized in Tris–HCl buffer (45 ml, 50 mM, pH 8.0) containing β-mercaptoethanol (5 mM), Triton X-100 (0.2%) and polyvinyl polypyrrolidone (2%, w/v). Acid-washed sand was used to grind the aleurone tissue. The homogenate was centrifuged at 20 000 × *g* for 15 min at 4°C. The supernatant was collected and subjected to ammonium sulphate fractionation (30–50% saturation). The partially purified

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fraction was desalted on a Sephadex G-25 column (1.8 cm × 9.0 cm) (G-25 fraction). The procedure in [6] was followed for the assay of poly(A) polymerase activity. The incorporation of [^{3}H]ATP (4 μCi/incubation mixture, spec. act. 1500 mCi/mmol) into the acid-precipitable polynucleotide product served as a measure of enzyme activity.

2.2. *Enzyme extraction and the assay of α-amylase activity*

The prewashed (5 h) half-seeds of wheat were imbibed for 48 h in the dark. The effect of Act D (100 μg/ml) and cordycepin (500 μM) was tested on the α-amylase activity in GA_3-treated wheat half-seeds and in isolated aleurone layers. The effect of these drugs was also examined on α-amylase activity secreted into the external medium during imbibing GA_3-treated half-seeds. The half-seeds (1.0 g) and excised aleurone layers (0.8 g) were homogenized in acetate buffer (5 ml, 1 mM, pH 5.0) containing $CaCl_2$ (0.2 mM) and spun at 20 000 × *g* for 15 min at 4°C. The crude extracts were exhaustively dialyzed against the homogenizing buffer. The imbibing medium, containing secreted α-amylase, was diluted with equal volume of homogenizing buffer and dialyzed. The method in [7] was adopted for the assay of α-amylase activity in the dialyzed crude extracts. This method was slightly modified as the pH of the incubation medium was adjusted to pH 5.0 instead of pH 4.8 and the assay was performed at 30°C in contrast to 25°C. One unit of α-amylase is defined as that amount of enzyme which hydrolyzed 10 μg starch in 3 min at 30°C in an incubation mixture of 2 ml.

2.3. *[^{14}C]Leucine incorporation into protein fraction*

The incorporation of [^{14}C]leucine (2 μCi/ml, spec. act. 203 mCi/mmol) into the acid-precipitable protein fraction was measured in wheat half-seeds according to [8]. The prewashed half-seeds were imbibed for 48 h in the dark in solutions of GA_3 (10 μM); GA_3 (10 μM) + cycloheximide (20 μg/ml); GA_3 (10 μM) + ABA (100 μM) and GA_3 (10 μM) + cordycepin (500 μM). The labelled [^{14}C]leucine was added at the beginning of imbibing in all experiments.

2.4. *Protein estimation*

Protein was estimated as in [9].

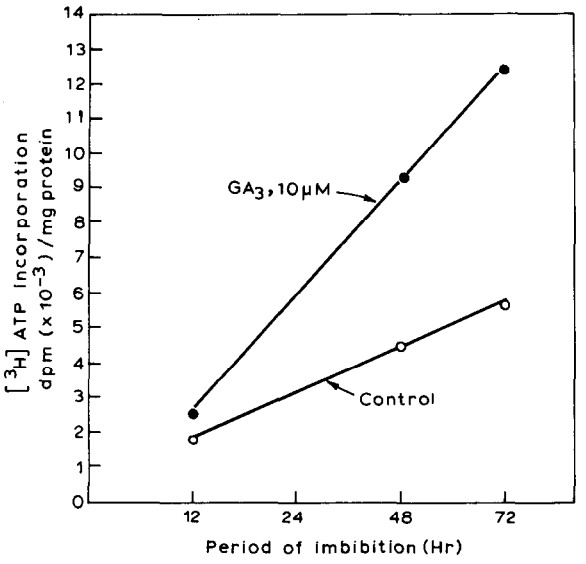

Fig.1. Time course study depicting the stimulation of poly(A) polymerase activity by gibberellic acid in isolated wheat aleurone layers.

