GIBBERELLIC ACID MEDIATED POST-TRANSLATIONAL MODIFICATION OF RNA POLYMERASE II IN EPICOTYLS OF DWARF PEA (PISUM SATIVUM L.)

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ABSTRACT

Gibberellic acid (GA_3) has been shown to play a significant function in influencing gene expression in plant cells. In many plants (e.g., barley and wheat), application of GA_3 resulted with an upsurge in the proportional amounts of poly $(A)^+$ RNA. It has been shown in wheat aleurones and embryos that GA_3 influences activity of poly (A) polymerase, the enzyme responsible for poly- adenylation of mRNA. Thus, it appears that the regulation of this enzyme could be crucial for regulating the relative amounts of poly $(A)^+$ RNA in plant cells. Since application of GA_3 also promotes the synthesis of RNA, it was considered worthwhile to see whether GA_3 regulates the activity of RNA polymerase II in dwarf pea epicotyls. Prior to this study regulation of this enzyme by GA_3 has been reported in dwarf pea epicotyls. However, the effect of this phytohormone was perceived after 48hr of treatment. Therefore, it was difficult to precisely state whether it is a primary or secondary effect of the phytohormone. Also, the precise extent of impact of RNA polymerase by GA_3 was not known. With a view to elucidate the mechanism by which GA_3 regulates the activity of transcriptional enzymes, this problem was taken up in the dwarf pea epicotyl RNA polymerase II system. The research findings clearly show that the regulation of RNA polymerase II can be achieved within a short duration of time (2hr). A more systematic study has revealed that the phytohormone regulates RNA polymerase II activity at the post-translational level.

Interestingly, experiments with cell-free extracts of dwarf pea epicotyls have shown that the in vivo impact of GA3 may be reproduced in vitro by adding phytohormone. However, the GA3-mediated modulation of RNA polymerase II activity in vitro required preincubation of the enzyme fraction for at least 30 minutes. The *in vitro* approach further revealed that this response of GA_3 is highly specific as analogue of GA_3 namely GA_8, failed to stimulate RNA polymerase II activity. It has also been observed that ABA nullified the GA_3 response both *in vivo* and *in vitro.* Since the phytohormonal regulation of RNA polymerase II *in vitro* could be achieved within 30 min of hormone treatment, it is considered to be a primary response of the phytohormone in dwarf pea. There is evidence to show that GA_3 probably interacts with some regulatory protein factor which in turn activates RNA polymerase II. The fact that GA_3 failed to activate RNA polymerase II in partially purified preparation (DEAE-Sephadex A-25 fraction) of dwarf pea epicotyls, strongly suggests that the regulatory factor is probably dissociated from RNA polymerase II following fractionation on DEAE-Sephadex A-25. Thus, it is considered that some regulatory protein factor is necessary for the GA_3- mediated modulation of RNA polymerase II in dwarf-pea plant epicotyls. This investigation has opened a new avenue for understanding the role of GA3 in the post-translational regulation of RNA polymerase II in dwarf pea epicotyls. Further studies would be necessary to isolate and characterize this

regulatory protein factor for understanding the exact role of GA_3 in the regulation of RNA polymerase II at the molecular level.

INTRODUCTION

Transcription of nuclear genes in eukaryotic organisms is catalysed by nuclear Deoxyribonucleic acid-dependent Ribonucleic acid polymerases (ribonucleoside triphosphates: nucleotidyl transferase, a DNA dependent enzyme (E.C.2.7.7.6). The transcription of rRNA, mRNA, and tRNA is carried out by a single kind of DNA dependent RNA polymerase found in prokaryotes. In contrast, eukaryotes have unique mitochondrial and chloroplast RNA polymerases in addition to the DNA-dependent RNA polymerases found in their nuclei.

RNA polymerase II catalyses the generation of mRNA precursors. The enzyme catalysed reaction requires four nucleotide triphosphates (ATP, CTP, GTP, and UTP), DNA template, $Mn^{2+,}$ and $Mg^{2+,}$ ions.

Purification and subunit structure

While RNA polymerases have been identified from over 70 different eukaryotic species, much of the attention and research has been focused on yeast (Valenzuela *et al*., 1976), maize (Mullinix *et al*, 1973), Drosophila (Johnson *et al*, 1977), rooster (Wallin and Mäenpää, 1979), calf thymus (Schwartz *et al.*, 1974), rat liver (Yu, 1975), human Hela cells (Hossenloop and associates, 1975) and mouse myeloma tumours (Blair, 1981).

Since plant tissues contain significant levels of RNA polymerase II, this enzyme has been purified and characterized for its subunit structure from rye embryos as reported by Fabisz-Kijowska and associates, (1975), from wheat germ by Jendrisak and Burgess, (1975), from soybean embryonic axis by Guilfoyle and associates, (1980), from cauliflower inflorescence by Goto and associates, (1978) and from pea apices by Sasaki and associates, (1979).

It appears that RNA polymerase II has a very consistent and conserved component structure, that is about 220,000 and 140,000 mw (molecular weight) subunits, a singlet or doublet of about 40,000 mw, a singlet or doublet of about 27,000 mw, 2 subunits of about 20,000 mw, 2 subunits of about 17,000 mw, 2 subunits of about 16,000 mw, and a single polypeptide of about 14,000 mw make up the enzyme. This suggests that there are 11-14 subunits in each RNA polymerase II enzyme. Experiments on RNA polymerase II's ability to attach to template DNA have shown that both its big (mw 220,000) and tiny (mw 23,000) subunits are involved (Buhler et al., 1980). There are two separate functional forms of RNA polymerase II, one that is "free" and not connected with chromatin and another that is "engaged" and strongly attached to chromatin (Yu, 1975).

The classification of RNA polymerases is based on their chronological sequence as they elute out from DEAE-Sephadex A-25 according to Roeder and Rutter, (1969) or it is based on their sensitivity to α-amanitin as reported by Kedinger and colleagues, 1971; Chambon, (1975). Since RNA polymerases I, II, and III comprise a group of relatively big proteins with an overall acidic charge, they are able to form bonds with resin like DEAE-Sephadex A-25 which is anionic in nature. Subsequently, from this column, forms I, II, and III elute with ammonium sulphate in

concentrations ranging from 0.05–0.15M, 0.15-0.25M, and 0.25-0.35M, correspondingly (Roeder, 1976). Presumably due to a shared affinity for nucleic acids, RNA polymerases are similarly preserved on cationic exchange resins like phosphocellulose.

Separation of the three forms is also carried out on DEAE-Cellulose and CM-Sephadex (Roeder, 1976). Cochet-Meilhac and Chambon (1974) found that α-amanitin, which is a bicyclic octapeptide, interrupts the formation of RNA by interacting directly with the enzyme RNA polymerase instead of interacting with the template-DNA. The higher plant and animal type I RNA polymerase is susceptible to α-amanitin. However type II enzymes of higher eukaryotes are usually extremely susceptible (50% inhibition occurs at about 0.01-0.05 µg/ml) and type III enzymes become ineffective at extremely high levels of the toxic substance (50% inhibition occurs at approximately 10-20 µg/ml) (Roeder, 1976). However, there are some exceptions, for example yeast RNA polymerase I is sensitive to α-amanitin (Sentenac and Hall, 1982) whereas type III enzyme from cauliflower (Guilfoyle, 1976) and insects is insensitive (Sklar *et al.*, 1976).

Nucleases and proteases probably are component parts of RNA polymerase or they could be tenaciously associated with the functional subunits of the enzyme. According to Dahmus, in calf thymus (1981a) and according to Steler and Rose, (1983) in Morris hepatoma (3924A), a protein kinase activity copurifies with RNA polymerase II. Likewise, RNA polymerase II purified from seedlings of *Cucurbita pepo* showed close association of DNA endonuclease activity (Szopa *et al.,* 1985).

Phosphorylation of RNA-polymerases

Post-translational regulation of the enzyme RNA-polymerase II has been shown through phosphorylation both under *in vivo* conditions and also under *in vitro* conditions. *In vivo* phosphorylation of RNA-polymerase II has been reported in yeast for all the three enzymes by Breant and associates, (1983), for enzyme Il from wheat embryos by Mazus and coworkers, (1980), Hela cells by Dahmus, (1981b) and in rat C6 glioma cells by Lee and Jungmann, (1981). In wheat germ the largest subunit was phosphorylated (Mazus et al., 1980). This is in contrast to HeLa cells (Dahmus, 1981b), in which 3 smaller subunits as well as the largest subunit were phosphorylated.

In vitro post-translational phosphorylation by an endogenous protein kinase has been reported in yeast for all the 3 enzyme forms, by Breant and associates, (1983), in the calf thymus form I and form II by Dahmus, (1981), in calf ovary by Jungmann and colleagues, (1974), in Morris rat hepatoma cells 3924A by Jacob and Rose, (1980), in Novikoff rat hepatoma cell form II by Dahmus, (1976) and in rat liver form I enzyme, by Hirsch and Martelo, (1978).

Since *in vitro* and *in vivo* phosphorylation of the yeast enzymes results in the phosphorylation of the same subunit, phosphorylation must have a physiological role. It has been demonstrated that phosphorylation results in increase in the activity of RNA-polymerase II, isolated from wheat embryo, Novikoff hepatoma, calf thymus, calf ovary, and Morris hepatoma 3924A but it is not known whether or not this activation can be shown to occur *in vivo*.

Guilfoyle (1989) has isolated a protein kinase (200,000 mw) from wheat germ that is capable of phosphorylating 220,000 mw subunit of wheat, soybean, pea and human RNA polymerase II and this phosphorylation results in a mobility shift of protein band from 220,000 mw to 240,000 mw.

Effect of carcinogens

Six mouse ascites tumours and a solid tumour showed significantly high activity of RNA polymerases in comparison to the enzyme present in five mouse tissues (Blair, 1981) Chemical carcinogens that have been shown to increase activity of RNA polymerase II are aminoazo dyes, e.g., 4-dimethylaminoazobenzene(DAB) (Wu and Smuckler, 1971) and 3'-methyl-4-dimethylaminoazobenzene (3'-MEDAB) (Philips and Blunck, 1977) and polycyclic aromatic hydrocarbons, e.g., 3-methylcholanthrene (3-MC) (Liberator and Brescnick, 1981; Kleeberg et al., 1982) and TCDD or 2,3,7,8-tetrachlorodibenzo-p-dioxin (Kurl et al., 1982). Decrease in activity of RNA polymerase II has been shown by amnioazo dyes, e.g., 3'-MEDAB (Akao et al., 1974), aminofluorenes, e.g., N-hydroxy-AAF or N hydroxy-2-acetylaminofluorene (Zieve, 1972; Herzog and associates, 1975; Austin et al., 1980), actinomycin D (Yu, 1980), antibiotic fungal toxins, e.g., aflatoxin B1 (Saunders and coworkers, 1972; Neal, 1973; Yu, 1981), aflatoxin B1, B2, G1, G2 (Yu et al., 1982). Nickle compound nitrosamines and dimethyl nitrosamine (Herzog and Faber, 1976).

