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The use of transcription inhibitors in the study of the mechanism of abscisic acid action in germinating triticale caryopses

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Abstract

The study was conducted on germinating triticale (var. Grado) caryopses. The purpose of the experiments was to compare the effect of selected inhibitors of transcription with the action of abscisic acid during germination of caryopses. The following inhibitors were used: α-amanitin, cordycepin, cycloheximide and 5-fluorouracil. Studied were the synthesis of total and polyribosomal RNA, the process of polyribosome formation and the synthesis of ribosomal proteins. The effect of exogenous ABA, especially in the early stages of germination, was not similar to any of the four above inhibitors of transcription. After 12 h of imbibition at a lowered temperature and 3 h of germination, ABA caused a relatively low level of inhibition of RNA synthesis, whereas all of the inhibitors used halted RNA synthesis in embryos by about 50–60%. After 6 h of germination, the same proportion of polyribosomes in the total ribosome fraction (46%) was found in both the embryos from the control sample and treated with ABA. The use of inhibitors brought this figure down to below 40%. The conclusion is drawn that in the early stages of germination, regulation of protein synthesis by ABA in triticale caryopses must occur on a level other than transcription.

Key words: *triticale, germination, transcription inhibitors, abscisic acid*

INTRODUCTION

The regulation of gene expression in eukaryotic organisms is studied by various methods. Very instrumental here are inhibitors which act on specific stages of the synthesis and processing of RNA and on other

levels of regulation of protein biosynthesis. For example, α-amanitin is a widely used specific inhibitor of mRNA synthesis in both animal and plant cells, which acts on RNA polymerase II (Fiume and Weiland 1970, Jacob et al. 1970, Beermann 1971, Brändle and Zetsche 1973, Jendrisak 1980, Galling 1982, Fernandez-Gomez et al. 1984).

Cordycepin is used with good effect to inhibit the synthesis of RNA in plant cells (Delseney et al. 1975). This compound acts by inhibiting the polyadenylation of mRNA (Payne et al. 1978, Rodriguez et al. 1982). In the embryos of germinating lettuce seeds, nearly 60% inhibition of incorporation of ^{3}H-adenosine into poly A-RNA was found, while in germinating wheat embryos, the incorporation of radioactive precursors into poly A-RNA was inhibited by cordycepin by 82–83% (Spiegel and Marcus 1975, Tao and Khan 1976).

Cycloheximide, in turn, selectively inhibits the synthesis of rRNA while not affecting the synthesis of poly(A)$^+$ (Delseny et al. 1977). Because cycloheximide blocks the cytoplasmic ribosomal mechanism in eukaryotic cells, this compound is widely used as an inhibitor of protein synthesis (Baliga et al. 1969).

One of the most commonly used inhibitors of RNA synthesis in prokaryotic and animal cells is actinomycin D (Franklin 1963, Reich and Golberg 1964, Perry and Kelley 1970, Shan-ching Sung 1972, Ernst and Oleinick 1977, Wilson and Jones 1981). However, there are still controversies about the effectivity of this antibiotic in plant cells (Fraser 1975, Aspart et al. 1979, Galling 1982, Olmedilla et al. 1985).

The effect of the antimetabolite, 5-fluorouracil, on the synthesis of DNA and RNA has been studied for many years (Heidelberger 1965, 1972, Mandel 1969). It has been determined that this compound is incorporated into all types of RNA. It inhibits the process of maturation of ribosomal RNA (Wilkinson and Pitot 1973) and the synthesis of DNA (Danenberg 1977). Bex (1972) found that in maize coleoptiles, 5-fluorouracil inhibits the incorporation of cytidine into mature cytoplasmic RNA, while the synthesis of the 2.2×10^6 D precursor molecule is not inhibited by it. Other authors (Ingle 1968, Lerbs and Wollgiehn 1975, Lerbs et al. 1980) have shown that 5-fluorouracil inhibits the formation of cytoplasmic rRNA more than of chloroplast rRNA.

The molecular nature of the physiological block through which abscisic acid (ABA) prevents the initiation of germination processes still remains unknown. It is generally assumed that ABA action lies in the disturbance in mRNA synthesis, post-transcriptional modification or translation (Walton 1977, 1980, Bewley and Black 1982, Ho 1984, Weidner 1986). The mechanism of action of compounds such as α-amanitin, cordycepin,

cycloheximide and 5-fluorouracil is well documented. The aim of this study was to compare the effects of the listed inhibitors with that of ABA during the germination of triticale caryopses in order to uncover the mechanism of ABA action in this process. The effects of ABA and inhibitors on the growth and germination capacity of embryos as well as on the synthesis of total and polyribosomal RNA, formation of polyribosomes and the synthesis of ribosomal proteins in the initial stages of germination of triticale caryopses were studied.