3. Results

3.1. *Effect of GA_3 and ABA on poly(A) polymerase activity*

The imbibing of embryo-less wheat half-seeds in a solution of GA_3 (10 μM) resulted in a 2–2.5-fold stimulation of poly(A) polymerase activity in the isolated aleurone layers. Time course studies revealed a distinct increase in poly(A) polymerase activity in GA_3-treated aleurone layers over that of the controls (fig.1). This stimulatory effect of GA_3 on poly(A) polymerase activity was nullified by the simultaneous addition of ABA (100 μM; table 1). The inhibitory

Table 1
Inhibition of GA_3-stimulated poly(A) polymerase activity by abscisic acid and cycloheximide in isolated wheat aleurone layers

Additions	Poly(A) polymerase activity ([^{3}H]ATP incorporation)	
	dpm/mg protein	Relative activity
Control	4042	1.00
GA_3, 10 μM	9406	2.33
+ ABA, 100 μM	4628	1.14
+ cycloheximide, 20 μg/ml	2800	0.69

Table 2
Effect of abscisic acid, cycloheximide and cordycepin on [^{14}C]leucine incorporation into the acid-precipitable protein fraction in GA_3-treated wheat half-seeds

Additions	[^{14}C]Leucine incorporation	
	dpm/mg protein	Percent inhibition
GA_3, 10 μM	3147	–
+ ABA, 100 μM	3029	3.6
+ cycloheximide, 20 μg/ml	397	87.4
+ cordycepin, 500 μM	2698	14.2

effect of ABA seemed highly specific, since it did not appreciably decrease the [^{14}C]leucine incorporation into the acid-precipitable protein fraction (table 2). However, no inhibition of poly(A) polymerase activity was observed in aleurone layers when ABA (100 μM) was added alone to the control wheat half-seeds (table 3).

3.2. *Effect of cycloheximide and amino acid analogues on poly(A) polymerase activity*

The GA_3-stimulated poly(A) polymerase activity was completely inhibited by treatment with cycloheximide (20 μg/ml) in isolated wheat aleurone tissue. In fact, the level of enzyme activity was below that of the control (table 1). The drug also strongly retarded the [^{14}C]leucine incorporation (87% inhibition) into the acid-precipitable protein fraction (table 2). Inhibition of poly(A) polymerase activity was also observed in aleurone tissue when cycloheximide (20 μg/ml) was added alone to the control wheat half-seeds (table 3). The addition of 6 amino acid analogues (1 mM each) also curtailed the entire

Table 3
Effect of abscisic acid and cycloheximide on poly(A) polymerase activity in isolated wheat aleurone layers

Additions	Poly(A) polymerase activity ([^{3}H]ATP incorporation)	
	dpm/mg protein	Relative activity
Control	5954	1.00
ABA, 100 μM	6458	1.08
Cycloheximide, 20 μg/ml	2855	0.48

Table 4
Inhibition of GA_3-stimulated poly(A) polymerase activity by amino acid analogues in isolated wheat aleurone layers

Additions	Poly(A) polymerase activity ([^{3}H]ATP incorporation)	
	dpm/mg protein	Relative activity
Control	3084	1.00
GA_3, 10 μM	7544	2.45
+ six amino acid analogues, 1 mM each	1764	0.57
+ six amino acid analogues, 1 mM each + six amino acids, 2 mM each	5192	1.68

GA_3-promoted poly(A) polymerase activity in isolated aleurone layers. This inhibitory response was substantially relieved by the simultaneous addition of the 6 corresponding amino acids (2 mM each; table 4). These experiments clearly indicated the absolute requirement of de novo protein synthesis for the GA_3-induced poly(A) polymerase activity in isolated wheat aleurone layers.

3.3. *Effect of Act D and cordycepin on poly(A) polymerase and α-amylase activities*

Although translational activity was mandatory for the GA_3-mediated poly(A) polymerase activity, it still remained to be seen whether the hormonal control was dependent on new transcriptional activity. This was examined by testing the effect of Act D (100 μg/ml) and cordycepin (500 μM) in GA_3-treated wheat half-seeds. The hormone-stimulated poly(A) polymerase activity was completely insensitive to the inhibitory action of Act D and cordycepin[+] in isolated wheat aleurone layers (table 5). The lack of inhibitory response of transcriptional inhibitors towards poly(A) polymerase could be ascribed to their inef-

[+] In other experiments, we observed that the addition of cordycepin (500 μM) to the GA_3-treated wheat half-seeds brought about a stimulation (20–40%) of poly(A) polymerase activity in isolated aleurone layers over that witnessed with GA_3 alone. We suggest that the drug possibly blocked the transcription of some inhibitory factor of poly(A) polymerase and this could account for the additional increase in enzyme activity

Table 5
Effect of actinomycin D and cordycepin on GA_3-stimulated poly(A) polymerase and α-amylase activities in isolated wheat aleurone layers