Hormonal regulation of RNA polymerases

RNA polymerase activities may be regulated in different ways, such as by alteration in the levels of enzymatic protein molecules, or by stimulation of preformed enzymatic protein molecules. Increased availability or modulation of template DNA could also modulate RNA polymerase activity. There is evidence to show that more than one mode of control could be operational for the regulation of activity of the enzyme RNA polymerase-Il. Varying degrees of control are however difficult to distinguish (Guilfoyle, 1980).

In animal systems, preferential RNA-polymerase II stimulation has been reported by treatment with hormone ACTH (Furhman and Gill, 1976). Increase has been demonstrated in the proportion of molecules of RNA polymerase II per DNA by administration of oestradiol to immature rats (Courvalin and associates, 1976). Oestrogen also modulates bird liver of RNA polymerase II (Kastern and associates, 1981).

In plant systems, auxin (a phytohormone) has been observed to boost total RNA transcription in many plant tissues (Key, 1969). Additionally an enhancement in the abundance of mRNAs containing poly (A) sequences has also been reported after an in vivo auxin treatment (Teissere et al., 1975). In vitro enhancement of RNA levels in response to auxins has been proven by isolated nuclei, chromatin-fibers and also by highly pure RNA polymerase enzyme preparations. The abnormal proliferation and buildup of rRNA in developed tissues of soybean hypocotyl are linked to an increased expression of chromatin-bound RNA polymerase enzyme according to O'Brien and colleagues, (1968). An upsurge in the amounts of RNA polymerase I and RNA polymerase II upon auxin application occurs in conjunction with de novo enzyme synthesis (Guilfoyle, 1980). However, no discernible change in subunit structure or pattern of insertion of 35S-methionine into RNA polymerase subunits was observed by the treatment. Thus, during

physiological or developmental transitions, increase in levels (and not alteration in the subunit structure) of RNA polymerase I and RNA polymerase II may influence, in part, the transcription rates (Guilfoyle, 1980).

Increased activity of RNA polymerase that is bound to chromatin has been reported in GA_3-treated dwarf pea epicotyls (McComb et al. 1970) and soybean hypocotyls according to Hou and Pillay, (1975). However, the activity of RNA polymerase bound to chromatin was reported to be neither enhanced nor inhibited by *in vitro* addition of GA_3 (McComb et al., 1970; Hou and Pillay, 1975)

The GA_3-dependent activation of RNA synthesis has been shown to be brought about by an increase in RNA polymerase activity (Tomi et al. 1983a) together with an enhancement of template activity of chromatin (Tomi et al., 1983b). Enhancement of RNA polymerase and II activities was observed in GA_3-treated pea epicotyls, however there was no detectable change in properties of RNA polymerase II in control and GA_3-treated pea epicotyls. *In vitro* addition of purified and partially purified enzyme from GA_3-treated tissue to purified and partially purified enzyme respectively from control tissue had no inhibitory or stimulatory effect. Enhancement in activity of the enzyme was therefore not due to the a factor being present (Tomi et al., 1983a).

Phytohormonal regulation of polyadenylated RNA levels

In pea, GA_3 increases the levels of total RNA (Broughton, 1968). Quantitative and qualitative alteration in the amounts of RNA have been reported within nuclei from GA_3-treated light grown dwarf plants of pea (Johri and Varner, 1967). However, treatment of purified nuclei with GA_3 showed no increase in RNA synthesis. Probably, some factor(s) localized in the cytosol or nucleus is mediating the GA3-elicited ultimate response, which is then measured as enhancement in the synthesis of RNA. The failure of isolated nuclei to elicit the response to GA3 treatment is possibly the result of loss of this component during isolation of nuclei.

GA_3 treatment causes preferential increase in the synthesis of corn mRNA (Wasilewska and Kleczkowski, 1974; Wasilewska and kleczkowski, 1976). In the cell-free wheat germ system, the translational activity per mRNA unit was much greater in the poly (A)+ mRNA population isolated from GA3-treated pea epicotyls than in the control group (Wasilewska et al., 1987). Based on CDNA poly $(A)^+$ MRNA hybridization studies, Dzhokhadze et al., (1989) concluded that GA_3 may activate new types of mRNA and probably increase synthesis of some poly$(A)^+$ mRNA populations. Both increases and decreases in the levels of specific polypeptides have been reported in pea within 30 min of treatment of light grown pea epicotyls with gibberellin (Chory et al., 1987). These changes in the populations of translatable mRNA in the presence and absence of gibberellin were measured by translation in rabbit reticulocyte extract and separated by 2-D gel electrophoresis. These gibberellin induced changes in expression of gene products could be due to changes in rate of transcription, stability of mRNA or increased efficiency of translation of certain mRNAs (Chory et al., 1987). The authors did not mention whether the GA_3-mediated increase in RNA synthesis was due to stimulation of type I, type Il or type III RNA polymerases.

In the present investigation, stimulation of RNA polymerase II activity was achieved in dwarf pea epicotyls within 2-4 hr of treatment with GA_3. Protein synthesis was not necessary for the GA_3-regulated RNA polymerase II activity. Stimulation in the activity of RNA polymerase II was also reported by the addition of GA_3 (1 µM) during extraction of enzyme fraction from dwarf pea epicotyls. Thus, it appears that GA_3 influences the activity of RNA polymerase II at the post-translational level. *In vivo* regulation of the enzyme activities was also mimicked *in vitro* by the inclusion of GA_3 to cell-free extracts of dwarf pea epicotyls. This response was observed within 30min of preincubation of enzyme extracts even with extremely low concentrations of GA_3 (10^{-15} M). The GA_3-mediated activation of RNA polymerase II activity was highly specific, since *in vitro* addition of the biologically inactive analogue of GA_3 (namely GA_8) failed to activate this enzyme. The *in vitro* activation of RNA polymerase II by GA_3 was witnessed both in the ammonium sulphate fractional precipitate ($(NH_4)_2SO_4$, 30-50% saturation) and polymin-P fraction. However, GA_3 failed to activate RNA polymerase II in the DEAE-Sephadex A-25 fraction. This suggested the involvement of some regulatory protein component in the GA_3-mediated activation of RNA polymerase II. Apparently, the factor gets dissociated from RNA polymerase II after DEAE-Sephadex ion-exchange chromatography.

MATERIALS AND METHODS

RESEARCH MATERIAL

The seeds of Pisum sativum L. (pea) both dwarf (var Arkel) and tall (var. B.V.) were sourced from Indian Agricultural Research Institute, Experimental Station, Karnal (India)

GERMINATION OF SEEDS

The pea seeds were rinsed in running water and then steeped for six hours at 25 degrees Celsius in sterile distilled water. The ingested seeds underwent a 10-minute surface sterilization with $HgCl_2$ (0.02%) and subsequent washing in distilled water. The water imbibed pea seeds were spread on a thick layer of sterilized absorbent cotton in plastic trays and kept for germination in a growth chamber at 25°C for 48 hr in the dark. The germinated seedlings (48 hr old) were then transferred to continuous light (8000+/-100 lux) for the next four days.

HARVESTING OF TISSUE

Pea epicotyls were hand clipped from 6-day-old seedlings. The epicotyls were then segmented into the basal (1 cm) and the apical (1.5 cm) part. The apical and basal portions of the epicotyls were kept in separate beakers under chilled conditions (4°C), washed with distilled water, blotted dry, submerged in liquid Nitrogen (-195.8°C) to freeze and subsequently stored at -70°C.

EXTRACTION AND ISOLATION OF ^{32}P-LABELLED POLY $(A)^+$ RNA

An atomizer was used to spray a solution of GA_3 (10^{-6}M) onto the 4-day-old seedlings. Epicotyls were harvested from both untreated and GA_3-treated plants after 48 hours (10 g total). The epicotyls' lower portions were thinly cut and placed in conical flasks with ^{32}Pi (carrier-free, 1mCi per flask) along with chloramphenicol (50 g/ml) in a total volume of 10 ml. Brunswick Shaker was used to incubate the tissue at 25 degrees Celsius in the dark for 6 hours. The tissue

was then rinsed in distilled **H_2O**, flash-frozen in liquid N_2 at **-195**°C, and kept at -70°C for later use.

Following this, the apical segments were treated to enable the separation and subsequent extraction of ^{32}P -labelled poly $(A)^+$ RNA.

Nucleic acid extraction: The frozen tissue was homogenized to a fine powder in liquid N_2 and four volumes of extraction buffer (Tris-HCI, 50mM pH 8.0; NaEDTA, 25mM; NaCl, 150mM; SDS, 1%, w/v), was added. To this slurry, an equal volume of PBS solution, (prepared by mixing phenol 200ml with buffer 50ml, containing Tris. HCl,50mM, pH 8.0; NaEDTA 25mM and NaCl 150mM, by shaking the mixture for 2-3 hr and then adding an equal volume of chloroform to it) was added. The mixture was stirred for 15 min at room temperature and then chilled for 20 min. The mixture was centrifuged and the aqueous phase was removed. The interphase layer was drawn out from the centrifuge bottle and mixed with an equal volume of PBS solution. After stirring the sample for 10 min, at room temperature, it was chilled for 20 min and centrifuged for 15 min at 10,000 rpm. The aqueous phase was again removed. The combined aqueous phases were re-extracted with equal volumes of PBC solution. There was no protein left in the interphase layer. To this, 0.2M potassium acetate was added and the nucleic acids were precipitated overnight at -20°C by the addition of 2 volumes of ethanol. The precipitated nucleic acids were collected by centrifugation (10 min at 10,000 rpm and 2°C) and washed 4 times with Tris-HCl buffer (20mM, pH 7.6) containing 3M sodium acetate. The precipitate essentially contains RNA and traces of DNA. The precipitate was redissolved in Tris-HCI (20mM, pH 7.6) and the nucleic acids were precipitated at -20°C, overnight by adding 3 volumes of chilled ethanol. The precipitate, collected after centrifugation, was lyophilized for removing alcohol.