MATERIAL AND METHODS

The experiments were conducted on triticale caryopses of the Grado variety, supplied by the Plant Cultivation Station in Choryń. The caryopses were washed with tap water and placed for 3 min in a 1% solution of sodium hypochlorite. After sterilization, the caryopses were washed with sterilized water and their surfaces dried. The sterilized material was then subjected to 12 h of imbibition at 0°C. Imbibition in some samples was conducted in the presence of abscisic acid (ABA) and inhibitors of RNA synthesis. Abscisic acid was used at concentrations of 10^{-6} M, 10^{-5} M and 10^{-4} M, cordycepin, 5-fluorouracil (5-FU) and cycloheximide at a concentration of 10^{-4} M and α-amanitin at 1.2×10^{-5} M. The concentrations of the inhibitors used were taken from data in the literature cited in the introduction.

After preimbibition, the swollen caryopses were germinated in Petri dishes in the dark at a temperature of 21–22°C. Germination was conducted in the presence of chloramphenicol (10 μg cm^{-3}) and abscisic acid or the appropriate inhibitors of RNA synthesis. During germination, the following radioactive precursors were also used: 5-^{3}H-uridine, 8-^{3}H-adenosine and ^{14}C-hydrolysate of amino acids at a concentration of 0.8 MBq cm^{-3}. The radioactive compounds were purchased from Chemapol UVVVR — Czechoslovakia. After the assigned time of germination, embryos were isolated from the caryopses which had been cooled on ice, the remaining unincorporated compounds were rinsed from their surfaces, the surfaces dried and the embryos stored at −25°C for further analyses.

The total ribosome fraction (polyribosomes + monosomes + ribosome subunits) were isolated from the embryos according to the method previously described (Weidner 1984). For this purpose, about 2 g of the plant material were homogenized in buffer "A" (0.2 M sucrose, 200 mM Tris-HCl (pH 8.5), 30 mM $MgCl_2$, 60 mM KCl. The homogenate was centrifuged at 29000 × g in a Janetzki (K-70) centrifuge. The supernatant was transferred onto a 4 cm^3 layer of 1.5 M sucrose in buffer "B" (40 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 20 mM KCl) in 65 Ti rotor tubes

and centrifuged in a Spinco L-3-40 ultracentrifuge at 95000 × g for 90 min. The pellet (about 1 mg) was suspended in 1 cm^3 of buffer "B" and layered onto the surface of a sucrose concentration gradient. Polyribosomes were fractionated by centrifugation at 122 000 × g in a SW-41 rotor for 75 minutes. In order to determine the amount of ribosomes, it was accepted that the absorption of a 1% solution of ribosomes in a cuvette with a 1 cm optical path, at 260 nm, equals $E_{1\%}^{1cm} = 135$ (Gualerzi and Cammarano 1969).

Isolation and fractionation of total RNA was done according to the method of Tanifuji et al. (1970) as modified by Takaiwa and Tanifuji (1978). A sample of 200 embryos was homogenized in 10 cm^3 of buffer "A" of the following composition: 0.1 M Tris-HCl (pH 9.0), containing 0.1 M NaCl, 1% bentonite, 2% SDS and 100 μg cm^{-3} polyvinyl sulfate. An equal volume of a mixture of m-cresol-phenol-water (10:70:20, V/V/V) was added to the homogenate and 8-hydroxyquinoline was added to a final concentration of 0.1%. The suspension was agitated and after centrifugation at 5000 × g, the aqueous phase collected. Next, the aqueous phase was deproteinized three times with a mixture of chloroform-phenol, and ribonucleic acids were precipitated with cold ethanol. The RNA preparations were dissolved and ultracentrifuged in a linear sucrose concentration gradient (5–20%) containing 25 mM Tris-HCl (pH 9.0), 50 mM NaCl and 5 mM EDTA (ethylene diamine tetraacetic acid). The samples were centrifuged for 5 h at 196 000 × g in a Spinco L-3-40 centrifuge using a SW-41 rotor. After centrifugation, the contents of the tubes were separated into approx. 40 fractions and their absorption measured at 260 nm. RNA was precipitated by adding an equal volume of 10% trichloroacetic acid and collected on fiberglass filters.

Radioactivity measurements for both methods used were made with a Beckman LS-1801 scintillation counter.

All of the results presented in this paper are the mean values obtained from 3–5 independent experiments.