Additions	Poly(A) polymerase act. ([^{3}H]ATP incorporation)		α-amylase activity	
	dpm/mg protein	Relative activity	Enzyme (units/mg protein)	Relative activity
Control	5000	1.00	69.9	1.00
GA_3, 10 μM	10 448	2.09	571.6	8.18
+ Act D, 100 μg/ml	9816	1.96	536.3	7.67
+ cordycepin, 500 μM	10 072	2.01	70.4	1.01

fective penetration into the aleurone layers. This objection could be overruled, provided one could show the strong inhibition of some other GA_3-induced enzyme by Act D or cordycepin in wheat aleurone layers. This possibility was tested by studying the effect of the two drugs on GA_3-induced α-amylase activity. Whereas Act D* (100 μg/ml) was ineffective in blocking the GA_3-stimulated α-amylase, cordycepin (500 μM) completely inhibited the hormone-induced enzyme activity in isolated wheat aleurone layers (table 5) and also in wheat half-seeds. In addition, cordycepin strongly blocked (88% inhibition) the GA_3-triggered α-amylase secretion into the incubation medium (not shown). The drug did not seem to significantly interfere with protein synthesis as there was a minor decrease (14%) in [^{14}C]leucine incorporation into the acid-precipitable protein fraction (table 2). Thus, our data obtained with cordycepin clearly indicated that fresh transcriptional activity, though necessary for the formation of the GA_3-induced α-amylase, could be completely dispensed with for the GA_3-promoted poly(A) polymerase activity. We consider that the hormone-directed synthesis of poly(A) polymerase is translated from its conserved message.

* We observed a meagre inhibition of 10–30% of [^{14}C]uracil incorporation into the RNA fraction when Act (100 μg/ml) was added to GA_3-treated wheat half-seeds. This indicated inadequate penetration of the drug in the aleurone tissue and could explain the lack of inhibition of GA_3-induced α-amylase activity. Other workers have also reported poor inhibition of RNA synthesis by Act D in plant tissues due to slow penetration of the drug (see review [15]). However, Act D (100 μg/ml) penetrated excised germinating wheat embryos in effective concentrations and brought about severe inhibition (70%) of [^{14}C]uracil incorporation into the RNA fraction [8]

4. Discussion

This investigation has revealed that poly(A) polymerase activity in wheat aleurone layers is modulated by GA_3 and ABA. The total inhibition of GA_3-stimulated poly(A) polymerase activity by cycloheximide and 6 amino acid analogues clearly pointed out the necessity of de novo protein synthesis. However, the hormone-triggered poly(A) polymerase activity was not at all inhibited by cordycepin, a potent inhibitor of transcription. The effectiveness of cordycepin in wheat germ aleurone layers was demonstrated by showing the total inhibition of GA_3-induced development of α-amylase activity by cordycepin. Strong inhibition of GA_3-induced formation of α-amylase by cordycepin in barley aleurone layers was also reported in [10,11]. Cordycepin also strongly inhibits RNA synthesis in different plant tissues [12,13] including wheat half-seeds [14]. Thus, our cordycepin experiments favour the view that wheat aleurone layers contain a conserved message for poly(A) polymerase which is capable of supporting enzyme protein synthesis. Although conserved messengers for general protein synthesis have been reported in mature seeds of many plants [15–17], there are very few reports on conserved messages for individual enzyme proteins [8,14,18]. Among these, our knowledge is rather meagre about the control mechanisms which operate during the active translation of long-lived messengers in germinating embryos. In sea urchin eggs, the mechanism responsible for an increased rate of protein synthesis from the conserved messengers at fertilization is still obscure [19]. Our study of poly(A) polymerase activity in wheat aleurone layers revealed that the expression of conserved message of this enzyme is regulated by the phytohormones. We propose that GA_3 controls the activation of dormant, conserved message of poly(A) polymerase which then supports increased enzyme synthesis. Abscisic acid inhibits the expression of poly(A) polymerase, possibly by preventing the GA_3-elicited activation of its preformed messenger. Thus, the regulation of poly(A) polymer-

ase by the 2 phytohormones operates at the post-transcriptional level.

Curiously enough, GA_3 seems to regulate poly(A) polymerase and α-amylase activities in wheat aleurone layers at different control points: the induction of α-amylase requires both translational [20] and transcriptional activities, while the stimulation of poly(A) polymerase depends exclusively on de novo protein synthesis.

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