Isolation of poly $(A)^+$ RNA: The RNA samples were solubilized in Tris-HCI. (10mM, pH 7.6) heated at 60 degrees Celsius for a minute and chilled instantly. The RNA sample solution was then diluted to achieve the desired concentration of 0.2M NaCl. ^{32}P -labelled RNA samples, equivalent in radioactivity ($4x10^5$ dpm), were recovered from both control (untreated) and GA_3-treated seedlings. Fractionation was performed on oligo (dT)-cellulose column (5mm x13mm) equilibrated with a binding buffer (Tris-HCI (20mM, pH7.6 + NaCI (0.5M) at 4°C at a flow rate of 3-4 ml min-1. At 37 degrees Celsius, 0.5 ml portions of the elution buffer (Tris-HCI, 10 mM, pH 7.6) were used to elute the radioactively labeled poly $(A)^+$ RNA.

IN VIVO GA_3 TREATMENT

The apical and the basal segments of the dwarf pea epicotyls (10-15 gms) were placed in flasks (containing 30 ml of buffered solution containing PO_4 buffer 10mM, pH 6.6 and sucrose 2%) in the presence of GA_3 (10^{-6} M)and also in the absence (control). The flasks were shaken (at 100 rpm) for 4 hr at 25°C, in the presence of light. The tissue was washed with distilled water, blotted dry, submerged in liquid Nitrogen (-195.8°C) to freeze and subsequently stored at -70°C.

TIME COURSE OF GA_3 - TREATMENT (*IN VIVO*) OF DWARF PEA EPICOTYLS

The clipped basal segment of light grown epicotyls were incubated with GA_3 (10^{-6} M) and in the absence (control), for 1 to 6 hr at 25°C. Ammonium sulfate fraction precipitate was sourced from

the samples (between 30 and 50 percent satn). RNA polymerase enzymatic activity Il was measured in both.

IN VIVO TREATMENT OF DWARF PEA EPICOTYLS WITH GAS

Dwarf pea epicotyls were incubated in presence of different concentrations of GA_3 (10^{-12} M to 10^{-6} M) for 4 hr. The control set was incubated in absence of GA. Ammonium sulfate fraction precipitate that was obtained from the samples (between 30 and 50 percent saturation). RNA polymerase enzymatic activity Il was measured in both.

TREATMENT OF TISSUE WITH CYCLOHEXIMIDE

RNA polymerase II activity was examined in enzyme extracts (ammonium sulphate fraction ppt 30 - 50% saturation) sourceded from the clipped basal tissue, incubated with GA_3 (10^{-6} M) and in the absence (control) and compared with enzyme activity from tissue grown in GA_3 (10^{-6} M) + CHI (20 **µ**g/ml).

TREATMENT OF TISSUE WITH AMINO ACID ANALOGUES

Dwarf pea epicotyls (basal segments) were incubated for 4 hr at 25°C in presence of GA3 (10^{-6} M), in the absence (control) and in GA_3 (10^{-6} M) + amino acid equivalent-analogues (2 mM each of D-fluoro-DL-phenylalanine, DL-7-azatryptophan, DL-methionine, L-canavanine, L-hydroxyproline and L-thioproline). The activity of RNA polymerase II was assessed in the ammonium sulphate fractional precipitate (30-50% saturation) sourced from the GA_3-treated and control-untreated dwarf pea epicotyls.

TREATMENT OF TISSUE WITH ABSCISIC ACID

The clipped basal segments of epicotyls were incubated with 10^{-6} M GA_3 (in presence) or without GA_3 (in absence or control), ABA (10^{-6} M) and in GA_3 (10^{-6} M) + ABA (10^{-5} M) for 4 hr at 25°C. Activity of RNA polymerase II was determined in $(NH_4)_2SO_4$ fraction precipitate (30 - 50% satn.) of these sets subsequently.

PREPARATION OF AMMONIUM SULPHATE FRACTIONAL PRECIPITATE

In a solution of 50 mM, Tris-HCL buffer (pH 7.3, in the ratio of 1:3 w/V) containing -mercaptoethanol (5 mM) and polyvinyl polypyrrolidone 2% (w/v), the tissue was homogenized using sand as an abrasive. After filtering the homogenate through four layers of muslin fabric, the filtrate was subjected to centrifugation at 12,000 rpm for 15 minutes,

The supernatant part was subjected to ammonium sulphate fractionation (30 -50% saturation). The precipitated proteins were suspended in a small aliquot of buffer and dialyzed against Tris-HCI buffer (10 mM, pH 7.3) containing β-mercaptoethanol (5mM) and glycerol (15%). The desalted ammonium sulphate fractional precipitate was centrifuged at 12,000 rpm for 15 min. The clear supernatant was used for the assay of RNA polymerase II activity. All steps of enzyme preparation were carried out at 4°C.

EVALUATION OF RNA POLYMERASE ACTION

The evaluation reaction mixture (125ul) contained 60 mM Tris-HCl buffer (pH 8.0 at 25°C) $MnCl_2$ (1 mM), $MgCl_2$ (20 mM), $(NH_4)_2SO_4$ (50 mm), heat-denatured calf thymus DNA (50ug). DTT (4 mM), ATP, GTP and CTP (0.8 mM each), [^{3}H]-UTP (0.8 mM, 22 dpm pmol) and the enzyme fraction. The evaluation mixture was incubated for 15 minutes at 30°C. Thereafter, a small amount (40 µl, in duplicate) of the reaction mixture was applied to a 2.5cm paper discs (Whatman 3 MM) and soon after dipped in chilled 5% TCA containing 0.02M sodium pyrophosphate. The unbound radioactivity was eliminated by intensive washing (x 4) with chilled TCA (5%) followed by a wash (x 1) with equal proportions of diethyl ether and ethanol (1:1) and finally an additional rinse in diethyl ether. The radioactivity incorporated in acid precipitable fraction was determined. The activity of RNA polymerase II was expressed as picomoles of UMP incorporated mg protein.

IN VITRO ADDITION OF GA_3

Gibberellic acid (GA_3, 10^{-6} M) was added to the buffer being used for homogenizing basal segments of light grown dwarf pea epicotyls. Activity of RNA polymerase II was evaluated in the dialyzed ammonium sulphate solution precipitate (30-50% saturation) and compared with activity of the enzyme from untreated (control) epicotyls. Ammonium sulphate solution precipitate (30 -50% saturation) from the untreated (control) apical and basal segments of light grown dwarf pea epicotyls were preincubated in presence of GA_3 (10^{-6}M) for 45 min at 4°C. Control for this was incubated in absence of GA_3. An aliquot (70 µl) from both treated and untreated incubation mixtures was then used for determining RNA polymerase II activity. Ammonium sulphate solution precipitate (30 - 50% saturation) from the untreated (control) basal segments of light grown tall pea epicotyls were preincubated in presence of GA_3 (10^{-6}M) for 45 min at 4°C. Control for this was incubated in absence of GA_3. RNA polymerase II activity in treated and untreated enzyme extracts of tall pea was compared with the treated and untreated enzyme extracts prepared from dwarf pea.

IN VITRO ACTIVATION BY DIFFERENT CONCENTRATIONS OF GAs

Activity of RNA polymerase II was determined in ammonium sulphate fractional precipitate (30 50% saturation) obtained from basal segments of untreated pea epicotyls after pre-treating it with different concentrations of GAS (10^{-15} M to 106 M)) at 4°C for 45 min. The activity in these sets was assessed in comparison to that in the control (untreated) sample.

PREINCUBATION OF ENZYMATIC FRACTION WITH GAs FOR DIFFERENT INTERVALS OF TIME

Ammonium sulphate fractional precipitate (30-50% saturation) from untreated basal segments was pre-treated with GAs (10^{-6} M) at 4°C, for different intervals of time (0 time to 50 min). RNA polymerase II activity was determined in these sets and compared with that in the untreated enzyme fraction,

IN VITRO TREATMENT WITH ABSCISIC ACID

Activity of RNA polymerase II was determined in ammonium sulphate fractional precipitate (30 50% saturation) after pre incubating it with GA_3 (106 M), GA_3 (106 M) + ABA (105 M) and ABA (105 M) for 45 min, at 4°C and was compared with the untreated sample.

IN VITRO TREATMENT WITH GAs

RNA polymerase II efficiency was determined in ammonium sulphate fractional precipitate (30 - 50% saturation) after pre-treating it with various concentrations of GAs (10^{-10} M and 10^{-5} M) and compared with activity in untreated sample and in GA_3 treated sample (10^{-10} M and 10^{-15} M).

PRODUCT CHARACTERIZATION

The reaction product of RNA polymerase II from control and GA_3 (*in vitro*) treated enzyme fraction was incubated with RNase A (100 µg and 200 µg) for 60 min at 37°C. The reaction product was precipitated with TCA (5%) for determining the sensitivity of the reaction product. Omission of RNase A from the reaction mix served as control.

α-AMANITIN SENSITIVITY

The basal segments of dwarf pea epicotyls were subjected to homogenization in buffer A (250 mM Tris-HCI, pH 7.3; 0.2 mM EDTA; 75 mM $(NH_4)_2SO_4$; 5mM $MgCl_2$; 50 mM β mercaptoethanol) with the addition of of acid rinsed sand and 2% PVP (w/v). The resulting suspension was strained using four sheets of muslin fabric and subjected to centrifuge at 20,000 g for 15 minutes. The supernatant was subsequently administered to ammonium sulphate precipitation (30-50% satn.). This fraction was dialyzed overnight against a Tris-HCI buffer designated as buffer B comprising 50 mM Tris-HCI, pH 7.3; 0.1 mM EDTA; 0.5 mM $MgCl_2$; 5 mM β-mercaptoethanol and 15% glycerol. The dialyzed ammonium sulphate fractional precipitate (30 - 50% saturation) was incubated in absence and presence of GA_3 (10^{-6} M) for 45 min at 4°C. The ammonium sulphate concentration in these fractions was adjusted to 0.05 M according to conductivity measurements. Both the fractions (100 mg protein each) were individually introduced onto DEAE-Sephadex A-25 columns by the ratio of 5 mg of protein sample per ml of column matrix, prepared with buffer B containing 0.05 M ammonium sulphate at an elution rate of 1 min per ml. The columns were then washed with 2-bed volumes of the same buffer B containing 0.05M ammonium sulphate, and 2-bed volumes of buffer B containing 0.15 M ammonium sulphate. RNA polymerase I was eluted with 2-bed volumes of buffer B containing 0.25 M ammonium sulphate. The proteins in the eluates were concentrated by ammonium sulphate precipitation (0-65% satn.) and desalted by dialyzing overnight in buffer B.