RESULTS

The effects of abscisic acid (ABA) and inhibitors of RNA synthesis on the increase of fresh weight and germination capacity of isolated embryos and on the germination capacity of whole, intact triticale caryopses are presented on Fig. 1. ABA, used in three concentrations (10^{-6}, 10^{-5} and 10^{-4} M) exerted a significant influence on the germination of the caryopses. This effect increased with rising concentrations of the phytohormone. As regards the effects of the inhibitors, at the concentrations

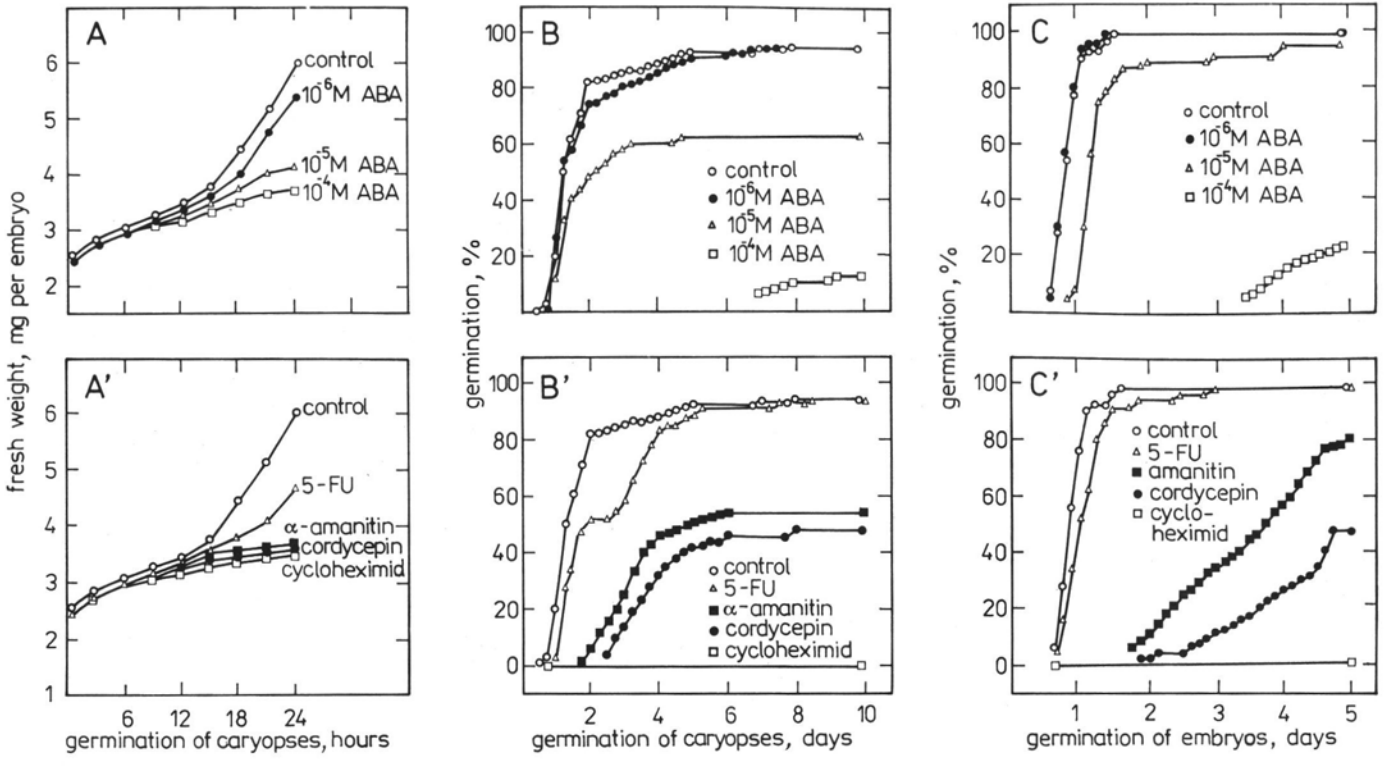

Fig. 1. The effect of abscisic acid (ABA) and inhibitors of RNA synthesis on: A, A′ — increase of fresh weight of embryos in the initial stages of germination, B, B′ — the germination capacity of whole caryopses, C, C′ — the germination capacity of isolated embryos. Germination of caryopses and isolated embryos was preceded each time by 12 h of inhibition of caryopses at 0°C, in the presence of ABA or inhibitors of RNA synthesis. All of the data on the figure are the mean values obtained in five independent experiments

used, cycloheximide had the decidedly greatest inhibitory effect, since it completely halted the germination of both isolated embryos and whole caryopses. Germination was also strongly inhibited by α-amanitin and cordycepin, but cordycepin was the stronger inhibitor, especially during germination of isolated embryos. Germination was inhibited the least by 5-fluorouracil (5-FU).