The desalted 0.25 M eluates from the two columns were incubated in the presence of α-amanitin (0.05 µg/ml) for 10 min at 4 degrees Centigrade, prior to the assay for RNA polymerase II activity. Activity of RNA polymerase II in the α-amanitin treated sample was compared with activity in absence of α-amanitin (control).

RNA POLYMERASE II PARTIAL PURIFICATION

Step 1. Extraction: 500g pre-frozen base sections form dwarf pea epicotyls were subjected to homogenization with acid-washed sand, 2% PVP (w/V) and 600 ml of buffer A. The resulting

mixture was passed through 4 sheets of muslin fabric followed by for 15 minutes of centrifugation at 20,000 g .The clear fluid hence produced was fractionated using polymin-P.

Step II. Polymin-P fraction: The supernatant component was treated with polymin-P (10%) with gentle stirring to obtain a final concentration of 0.7%. The precipitated nucleic acid fraction along with the bound proteins was collected by centrifugation at 20,000g for 15 min. The pellet fraction was washed with 350ml of buffer C (50 mM Tris-HCI, pH 7.3; 0.1 mM EDTA; 5 mM β-mercaptoethanol) followed by centrifugation at 20,000 g for 15 min. The washed pellet fraction was suspended in buffer C (250 ml) containing ammonium sulphate (0.5 M) for the extraction of proteins. The supernatant fraction obtained after centrifugation, was subjected to ammonium sulphate precipitation (0-65% satn.). The precipitated proteins were collected by centrifugation at 20.000 g for 30 min. The polymin-P fraction thus obtained was dialyzed using the same buffer B overnight.

Step III. DEAE-Sephadex chromatography: The concentration of ammonium sulphate in the desalted polymin-P fraction was set at 0.05 M utilizing conductivity measurements, and 5 mg of protein per ml of column matrix was introduced on a DEAE-Sephadex A-25 column that had been in equilibrium with 0.05 M ammonium sulphate in buffer B. The column's flow rate was adjusted to 1 min per ml. The column was rinsed with two bed volumes of buffer B with 0.05 M of ammonium sulphate and then with 0.15 M of ammonium sulphate in buffer B. With 2 bed volumes of buffer B containing 0.25 M ammonium sulphate, the RNA polymerase II-active fraction was recovered.

GENERAL METHODOLOGY

BRADFORD'S METHOD FOR PROTEIN ESTIMATION

Preparation of Coomassie Brilliant blue reagent

Coomassie brilliant blue G-250 (100 mg) was dissolved in ethanol (95%, 50 ml) and orthophosphoric acid (85% w/v, 100 ml) was added to it. The resulting solution was diluted to one litre for obtaining a final concentration of

CBB G250: 0.01% (w/v)

Ethanol (95%) 4.7% (w/v)

Orthophosphoric acid 8.5% (w/v)

The reagent was passed through 2 layers of Whatman 1 MM paper before use.

Protein Estimation

Dilutions of BSA were made in the range of 10-100 µg in a final volume of 100 µl with distilled water. Protein concentration in the sample was determined by making appropriate dilutions. To 100 µl sample volumes, 5 ml of CBB reagent was added and rapidly mixed on a vortex. The absorbance was measured after 5 min at 595 nm. The blank contained 100 µl of distilled water and Bradford's reagent (5 ml) and was used to adjust 100% transmission.

Microprotein Estimation

Protein solution containing 1-10 µg protein was diluted to 100 µl with distilled water. To each sample 1 ml CBB reagent was added and the contents were mixed on a vortex. The absorbance was measured at 595 nm after 10 min., against a blank that was prepared by taking 100 µl of distilled water and mixing it with 1 ml of Bradford's reagent

PROCESSING OF OLIGO (dT)

The powdered beads of oligo (dT) were soaked in Tris-HCI buffer (20mM, pH 7.6) for 2-3 hr at 4°C to allow the beads to swell. Columns of required height were packed and set to equilibrium with 20mM Tris-HCl buffer, pH 7.6, containing 0.5 M NaCl. After use, the column matrix was stored in 50% alcohol.

[H] UTP

Tritiated UTP was obtained from BARC (sp. activity 45 Ci/m mole) in 50% ethanol. The labelled compound was transferred to a sterilized glass beaker. The contents of the beaker were frozen in liquid N2 and freeze-dried (lyophilized) to remove ethanol. To this 10m Mole of UTP and 10 ml of Tris - HCI buffer (20 mM pH 7.2) was added to make a final concentration of 2 **µ**Ci/ 20 ml and specific activity 22 dpm/ pmol or 10 **µ**Ci / mole of UTP.

PROCESSING OF DEAE-SEPHADEX A-25

DEAE-Sephadex was soaked in distilled water for 4-5 hr. It was then charged with HCI (0.5 N) for 30 min with intermittent stirring and washed with distilled water to neutral pH on a Büchner funnel. The neutralized gel was suspended in NaOH (0.5 N) for 30min to convert it into its free base form. The gel was washed with distilled water to neutral pH. The gel was then suspended in the desired buffer and pH of the matrix adjusted to that of the buffer with the conjugate acid. The matrix was finally suspended in a fresh buffer.

PROCESSING OF DIALYSIS TUBINGS

Dialysis tubings were boiled for 30 min in Na_2CO_3 (1%) containing Na_2EDTA (1 mM). Thereafter, tubings were rinsed repeatedly (7-8 times) in hot distilled water. The tubings were stored in sodium azide (0.02%) at 4°C.

PURIFICATION OF SALTS AND SOLVENTS

Ammonium Sulphate

A supersaturated solution of ammonium sulphate was prepared at 80°C. The undissolved salt and other impurities were removed by filtration. The filtrate was collected over ice and chilled overnight Crystals of ammonium sulphate were collected over a Büchner funnel and oven dried at 50°C.

Solvents

Ethanol and toluene were routinely distilled at 79°C and 110°C respectively.

MEASUREMENT OF RADIOACTIVITY

Each filter disc (oven dried at 60°C) with the adsorbed radioactive compound was placed in a vial containing 10 ml of scintillation fluid (Toluene: PPO POPOP; 11: 5.0 g: 0.3 g). The closed vials were wiped with tissue paper and radioactivity was measured in a Beckman liquid scintillation counter (LS 1801).

RESULTS

DWARF PEA EPICOTYLS ARE SUBJECT TO REGULATION *IN VIVO* BY GA_3 TARGETING POLY $(A)^+$ RNA AND RNA POLYMERASE II

In several GA_3 -responsive tissues like barley (Varner and Ho, 1976; Jacobsen and Zwar, 1974) and wheat aleurones (Baulcombe and Buffard, 1983), application of GA_3 increases the relative abundance of poly $(A)^+$ RNA, since dwarf pea epicotyls are known to be a GA_3 -responsive tissue, the levels of poly $(A)^+$ RNA in with (hormone treated) and without GA_3 treatment (control) dwarf pea epicotyls were determined. To accomplished this, the control and GA_3 -treated epicotyls were excised, nucleic acids were labelled *in vivo* by gently agitating the basal segments of epicotyls in presence of carrier free 32 Pi for 6 hr and poly $(A)^+$ RNA was purified by oligo (dT)-cellulose column affinity chromatography. There was a 2.54-times increase in the content of poly (A)+ RNA in GA3 -administered tissue over that of the controls (**Table 1**). In a parallel set of experiments, the excised basal segments of dwarf pea epicotyls from seedlings sprayed with GA_3 and sterile distilled H_20 (control) were processed for the preparation of ammonium sulphate fraction precipitate (30 - 50% Satn.). A twofold boost in polymerase II activities was detected in the phytohormone treated tissue (**Table 1**). Thus, it appears that modulation of the enzyme RNA polymerase II could be related to the relative proportion of poly$(A)^+$ RNA in dwarf pea epicotyls.

EARLY RESPONSE OF GA_3 IN THE ORDINANCE OF RNA POLYMERASE II IN DWARF PEA UNDER *IN VIVO* CONDITIONS

Previously it was reported that GA_3 stimulated RNA polymerase II activity in dwarf pea epicotyls after a prolonged treatment of phytohormone (48 hr) (Tomi et al., 1983a). Presently, stimulation of RNA polymerase II activity in dwarf pea epicotyls was achieved after a short duration treatment of GA_3 (2 hr). The basic approach was to excise basal segments of dwarf pea epicotyls (1 cm) and incubate the segments in solution of GA_3 (1uM) at 25°C for different duration of time (1-8 hr). The control epicotyls were incubated in solution without GA_3. RNA polymerase II activity was then assessed in the ammonium sulphate fractional precipitate (30-50% satn.) prepared from control and GA_3 -treated tissue. **Fig 1** shows that there is significant elevation in the activity of RNA polymerase II in GA3 -treated epicotyls while the controls showed no such time-dependent rise in enzyme activity. Thus within 4 hr of phytohormone treatment, there was a 2.2-fold stimulation of RNA polymerase II activity over that of the controls. These results suggest that increase in activity of RNA polymerase II in dwarf pea epicotyls following application of GA_3 is an early response of the phytohormone. A wide range of GA_3 concentrations were tested for studying activation of RNA polymerase II *in vivo*. Stimulation of enzyme activity could be achieved at a concentration as low as 10^{-8}M (**Table 2**).

Further it was observed that the GA_3 -mediated stimulation of RNA polymerase II expression was not hindered by the simultaneous addition of cycloheximide (200 µg/ml). Similarly, administration of six amino acid analogues (2mM each) along with GA_3 (1uM) failed to decrease the GA_3 -stimulated RNA polymerase II activity in the epicotyls of dwarf pea (**Table 3**). Thereby, the experiment with cycloheximide and amino acid equivalents strongly suggested that de novo generation of proteins was not needed for the GA_3 -mediated regulation of RNA polymerase II activity in dwarf pea epicotyls. However, abscisic acid (ABA, 10uM) nullified the stimulatory response of GA_3 on RNA polymerase II function. Incubation of pea epicotyls in ABA (10°M) alone showed no appreciable inhibition of ANA polymerase II activity over that of control (**Table 4**).

Two major facts emerged from the studies pertaining to regulation of RNA polymerase II by GA_3 in dwarf pea epicotyls under *in vivo* conditions. First, that the elevation of RNA polymerase II performance by the phytohormone was an early response (~ 2 hr) and second, that *de novo* generation of proteins was not needed for enhanced expression of RNA polymerase II in response to GA_3.

ORDINANCE OF RNA POLYMERASE II ACTIVITY BY GA_3 IN CELL FREE EXTRACTS OF DWARF PEA EPICOTYLS

In order to elucidate the mechanism of activation of RNA polymerase II by the phytohormone, GA_3 (106 M) was added during homogenization of control dwarf pea epicotyls. This resulted in elevated RNA polymerase II efficiency by approximately 2-fold **(Table 5**). This experimental approach gave a strong indication of promotion of RNA polymerase II by GA_3. Thereafter, the impact of GA_3 on RNA polymerase II activities in cell-free extracts of untreated dwarf pea epicotyls was checked. Addition of GA_3 to the cell free extracts (30 - 50% Satn., ammonium sulphate fraction precipitate) of dwarf pea epicotyls during the assay of RNA polymerase II showed no stimulation of the enzyme activity. However, preincubation of cell-free extracts with GA_3 (1µM) did stimulate RNA polymerase II activity. **Fig 2** depicts that preincubation of enzyme fraction with GA_3 (1 µM) at 4°C, significantly increased the activity of RNA polymerase II as a function of time. After 30min of preincubation with GA_3, there was about 3.0-fold stimulation of RNA polymerase II activity. Preincubation of enzyme fraction with GA_3 (1 **µ**M) beyond 30min, showed no further increase in enzyme activity *in vitro*. A wide range of GA_3 concentrations have been tested for studying activation of RNA polymerase II *in vitro*. Whereas the activation of RNA polymerase II by GA_3 could be achieved *in vivo* at a concentration of 10M (**Table 2**), the *in vitro* initialization of this enzyme could be observed even at extremely low concentrations of GA_3 (10^{-15} M) **(Table 6**). Apparently, the modulation of RNA polymerase II by GA_3 *in vitro* is a very sensitive physiological response of the phytohormone GA_3.

The *in vitro* activation of RNA polymerase II by GA_3 was effectively abolished by the simultaneous incorporation of abscisic acid (10^{-5} M) (**Table 7**). However, *in vitro* addition of ABA (10^{-5} M) alone did not decrease the activity of RNA polymerase II in comparison to the controls. Thus, this data suggests that *in vivo* regulation of RNA Polymerase II in dwarf pea epicotyls by GA_3 could be faithfully mimicked *in vitro* at extremely low concentrations of GA_3 (**Fig 3**).

The next objective was to study specificity of GA_3 -mediated activation of RNA polymerase II in dwarf pea epicotyls. The effect of GA_8 (10^{-10} M – 10^{-15} M) on the *in vitro* regulation of RNA polymerase ll was tested, since GAs is known to be a biologically inactive analogue of gibberellic acid. **Table 8** gives comparative information on the *in vitro* response of GA_3 and GA_8 on the activity of RNA polymerase II. Unlike GA_3, GA_8 failed to stimulate RNA polymerase II activation *in vitro* (**Table 8**) This experiment therefore clearly suggested that the regulation of RNA polymerase II by GA_3 is a very specific phytohormonal response in dwarf pea epicotyls.

The effect of GA_3, *in vitro* has also been tested on the polymin-P fraction, which essentially contains chromatin bound RNA polymerase II. The *in vitro* addition of by GA_3 (1 µM) to the polymin-P fraction, brought about 1.8-fold stimulation of RNA polymerase II activity. Further fractionation of polymin-P fraction on DEAE-Sephadex A-25, increased the specific activity of the enzyme. However, *in vitro* addition of GA_3 to DEAE-Sephadex A-25 fraction failed to stimulate RNA polymerase II activity in this fraction (**Table 9**). Thus, it appears that some factor associated with RNA polymerase II is eliminated after DEAE-Sephadex A-25 fractionation that is crucial for GA_3 -elicited activation of the enzyme (**Table 9**).

RNA polymerase II activity after ammonium sulphate fraction precipitation (30 - 50% satn.) was stimulated under *in vitro* conditions by pre incubating with GA_3 at 4°C for 45min and the enzyme fractions were subjected to DEAE-Sephadex A-25 column chromatography. The enhanced activity of RNA polymerase II was maintained even after DEAE-Sephadex A-25 ion-exchange chromatography (**Table 10**).

The three different types of RNA polymerases differ in their sensitivity to α-amanitin. RNA polymerase I is known to be insensitive to α-amanitin, RNA polymerase II is highly sensitive and RNA polymerase III is sensitive to high concentrations of the toxin (Chambon, 1975). Assay of the enzyme in the DEAE-Sephadex A-25 fraction in the presence of trace amounts of α-amanitin (0.05 µg/ml) resulted in 68% inhibition in its activity over the control. This showed that it was a type II enzyme that was GA_3 -responsive (**Table 10**).

The effect of GA_3 was also tested *in vitro* in extracts prepared from tall variety of pea. Whereas consistent stimulation of RNA polymerase II activity, was observed in cell-free extracts of dwarf pea epicotyls, there was no stimulatory response of GA_3 on RNA polymerase II activity in cell-free extracts of tall pea. Nevertheless, tall variety of pea exhibited equivalent RNA polymerase II specific enzyme activity to that of dwarf pea epicotyls treated with GA_3 (**Table 11**). It is postulated that high endogenous levels of GA_3 could be responsible for the high specific activity of RNA polymerase II in tall variety pea. Dwarf pea is known to lack endogenous gibberellin and thus depicts a lower specific activity of RNA polymerase II. Thus, the *in vitro* activation of RNA polymerase II by GA_3 in dwarf pea epicotyls has a physiological relevance, since increased activity of RNA polymerase II does exist in dwarf pea without exogenous addition of GA_3 (Table 11).

Enzymatic analysis confirmed that RNA is the final result of RNA polymerase II reactions. Treatment of the reaction product with RNase A (100 µg and 200 µg) resulted in a significant

loss of TCA precipitable radioactivity (**Table 12**). This is due to the enzymatic degradation of RNA products by RNase A.

Table 1. Relative abundance of polyadenylated RNA and enhancement of RNA polymerase II activity by GA_3 in dwarf pea epicotyls.

Treatment	32[P]-labelled poly $(A)^+$ RNA (dpm)	Relative radioactivity	RNA polymerase II activity (pmoles Ump incorporated/ mg protein)	Relative activity
Control	6125	1.00	3534	1.00
GA_3, 1 µM (*in vivo*)	15550	2.54	7093	2.0

Table 1.

4-day-old light grown dwarf pea seedlings were administered with GA3 (1µM), whereas the controls were sprayed with sterile distilled H_2O. The basal segments of pea epicotyls were clipped after 48hr of treatment, sliced into small sections (4mm in length) and transferred into flasks containing carrier-free ^{32}Pi (1mCi/flask, 10ml). Chloramphenicol (50µg/ml) was added as a bactericidal agent. The tissue was incubated in a Brunswick shaker for 6hr at 25°C in the dark. Total RNA was extracted as described in Materials and Methods. The [^{32}P]-labelled RNA (4x10-5dpm) from control and GA_3 treated samples were separated on oligo (dt) cellulose-column, for the isolation of poly$(A)^+$RNA. RNA polymerase II efficiency was evaluated in the ammonium sulphate fraction precipitate (30-50% satn.), prepared from untreated and GA_3-treated dwarf pea epicotyls.

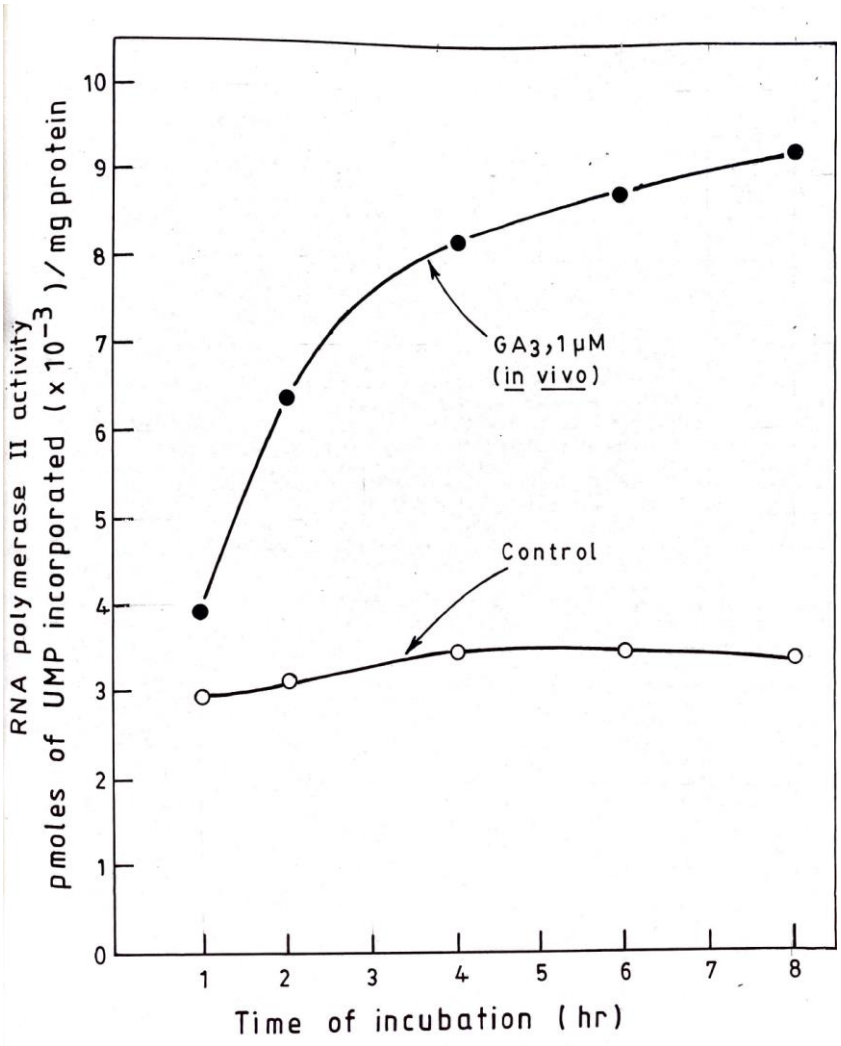

Fig 1. Enhancement of RNA polymerase II by the *in vivo* application of GA_3 to dwarf pea epicotyls.