Changes in the synthesis of RNA under the influence of ABA and inhibitors are presented on Fig. 2. In the experiments on RNA synthesis after 3 h of germination of caryopses, it was not possible to determine the radioactivity in the vicinity of the 4–5S RNA peak, due to the difficulty in removing from the embryos all of the unmetabolized precursor and low molecular weight products of its metabolism which were positioned on top of the gradient, contaminating the tRNA fraction. Abscisic acid, regardless of its concentration, caused relatively low inhibition of rRNA synthesis (25+18 S) after 3 h of germination. It equalled, for concentrations 10^{-6}, 10^{-5}, 10^{-4} M, approx. 8, 16 and 24%, respectively, while all of the used inhibitors of RNA synthesis inhibited the incorporation of labeled uridine into rRNA during the first 3 h of germination by approx. 50–60%. Similar tendencies were observed during 6 h germination of triticale caryopses in the presence of ^{3}H-adenosine. The incorporation of labeled adenosine into polyribosomal RNA was significantly lower in the samples treated with transcription inhibitors than in the sample treated with ABA (Fig. 3, Table 1). After preimbibition and 6 h of germination of the caryopses, the same proportion of polyribosomes in the total ribosome fraction (46%) was found in control embryos and those treated with ABA. The inhibitors, however, reduced the amount of polyribosomes in the total ribosome fraction to values below 40%. After 6 h of germination, cordycepin exhibited the strongest inhibition of polyribosome formation (Fig. 3, Table 1).

In the 24 h germination experiment, cycloheximide proved to be the strongest inhibitor. It inhibited RNA synthesis by 42% (Fig. 2) and ribosomal protein synthesis by up to 76% (Table 2, Fig. 4). During this period, abscisic acid at 10^{-6} M reduced RNA synthesis by about 11%, at 10^{-5} M, by about 20% and at 10^{-4} M, by about 27%. Cordycepin and α-amanitin inhibited this process by about 10 and 14%, respectively, and 5-fluorouracil by about 34% (Fig. 2). It was also shown that ABA strongly inhibited the synthesis of ribosomal proteins (by about 50%) and that this inhibition was much greater than that by cordycepin (38%), α-amanitin (33%) and 5-fluorouracil (22%). Only cycloheximide had a stronger inhibitory effect than ABA. It is accepted that cycloheximide acts more at the translation level, inhibiting the synthesis of proteins with the participation of ribosomes. As results from the experiments, cycloheximide is already active at the stage of association of ribosomal subunits. The proportion of subunits in the total ribosome fraction after 24 h of germination reached 22%

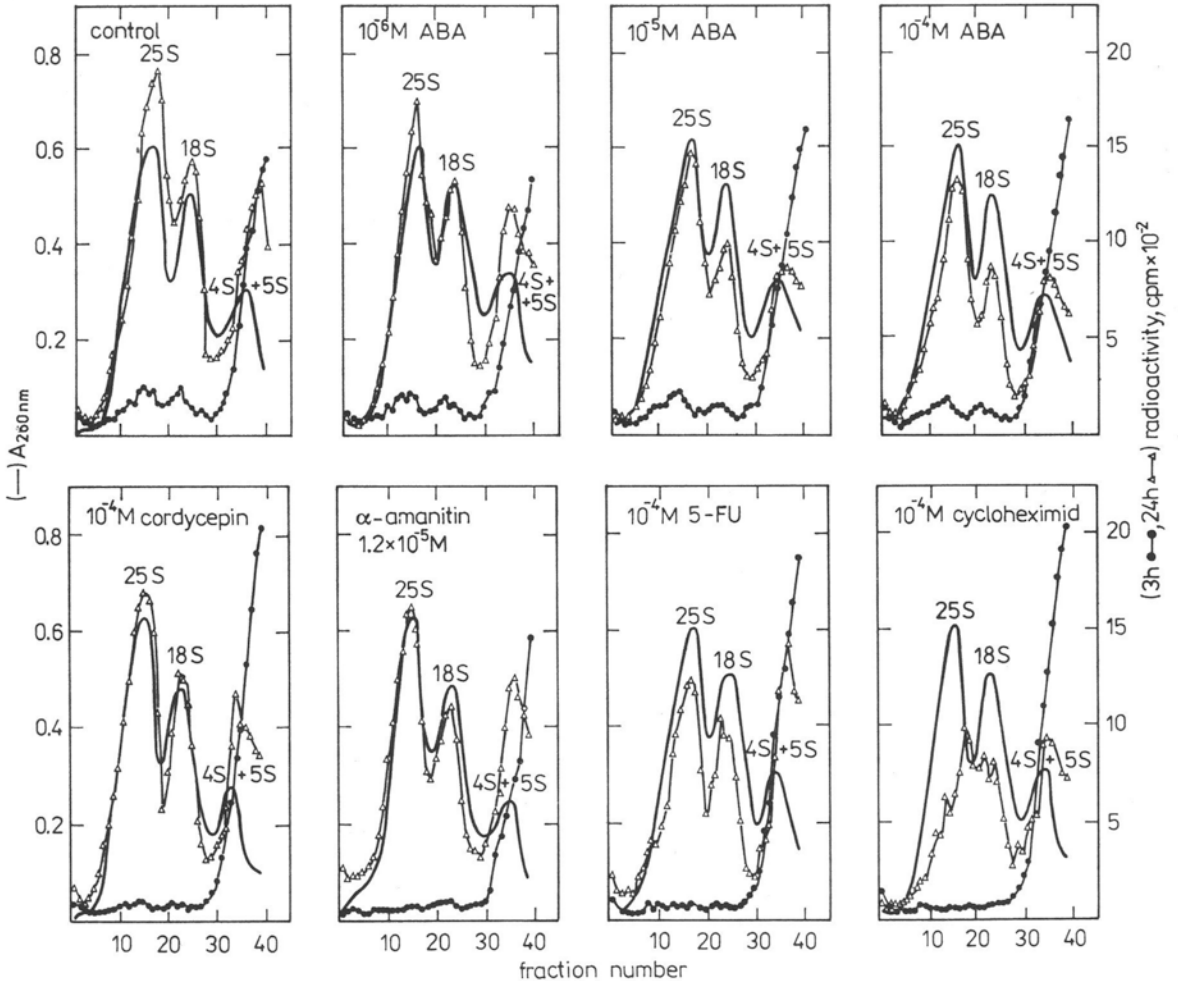

Fig. 2. Sedimentation profiles (in a 5–20% sucrose gradient) of total RNA isolated from triticale embryos. The embryos were subjected to preimbibition (12 h) and 3 or 24 h long germination. Preimbibition at a lowered temperature and germination were conducted in water (control) or in the presence of the appropriate concentrations of ABA and inhibitors of RNA synthesis. After the assigned period of germination, embryos were isolated (on ice) and used for isolation of total RNA. During germination, ^{3}H-uridine (0.8 MBq cm^{-3}) and chloramphenicol (10 μg cm^{-3}) were also used