The basal segments (1cm) of dwarf pea epicotyls harvested from 6-day-old-light grown pea plant seedlings were incubated in the presence and absence of GA_3 (1uM), for different intervals of time (1-8hr). RNA polymerase II activity was assessed in the desalted ammonium sulphate fractional precipitate (30-50% saturation), prepared from GA_3-administered and control dwarf pea epicotyls. There was an early stimulatory response of GAs on RNA polymerase II activity in dwarf pea epicotyls.

Table 2. *In vivo* enhancement in activity of RNA polymerase II by GA_3 in dwarf pea epicotyls.

Treatment GA_3	RNA polymerase II activity (pmoles of UMP incorporated/mg protein)	Relative activity
Control	3039	1.00
10^{-6} M	5346	1.75
10^{-8} M	5450	1.79
10^{-10} M	3763	1.23
10^{-12} M	2998	0.98
10^{-14} M	2895	0.95

Table 2. Basal 1 cm segments of the epicotyls of dwarf pea plants, harvested from 6-day-old plantlets, were incubated in different concentrations of GA_3 (10^{-6} M - 10^{-14} M) for 4hr at 25°C. The desalted ammonium sulphate fractional precipitate (30-50% saturation) obtained from GA_3 treated and untreated tissue was utilised for the assessing RNA polymerase II activity.

Table 3. *In vivo* stimulated RNA polymerase II activity by GA_3 in epicotyls of dwarf pea was insensitive to the action of cycloheximide and amino acid analogues.

Treatment	RNA polymerase II activity (pmoles of UMP incorporated/mg protein)	Relative activity
Control	3088	1.00
GA_3, 10^{-6} M	8617	2.79
GA_3, 106 M + CHI, 20 µg/ml	8335	2.69
GA_3, 10^{-6} M + 6 amino acid Analogues	8109	2.62

Table 3. Dwarf pea epicotyls (basal segments) were incubated along with GA_3(10^{-6}M); GA_3(10^{-6}M) + CHI (20µg/ml) and in GA_3(10°M) + 6 amino acid analogues (2mM each of D-fluoro-DL-phenylalanine, DL-7-azatryptophan, DL-methionine, L-canavanine, L-hydroxyproline and L-thioproline) for a period of 4hr at 25°C. Omission of GA_3 served as control. Ammonium sulphate fractional precipitate (30-50% saturation), extracted from these tissues was employed for assessing RNA polymerase II activities after extensive dialysis.

Table 4. Manipulation of RNA polymerase II activity by GA_3 and ABA in dwarf pea epicotyls.

Treatment	RNA polymerase II activity (pmoles of UMP incorporated/ mg protein)	Relative activity
Control	3363	1.00
GA_3 (10^{-6} M)	6147	1.82
GA_3 (10^{-6} M) + ABA (10^{-5} M)	2904	0.84
ABA (10^{-5} M)	2904	0.87

Table 4. Basal segments (1cm) of 6-day-old light grown seedlings were incubated in presence of 10^{-6}M GA_3, 10^{-6}M GA_3 + 10^{-5} M ABA and 10^{-5} M ABA for a period of 4hr al 25°C. Ammonium sulphate fractional precipitate (30-50% saturation) produced from tissues undergoing these treatments was used for the assay of RNA polymerase II activities.

Table 5. Activation of RNA polymerase II by GA_3, added during homogenization of dwarf pea epicotyls.

Additions	RNA polymerase II activity (pmoles of UMP Incorporated/mg protein)	Relative activity
Control	3350	1.00
GA3, 10^{-6} M (added during homogenization of tissue)	5965	1.78

Table 5. Ammonium sulphate fraction precipitate (30-50% satn.) was prepared from control dwarf pea epicotyls. In another set, GA_3 (10^{-6}M) was added during homogenization of dwarf pea epicotyls. Ammonium sulphate fractionation was performed on the homogenized sample. RNA polymerase II efficiency was assessed in both enzyme fractions.

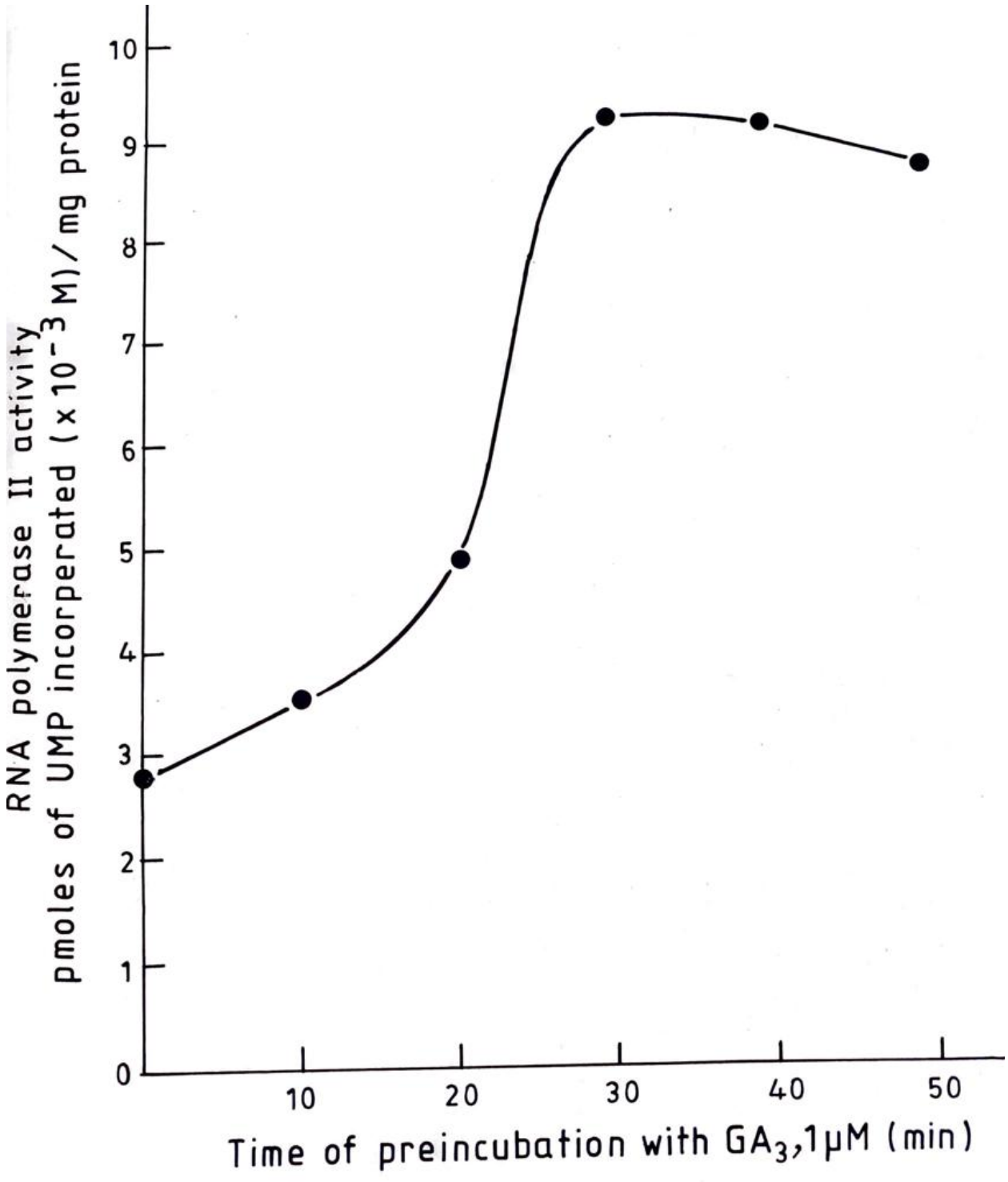

Fig 2. *In vitro* stimulation of pea RNA polymerase II as a function of time of preincubation of enzyme fraction with GA_3

The desalted ammonium sulfate fractional precipitate (30–50% saturation) obtained from reference basal 1 cm segments of dwarf pea epicotyls was preincubated with 1µM GA_3 at 4°C for periods of varying time durations (0–50 min). In these sets, RNA polymerase II activities were assessed at 30 °C for 15 min.

Table 6. RNA polymerase II *in vitro* activation by GA_3 in cell-free extracts of dwarf pea epicotyls.

Additions	RNA polymerase II activity (pmoles of UMP incorporated per mg protein)	Relative activity
Control	3842	1.00
GA3, 10^{-6} M	11016	2.86
GA3, 10^{-8} M	11024	2.86
GA3, 10^{-10} M	8565	2.22
GA3, 10^{-12} M	8418	2.19
GA3, 10^{-14} M	7009	1.82
GA3, 10^{-15} M	6942	1.80

Table 6. Ammonium sulphate fraction precipitate (30-50% satn.), was prepared from control dwarf pea epicotyls (basal segments). This cell-free enzyme extract was preincubated with different concentrations of GA_3 (10^{-6}M - 10^{-15}M) for 45 min at 4°c and then employed for the assessment of activity of RNA polymerase II.

Table 7. *In vitro* activation of RNA polymerase II by GA_3 and its counteraction by abscisic acid (ABA) in dwarf pea epicotyls.

Additions	RNA polymerase II activity (pmoles of UMP incorporated/ mg protein)	Relative activity
Control	3855	1.00
GA3, 10^{-6} M	7374	1.90
GA3, 10^{-6} M + ABA, 10^{-5} M	3316	0.86
ABA, 10^{-5} M	3623	0.94

Table 7. Ammonium sulphate fractional precipitate (30 50% saturation) obtained from the reference control dwarf pea epicotyls (basal segments) was preincubated in the presence of 10^{-6} M GA_3, 10^{-5} M ABA and 10^{-6} M GA_3 + 10^{-5} M ABA, for a period of 45 min at 4°C. These samples were then assessed for RNA polymerase II activity.