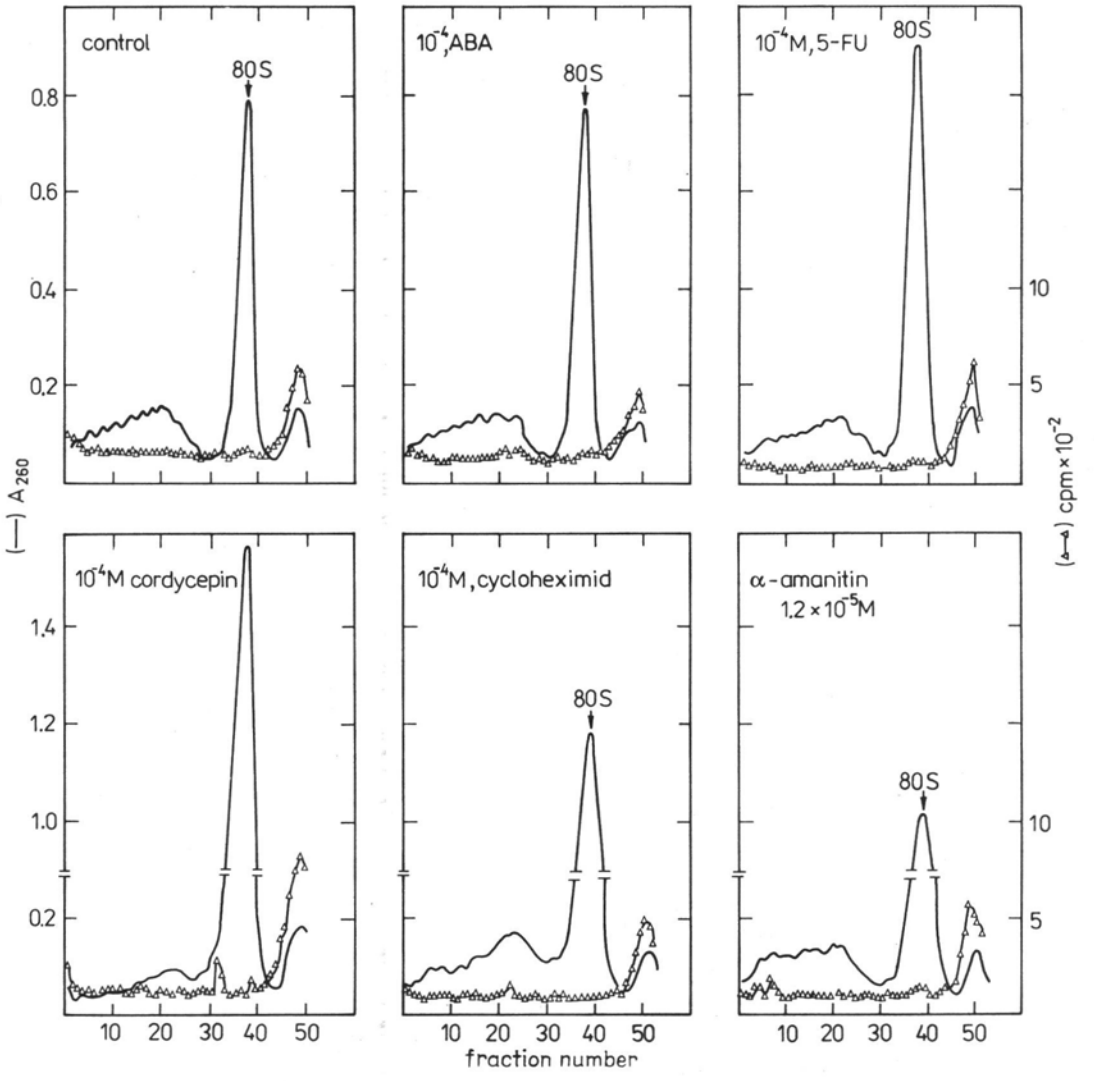

Fig. 3. Sedimentation profiles (in a 12.5–50% sucrose gradient) of polyribosomes isolated from triticale embryos. The caryopses were subjected to preimbibition (12 h) and 6 h germination. Preimbibition and germination were conducted in water (control) and in the presence of ABA and inhibitors of RNA synthesis. After the assigned period of germination, embryos were isolated from the caryopses and used for isolating the total ribosome fraction. During germination, ^{3}H-adenosine (0.8 MBq cm^{-3}) and chloramphenicol (10 μg cm^{-3}) were also used

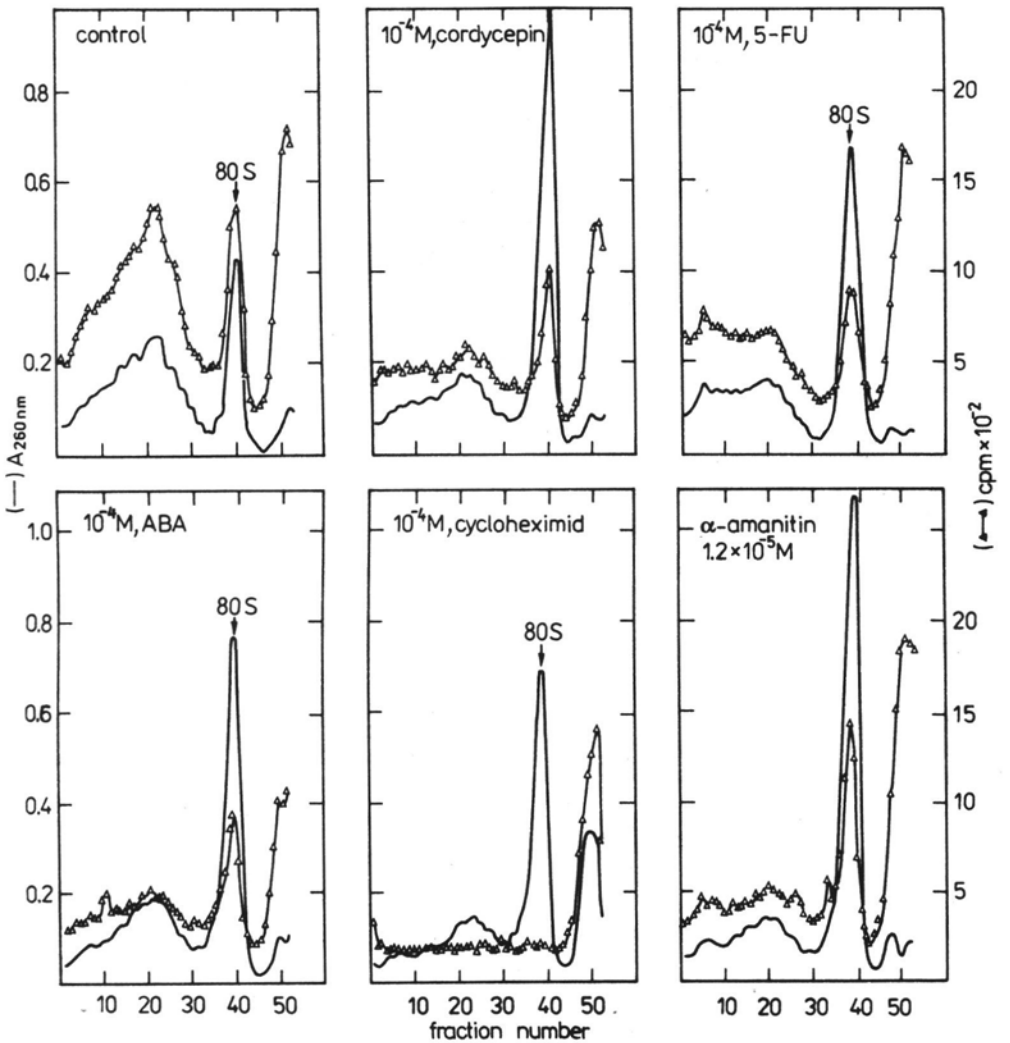

Fig. 4. Sedimentation profiles (in 12.5–50% sucrose gradients) of polyribosomes isolated from triticale embryos. The caryopses were subjected to preimbibition (12 h) and 24 h germination. Preimbibition and germination were conducted in water (control) and in the presence of ABA and inhibitors of RNA synthesis. After the assigned period of germination, embryos were isolated from the caryopses and used to obtain the total ribosome fraction. During germination, ^{14}C-hydrolysate of amino acids (0.8 MBq cm^{-3}) and chloramphenicol (10 μg cm^{-3}) were also used

in the sample treated with cycloheximide, while in the control sample was only about 5% (Table 2). The remaining inhibitors of RNA synthesis did not show any activity at this stage of protein biosynthesis. However, all of the studied compounds greatly lowered the proportion of polyribosomes in the total ribosome fraction (Fig. 4, Table 2) after 24 h of germination

Table 1

The effect of abscisic acid (ABA) and inhibitors of transcription on polyribosome formation in embryos and on the incorporation of ^{3}H-adenosine into embryo polyribosomal RNA during 6 h long germination of triticale caryopses

Treatment	cpm 1 mg^{-1} of the total ribosome fraction	Inhibition %	Percentage of ribosomal fractions		
			polyribosomes	monosomes	ribosomal subunits
Control	7262	–	46	43	11
ABA, 10^{-4} M	6511	10	46	44	10
Cordycepin, 10^{-4} M	5555	24	19	71	10
α-Amanitin, 1.2×10^{-5} M	5132	29	39	53	8
5-Fluorouracil, 10^{-4} M	5260	28	39	51	10
Cycloheximide, 10^{-4} M	4035	44	35	57	8

Table 2

The effect of abscisic acid (ABA) and inhibitors of transcription on polyribosome formation in embryos and on the incorporation of ^{14}C-hydrolysate of amino acids into ribosomal proteins of embryos during 24 h long germination of triticale caryopses

Treatment	cpm 1 mg^{-1} of the total ribosome fraction	Inhibition %	Percentage of ribosomal fractions		
			polyribosomes	monosomes	ribosomal subunits
Control	58430	–	70	25	5
ABA, 10^{-4} M	29061	50	47	47	6
Cordycepin, 10^{-4} M	36041	38	45	49	6
α-Amanitin, 1.2×10^{-5} M	39023	33	36	57	7
5-Fluorouracil, 10^{-4} M	45796	22	50	44	6
Cycloheximide, 10^{-4} M	13812	76	37	41	22

of the caryopses, which indicates that both ABA and the inhibitors reduced the intensity of protein synthesis. From the studies of Nowak et al. (1984), it is concluded that the proportion of polyribosomes in the total ribosome fraction (polyribosomes + monoribosomes + ribosomal subunits) is an unfailing indicator of the intensity of protein biosynthesis under various experimental conditions.

DISCUSSION

During imbibition, the effect of ABA was already observable as the lower uptake of water by the swelling embryos (Fig. 1). Schopfer et al. (1979) found that ABA reversibly halts the development of the embryo

during germination by inhibiting the uptake of water accompanying the growth of the embryo. In the opinion of these authors, the main effect of ABA in the inhibition of germination depends rather on the regulation of the uptake of water by tissues than on the regulation of gene expression. It should, however, be added that the differences observed by us in the uptake of water by imbibing embryos treated and not treated with ABA were relatively small and similar to the changes in the uptake of water evoked by the inhibitors of transcription used in this study.

As support for the thesis that ABA acts on the biosynthesis of nucleic acids, the results of experiments on the reversal of ABA-induced inhibition by growth stimulators, are often cited. In such experiments, the reversal of inhibition by cytokinins and gibberellins during the germination of pear embryos and elongation of bean axes, was related to an increase in RNA synthesis (Khan and Heit 1969, Sussex et al. 1975). In earlier studies (Weidner et al. 1984) on isolated embryos and whole triticale caryopses, a similar relationship was not observed.