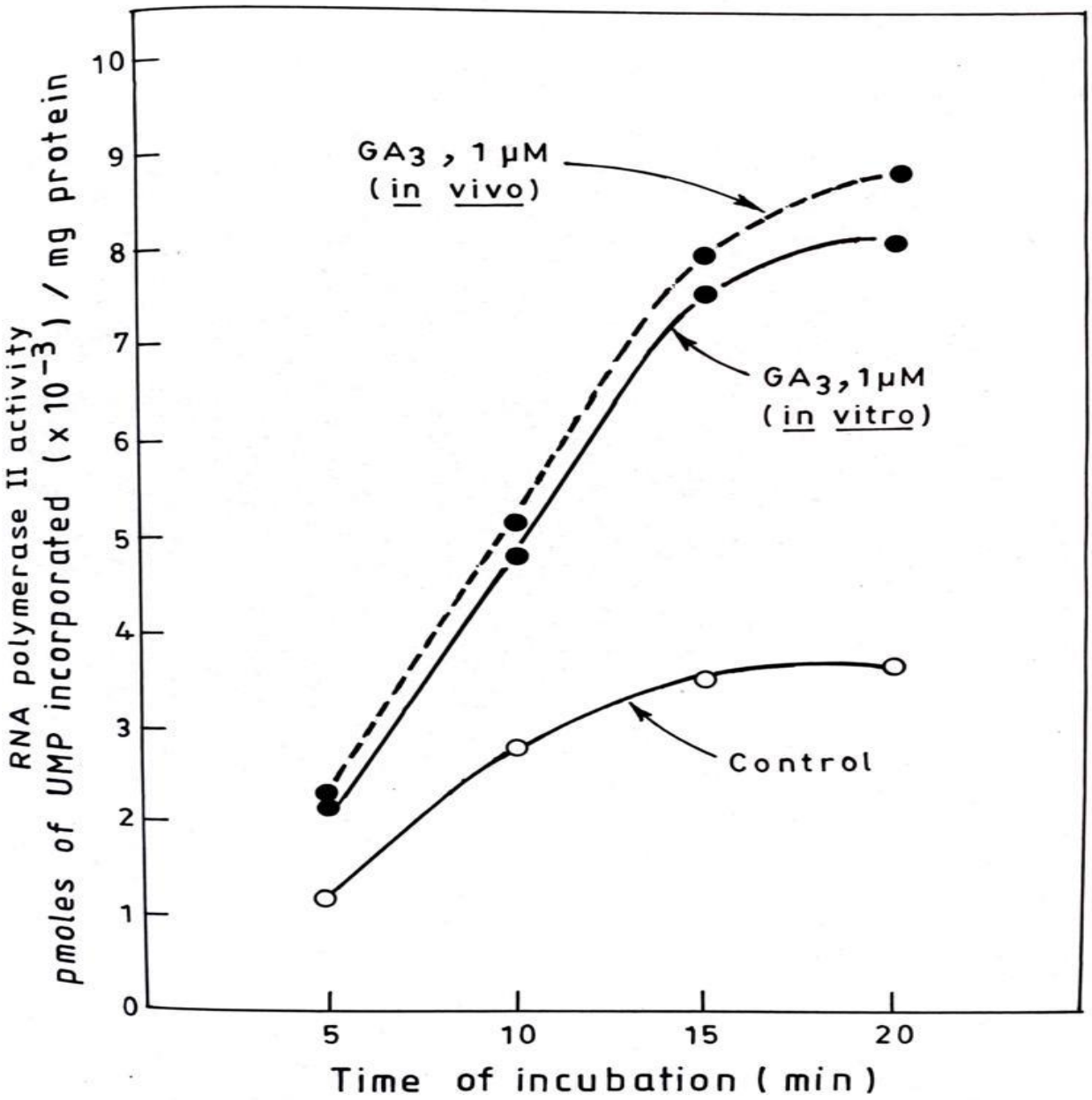

Fig 3. Kinetics of the GA_3-stimulated pea RNA polymerase II activity in dwarf pea epicotyls, under *in vivo* and *in vitro* conditions.

Basal segments of dwarf pea epicotyls were incubated in presence and absence of GA_3 (1µM). RNA polymerase II activity was then assessed in the ammonium sulphate fractional precipitate obtained from the control and GA_3 treated tissue. For studying the stimulation of RNA polymerase II (*in vitro*) by GA_3 the enzyme fraction from control dwarf pea epicotyls was preincubated with GA_3 for 45 min at 4°C and subsequently employed for assay of enzyme activity.

Table 8. The *in vitro* control of RNA polymerase II activity in dwarf pea epicotyls is influenced differently by GA3 and GA8.

Additions	RNA polymerase II activity (pmoles of UMP incorporated/ mg protein)	Relative activity
Control	3842	1.00
GA3, 10^{-10} M	8565	2.22
GAS, 10^{-10} M	3875	1.00
GA3, 10^{-15} M	6942	1.80
GAB, 10^{-15} M	3720	0.96

Table 8. Ammonium sulphate fraction precipitate (30-50% satn.), prepared from basal segments (1 cm) of control dwarf pea epicotyls, was exhaustively dialyzed. This cell-free extract was preincubated with GA_3 (10^{-10} M and 10^{-15} M) and with GA_8 (10^{-10} M and 10^{-15} M) for 45 min at 4°C and then employed to evaluate RNA polymerase II activity.

Table 9. Lack of stimulation of RNA polymerase II by GA_3 in DEAE-Sephadex A-25 fraction of dwarf pea epicotyls.

Steps of purification	RNA polymerase II activity (pmoles of UMP incorporated per mg protein)	
	Control	GA3,10^{-6} M. (*in vitro*)
Polymin-P fraction	1119	3448
DEAE-Sephadex A-25 fraction (0.25 M eluate)	10780	9853

Table 9. Polymin-P fraction and DEAE-Sephadex A-25 fractions were prepared from control dwarf pea epicotyls. The enzyme fractions were preincubated with GA_3 for 45 min at 4°C and then employed for the evaluation of RNA polymerase II activity.

Table 10. Sensitivity of RNA polymerase II activity towards α-amanitin.

	RNA polymerase II activity (pmoles of UMP incorporated/mg protein)			
Treatment	Control activity	Percent (*in vitro*)	GA_3 (10^{-6}M) activity	Percent
Control	8028	100	19039	100

(untreated)				
α-amanitin (0.05 µg/ml)	**2911**	**36.3**	**6979**	**36.7**

Table 10. Ammonium sulphate fraction precipitate (30-50% satn.) was prepared from control dwarf pea epicotyls. This enzyme fraction was incubated in the presence and absence of 10^{-6}M GA_3 for 45 min at 4°C. Both the fractions (100 mg protein each) were individually fractionated on DEAE-Sephadex A-25. The desalted DEAE-Sephadex eluates (0.25M) were preincubated with α-amanitin (0.05µg/ml) at four degrees Celsius for 10 minutes and then employed for testing the activity of RNA polymerase II.

Table 11. Differential regulation of RNA polymerase II activity by GA_3 in epicotyls of tall and dwarf pea.

	Dwarf pea		**Tall pea**	
Additions	**RNA polymerase II activity (pmoles of UMP incorporated per mg protein)**	**R.A.**	**RNA polymerase II activity (pmoles of UMP incorporated/mg protein)**	**R.A.**
Control	**3573**	**1.00**	**8015**	**1.00**
GA3, 10^{-6} M	**8518**	**2.38**	**9136**	**1.13**

RA. = Relative activity

Table 11. Ammonium sulphate fraction precipitate was prepared from basal segments of untreated tall and dwarf pea epicotyls. The cell-free enzyme extracts were preincubated with GA_3 (10^{-6}M) for 45 min at 4°C. RNA polymerase II efficiency was subsequently determined in these samples.

Table 12. Evaluation of reaction product of RNA polymerase II in dwarf pea epicotyls.

Treatment	**Control RNA polymerase II activity (pmoles of UMP Incorporated per mg protein)**	**GA_3 (*in vitro*) 10^{-6}M RNA polymerase II activity (pmoles of UMP Incorporated per mg protein)**
Untreated	**3391**	**6031**
RNaseA, 100 µg	**1320**	**2117**
RNaseA, 200 µg	**297**	**414**

Table 12. Ammonium sulphate fractional precipitate (30-50% saturation) prepared from control basal segments of dwarf pea epicotyls was preincubated in presence and absence of GA_3 (1µM) and used for assay of RNA polymerase II activity. The product of the reaction was characterized by testing its degradation with RNaseA (100µg and 200µg).

DISCUSSION

Previously, it was reported that relatively high concentration of GAs (10^{-14} M) stimulated RNA polymerase II activity in dwarf pea epicotyls after a prolonged treatment (48 hr) of tissue with this phytohormone (Tomi et al., 1983a). However, it was not ascertained whether GA_3 regulates the activities of RNA polymerase at the transcriptional or post-transcriptional level. A marked increase in the activity of chromatin bound FINA polymerase was observed in GA3-treated pea epicotyls (McComb et al., 1970) and vitro application of soybean hypocotyl (Hou and Pillay, 1975). However, GA_3 was shown to have no stimulatory or inhibitory effect on RNA synthesis. In the present investigation, it has been demonstrated that incubation of excised dwarf pea epicotyls (basal segments) in a solution of GA_3. (10^{-8} M) resulted in the enhancement of RNA polymerase II activity (2 - 3-fold) within 2 hr of treatment. Thus, the regulation of RNA polymerase I was achieved with much lower concentrations of GA_3 (- 10,0000-fold) in comparison to the earlier report (Tomi et al., 1983a) in dwarf pea epicotyls. In addition, the hormonal response was observed after a much shorter duration of GA_3 treatment (2 - 4 hr).

Subsequently, it was observed that the phytohormone-mediated stimulation of RNA polymerase II activity in dwarf pea epicotyls was not diminished by cycloheximide (20µg/ml) which is a potent inhibitor of protein synthesis. Similarly, administration of amino acid equivalents to **GA_3**-treated dwarf pea epicotyls, showed no inhibition of hormone regulated RNA polymerase II activity. Polypeptides synthesized in presence of analogues of amino acids are functionally defective. Thus, if protein synthesis was a prerequisite for GA_3-mediated stimulation of RNA polymerase II, the presence of amino acid analogues would have blocked the stimulatory response of GA_3 without any side effects of drugs like cycloheximide. Hence it became evident that the de novo protein synthesis was not required for the GA_3-induced stimulation of RNA polymerase ll in dwarf pea epicotyls. Since the stimulatory response of the phytohormone was witnessed after a short duration of treatment (2 hr) by adding GA_3, it was speculated that RNA polymerase II activity is regulated by GA_3 through the process of enzyme activation. Thereafter GA_3 was added during homogenization and extraction of enzyme fraction from control dwarf pea epicotyls. Interestingly, stimulation of RNA polymerase II activity was also observed by the presence of GA_3 during isolation of enzyme fraction. This strongly suggested that the **GA_3**-mediated stimulation of RNA polymerase II activities could be ascribed to enzyme activation. The next logical experimental approach was to test the effect of GA_3 in cell-free extracts of dwarf pea epicotyls. Addition of GA_3 (1µM) to ammonium sulphate fraction precipitate (30 - 50% satn.) showed no stimulation of RNA polymerase II *in vitro*. The enzyme fraction from basal segments of control epicotyls was then preincubated with GA_3 (1µM) for different durations of time at 4°C before the assay of enzyme activity. This approach gave exciting results. It seems that preincubation of enzyme fraction with GA_3 for at least 30 min is a prerequisite for

achieving regulation of RNA polymerase II *in vitro*. This then unequivocally proved that GA_3 indeed regulates RNA polymerase ll through the phenomenon of enzyme activation.