In the initial stages of germination of whole, intact caryopses, exogenous ABA caused only slight inhibition of RNA synthesis, while all of the inhibitors used in this study caused high, 50–60% inhibition of incorporation of labeled uridine into rRNA. Germination was always preceded by 12 h of imbibition at a lowered temperature in the presence of the studied compound in order to eliminate the eventual effect of slow penetration of some of the compounds through the plant tissues in the early stages of germination. This problem does not concern ABA since examples are known of uncommonly rapid reactions of plants to exogenous ABA (Ho 1984). This suggests that at least some of these processes are independent of nucleic acid-directed protein synthesis.

In a previous paper (Weidner et al. 1984), on the early stages of germination of isolated embryos, treating them with ABA did not cause any changes in the synthesis of RNA. However, a fall in the proportion of polyribosomes in the total ribosome fraction was found. In this paper, where the experiments were conducted on whole, intact caryopses, during the first six or even ten hours (unpublished results), no significant effect of ABA on the process of polyribosome formation was observed. It seems that the reasons for this lack of agreement should be sought in the disruption of the tie between the endosperm and embryo and in the mechanical damage of the seed coat during isolation, and the resulting from this, differences in the metabolic intensity of nucleic compounds in the whole seed and its its isolated parts (Weidner 1981). Grzelczak and Buchowicz (1977) have also found that even the cutting off of the embryo stimulates transcription. They conclude that the biochemical processes

which occur during the germination of isolated embryos cannot be extended to embryos germinating in whole, intact seeds.

Further experiments were conducted after 24 h of germination of the caryopses, and here too the effect of ABA on all of the indicators of the intensity of embryo growth was distinct. Abscisic acid at a concentration of 10^{-4} M inhibited the growth of the embryos by about 64%, the synthesis of ribosomal proteins by about 50%, the formation of polyribosomes by about 33% and the synthesis of RNA by about 27%. The mentioned lack of the influence of ABA on the formation of polyribosomes in embryos and, as can also be judged, also on protein biosynthesis in the early stages of germination of triticale caryopses finds confirmation in physiological studies on other seeds. Karsen (1976), in studies on *Chenopodium album*, divided the process of germination into three stages. He found that ABA inhibits only the third stage of germination in which the radicle disrupts all of the seed coats. Studies done on lettuce seeds, *Sinapis alba* and *Haplopappus gracilis* also suggest that ABA acts at a later stage (Bewley and Black 1982).

In summary, it should be stated that the effect of ABA (especially in the initial period of germination) is not similar to the effect of any of the four transcription inhibitors used in this study. These results are supported by observations on the regulation of physiological processes by ABA which are not sensitive to inhibitors of transcription, as well as the determined fact that ribosomes isolated from wheat embryos treated with ABA less actively synthesize protein than control ribosomes (Ho 1984). Thus, it results from this that the regulation of protein biosynthesis in germinating cereal grains controlled by ABA must take place on a level other than transcription.

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Zastosowanie inhibitorów transkrypcji do badań mechanizmu działania kwasu abscyzynowego w kiełkujących ziarniakach triticale

Streszczenie

Badania przeprowadzono na kiełkujących ziarniakach triticale odmiany Grado. Celem pracy było porównanie działania wybranych inhibitorów transkrypcji z działaniem kwasu abscyzynowego w procesie kiełkowania ziarniaków. Zastosowano następujące inhibitory: α-amanitynę, kordycepinę, cykloheksimid, 5-fluorouracyl. Badania obejmowały syntezę ogólnego i polirybosomalnego RNA, proces formowania się polirybosomów oraz syntezę białek rybosomalnych. Działanie egzogennego ABA, szczególnie w początkowym okresie kiełkowania, nie jest podobne do działania żadnego z czterech zastosowanych inhibitorów transkrypcji. Kwas abscyzynowy powodował, po 12 godz. imbibicji w obniżonej temperaturze i 3 godz. kiełkowania, stosunkowo niewielką inhibicję syntezy RNA. Wszystkie stosowane inhibitory syntezy RNA natomiast hamowały syntezę RNA w zarodkach o ok. 50–60%. Po 6 godz. kiełkowania stwierdzono w zarodkach ziarna próby kontrolnej i traktowanej ABA taki sam udział polirybosomów w ogólnej frakcji rybosomalnej (46%), a zastosowanie inhibitorów zmniejszało udział polirybosomów w ogólnej puli rybosomów do wartości mniejszych niż 40%. Wyciągnięto wniosek, że w początkowym okresie kiełkowania regulacja biosyntezy białka przez ABA w zarodkach ziarników triticale musi przebiegać na innym poziomie niż poziom transkrypcji.