GA_3-mediated stimulation of RNA polymerase II activity under *in vivo* conditions was strongly inhibited by the simultaneous addition of ABA (10 **µ**M). It was worth investigating whether ABA could be effective in the negative regulation in our *in vitro* system. Thus, addition of ABA along with GA_3 *in vitro* proved to be equally effective in blocking the stimulatory effect of **GA_3**. It is therefore considered that RNA polymerase II can be activated by GA_3 and this response is counteracted by ABA in dwarf pea epicotyls.

With a view to ascertain the specificity of **GA_3**-mediated regulation of RNA polymerase II in dwarf pea epicotyls, the effect of the biologically inactive analogue of GA_3, namely GA_8 was tested for *in vitro* regulation of RNA polymerase II. The experimental outcomes have clearly shown that regulatory response of GA_3 is highly specific as no stimulation of RNA polymerase II was witnessed when GA_8 was present.

So far, the effect of GA_3 was tested at a dosage level of 10^{-6}M for demonstrating the *in vitro* activation of RNA polymerase II in dwarf pea epicotyls. However, addition of lower concentrations of GA_3 (10^{-8}. 10^{-15} M) proved equally effective in the *in vitro* activation of RNA polymerase II. To our knowledge, there is no other report in plant systems, where femtomole concentrations of GA_3 (10^{-15} M) could bring about the modulation of enzyme activity.

Briefly then, it has been possible to mimic the *in vivo* regulation of RNA polymerase II under *in vitro* conditions in dwarf pea epicotyls. Thus, dwarf pea epicotyl has proved to be an excellent system for demonstrating the post-translational regulation of RNA polymerase II. The fact that GA_3 is indeed crucial for the regulation of RNA polymerase II in pea, became further evident when the effect of GA_3 in dwarf and tall varieties of pea was compared. Whereas GA_3 promoted stimulation of RNA polymerase in dwarf pea epicotyls, there was no stimulatory response in tall pea epicotyls. This is not surprising in view of the fact that the tall pea plants have high levels of endogenous GAs as compared to that of dwarf pea. Moreover, the specific activity of RNA polymerase in control tall pea epicotyls (sp. act=8,015) was significantly higher than that observed in control dwarf pea epicotyls (sp. act. =3,573) Nevertheless, the specific activity of RNA polymerase II was quite comparable in control tall pea epicotyls and GA_3 treated dwarf pea epicotyls. In all likelihood high specific activity of RNA polymerase II in the tall variety is due to endogenous high levels of **GA_3**. Clearly, the **GA_3**-regulated RNA polymerase ll activity in dwarf pea epicotyls represents a physiological system, in view of observations with tall pea epicotyls.

Partially purified enzyme fraction (DAEA-Sephadex A-25 fraction) exhibited no stimulation of RNA polymerase II under *in vitro* conditions (in presence of GA3). Thus, it appears that GA_3 is not responsible for directly interacting with the enzyme molecule, but probably with some regulatory protein factor which gets eliminated during ion-exchange chromatography. Further studies would be desirable to isolate and characterize this regulatory protein factor for elucidating the phytohormonal regulation of RNA polymerase II at the molecular level. Nevertheless, this investigation has made a conceptual advancement over the existing knowledge

in dwarf pea epicotyls by establishing that RNA polymerase II is regulated at the post-translational step by **GA_3**. In addition, specificity of the phytohormonal response is explicit by the lack of stimulatory response by GA_8.

What could be the biological significance of the modulation of RNA polymerase by GA_3 in dwarf pea epicotyls? In this context, it may be stressed that in several plants, for example maize seedlings (Wasilewska and Kleczkowski, 1976), hazel seedlings (Shanon and Jarvis, 1982) and castor beans (Martin and Northcote, 1982), GA_3 promotes the relative abundance of poly $(A)^+$ RNA. Hence, higher levels of poly $(A)^+$ RNA have been reported in barley aleurone (Jacobsen and Zwar, 1974a) and wheat embryos Lakhani and Sachar, 1985). In particular, significant increase in the levels of translatable poly $(A)^+$ RNA was reported in GA3-incubated dwarf pea epicotyls (Chory et al., 1987). We postulate that the increased levels of poly $(A)^+$ RNA by GA_3 could be achieved through the regulation of poly (A) polymerase and RNA polymerase II, which are crucial in synthesis of poly $(A)^+$ RNA. The regulation of poly (A) polymerase by GA_3 has already been reported in wheat aleurones (Berry and Sachar, 1982), wheat embryos (Lakhani and Sachar, 1985) and dwarf pea epicotyls (Berry and Sachar, 1983). This RNA processing enzyme is responsible for the polyadenylation of mRNA fraction. Inhibition of poly (A) polymerase activity by cycloheximide and ABA in **GA_3**-treated tissue resulted in parallel reduction in the levels of poly$(A)^+$RNA.

Presently, it is suggested that in addition to the modulation of poly (A) polymerase, the post-translational regulation of RNA polymerase II by GA_3 could be responsible for the increased levels of pre-mRNA. However, it may be emphasized that GAs has a pleiotropic role in the regulation of gene expression. In addition to its role in post-translational regulation of RNA polymerase ll, there is ample evidence to show that this phytohormone controls the expression of many genes at the transcriptional level, possibly by interacting with the upstream sequences of α-amylase genes in rice aleurones (Ou-Lee et al., 1988). However, the molecular mechanism by which GA_3 operates at the transcriptional level is not well understood.

There are several reports which claim post-translational regulation of enzymes by **GA_3**. In barley aleurones, GA_3 was shown to be responsible for the activation of zymogen β-amylase by the proteolysis and disulphide bond cleavage of the dormant enzyme (Shinke and Mugibayashi, 1972). In embryo-less half-seeds of wheat, GA_3 treatment has been shown to stimulate the activity of monophenolase (Berry and Sachar, 1982) by influencing the pH optimum, thermostability, response to ionic strength, electrophoretic mobility and molecular properties. Enhancement in activation of acid phosphatase was demonstrated in GA_3 incubated barley endosperm (Bailey et al., 1976). However, the precise mechanism by which GA_3 activates the preformed enzyme molecules has not been studied in depth.

SUMMARY

In vivo spray application of GA_3 to light grown dwarf pea epicotyls (48 hr). brought about a 2.5-times increase in the amounts of poly (A) RNA over the untreated-controls. This was paralleled by a 2-fold enhancement of RNA polymerase II activities in dwarf pea epicotyls. An early response of GA_3 for the regulation of RNA polymerase II was observed in the basal segment of

pea epicotyls. The excised basal segments of light-grow dwarf pea epicotyls were immersed in a solution of GAs (10^{-6}M) for 2-8 hr. There was significant stimulation (about 1.8 - 2.5-times) of RNA polymerase II activity after incubation of epicotyls for 4 hr in GA_3. A similar response was also observed at lower concentrations of GA_3 (10° M). Abscisic acid (ABA) effectively counteracted the GA3-mediated stimulation of RNA polymerase II in dwarf pea epicotyls. Both cycloheximide (20 µg/ml) and six amino acid analogues (2 mM each) failed to repress the GAs-mediated stimulation of RNA polymerase II activity in dwarf pea epicotyls. This suggested that de novo protein synthesis was not a pre-requisite for the phytohormone mediated activation of RNA polymerase II in dwarf pea epicotyls.

Activation of RNA polymerase II (1.8 - 2.0-fold stimulation) has been achieved *in vitro* by the addition of GA_3 (10^{-6}M) to cell-free extracts, prepared from the basal part of pea epicotyls. This *in vitro* response of GA_3 on RNA polymerase II could be achieved only when the enzyme fraction was preincubated with GA_3 for a period of 30 min at 4°C.

The *in vitro* activation of RNA polymerase was also achieved with extremely low concentration of GA_3 (10^{-15} M). In contrast, the *in vivo* stimulation of RNA polymerase II required relatively high concentration of GA_3 (10^{-8} M). The *in vitro* activation of RNA polymerase II by GA_3 was a highly specific response, since GA_3 (10^{-10} M) a biologically Inactive analogue of GA_3, failed to enhance the activity of this enzyme. The *in vitro* stimulation of RNA polymerase II activity by GA_3 (10^{-6} M) was significantly inhibited (80%) by the concomitant addition of abscisic acid (ABA, 10^{-5} M). Cell-free extracts, prepared from tall pea epicotyls showed no enhancement of RNA polymerase II acclivity by the *in vitro* addition of GA_3. Further it was observed that the specific activity of ANA polymerase II in $(NH_4)_2SO_4$ fraction precipitate from tall pea epicotyls was comparable to the enzyme activity within GA_3-treated dwarf pea epicotyls. This suggested that high endogenous levels of GA_3 could be responsible for the lack of further stimulation of RNA polymerase II activity in tall pea. The ^{3}H-labeled reaction product of RNA polymerase ll was sensitive to RNase A treatment. Addition of low concentration of α-amanitin (0.05µg/ml) to the assay mixture of RNA polymerase II significantly decreased (68% inhibition) the inclusion of (H]-AMP into the TCA precipitable reaction byproduct both in untreated control and GA_3-treated pea epicotyls. The sensitivity of RNA polymerase II to the low concentration of this toxin suggested that GA_3 predominantly regulates the activity of RNA polymerase II in dwarf pea epicotyls. The *in vitro* stimulation of RNA polymerase II by GA_3 was not observed in the DEAE-Sephadex A-25 fraction. Conceivably, some factor which may be crucial for GA_3-mediated activation of RNA polymerase II was separated from the enzyme on ion-exchange chromatography on DEAE-Sephadex A-25. Thus the *in vivo* stimulation of RNA polymerase II by GA_3 in dwarf pea epicotyls could be mimicked *in vitro* by the addition of GA_3 to cell-free extracts of pea epicotyls. The relative abundance of poly $(A)^+$ RNA could be achieved via the phytohormonal regulation of poly (A) polymerase (Berry and Sachar,1983) and RNA polymerase II.

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