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An unusual minor protein appearing in embryonic axis cells of haricot bean seeds following germination process stimulated by 6-methylthiouracil

Victoria A. Tsygankova, Vladimir N. Zayetz, Larisa A. Galkina[1], Ludmila P. Prikazchikova[1], Yaroslav B. Blume

Institute of Cell Biology and Genetic Engineering, National Academy of Sciences of Ukraine
Acad. Zabolotny str., 148, Kyiv, 252143
[1] Institute of Bioorganic Chemistry and Petrochemistry, National Academy of Sciences of Ukraine
Murmanska str., 1, Kyiv, 252600

Using the two-dimensional polyacrylamide gel electrophoresis approach, an unusual ≈ 30 kDa protein was found in embryonic axis cells of haricot bean seeds following seed germination process stimulated by 6-methylthiouracil. No similar protein was found both in control and lutidine N-oxide stimulated seeds. The synthesis of an additional low molecular weight protein was also detected in a cell-free system prepared from rabbit reticulocytes in the presence of poly(A)$^+$RNA isolated from 6-methylthiouracil stimulated embryonic axes of haricot been seeds. At the same time the lutidine N-oxide was found to stimulate drastically the total polypeptide synthesis in an in vitro system prepared from wheat embryo in the presence of a «standard» poly(A)$^+$RNA preparation, no similar effect of the 6-methylthiouracil having been seen. The ratio of informosomes, free and incorporated into polyribosomes, was investigated following RNP-particles fractionation in a preformed CsCl gradient; the 6-methylthiouracil seed stimulation was shown to induce the development of an additional peak of synthetically active informosomes, their buoyant density being 1.46 g/cm^3. The 6-methylthiouracil stimulated seed germination causes a significant shortening of haricot plant ontogenesis period without any harmful changes of plant phenotype, the lutidine N-oxide stimulation leads, however, to deformed accelerated vegetative organ appearance accompanied by no reproductive organ development. Nature of 30 kDa protein as well as some problems concerning the correlation between different stimulator-induced cellular gene expression changes taking place during early postembryogenesis and further processes of haricot bean plant growth and development are discussed; some possible practical consequences of our experiments are also mentioned.

Introduction. Due to vast biological screening we have earlier shown [1] such substances as lutidine N-oxide (LNO, ivin-yan) and 6-methylthiouracil (methyur, 6-MTU) accelerate sharply haricot bean seed germination. Our conviction is an embryonic axis of haricot bean plant (morpho-physiological and biochemical characteristics of embryonic axis set, formation and germination, its growth as well as development during embryogenesis and early postembryogenesis, i. e. during haricot bean seeds maturation and germination were studied in detail [2—4]) to be a suitable model permitting to investigate several actual problems of plant physiology and biotechnology, namely:

(*i*) to answer some questions concerning the mechanism of plant growth stimulator effect (these compounds are strongly different from natural plant hormones in their chemical structure), to understand if synthetic stimulators act through the cellular receptor systems similarly to plant hormones or interact directly with some target molecules on the level of cellular regulatory system (i. e. at the level of genetic control or phytohormonal regulation of plant growth and morphogenesis);

(*ii*) to understand if the artificially stimulated

plant growth causes any harmful changes affecting the sequence of normal morphogenetical events during the early postembryogenesis (mature fully differentiated organs from primary non-differentiated embryonic axis organs — root, hypocotyl and leaf, beginning to form in this period) and if any drastic growth stimulation realized during the first developmental stages is able to impair the balanced growth and development of plant vegetative and reproductive organs during further plant ontogenesis stages (for example, acceleration of vegetative organ growth and inhibition set as well as formation of plant reproductive organs).

To solve such problems as fully as possible we decided to get at first two principal goals:

(*i*) to understand the mechanisms concerning the effect of two synthetic growth stimulators — 6-MTU and LNO changing cellular gene expression in embryonic axis cells at early postembryogenesis stage and programming plant growth and development;

(*ii*) to study the consequences of artificial haricot seed germination on following plant vegetation.

Materials and Methods. *Seeds.* In our experiments seeds of haricot beans (*Phaseolus vulgaris L.*) of the variety «Bielozernaya» were used. Ethanol-sterilized seeds were put for germination in a thermostat at 26 °C; they were incubated between layers of filter paper moistened by distilled water or by 2 % solutions containing plant growth regulators. After the seed incubation, embryonic axes were separated from cotyledons, washed by distilled water and divided into three parts (each containing 100 axes) aimed for isolation of proteins, RNA, and RNP-particles.

Protein extraction. The tissue samples of embryonic axes were frozen in liquid nitrogen and powdered carefully in a china mortar. 10 volumes of preliminary cooled to 0 °C extracting buffer consisting of 30 mM tris-HCl, pH 8.7, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (disodium salt), 1 mM ascorbic acid, 5 mM $MgCl_2$, and 10 mg polyvinylpyrrolidone were added to the triturated material. The extract obtained was twice run (during 10 min and 15 min) in a centrifuge at 35.000 *g*. The 3/4 v acidified acetone (pH 4.5), containing 0.07 % 2-mercaptoethanol was added to supernatant fluid. This mixture was strongly shaken and incubated at −20 °C during 1 h. The denatured proteins were then pelleted by centrifugation at 35.000 *g* during 10 min. The pellets obtained were dried using a vacuum evaporator and dissolved in a buffer containing (50 μl per 1 mg of dry precipitate): 8 M urea, 5 mM K_2CO_3, 0.5 % DTT, 2 % amphollynes solution — carrier ampholytes, their pH range being 3.5—9.5, 2 % Triton X-100 (MKSD-buffer). The soluble protein extract was separated from insoluble material by probes centrifugation (3 min at 15.000 *g*) and then kept at −20 °C until use.

Two-dimensional gel electrophoresis of proteins was realized according to the method described by O'Farrell [5] with several modifications. The protein fractionation in the first direction was made in a 5 % polyacrylamide laminar gel (C = 4 % w/w). The gel was polymerized in a 8 M urea solution supplemented by 2 % Triton X-100 and 2 % ampholines mixture (containing pharmalytes with pH ranges 3.5—9.5 and 5.0—7.0, their ratio being 3:1) to stabilize the central pH gradient zone. The gel was 1 mm wide, its size being 140 × 80 mm. 0.02 M NaOH was used as a cathode electrode solution, and 0,01 M H_3PO_4 was taken as an anode one. The gel pre-focusing was made at 400 V during 30 min. The protein electrophoresis was realized during 2 h at 1200 V in a cooled chamber. A gel strip with marker proteins was cut out and stained for marking gel lanes, all other ones containing protein fractions were polymerized in a polyacrylamide gel with a 5—15 % concentration gradient to fractionate the proteins in the second direction realized in a buffer system described by Laemmli [6]. This gel was 1.5 mm wide, its size being 145 × 145 mm. As an electrode buffer a tris-glycine solution (pH 8.3) supplemented with SDS was used. The proteins entered to the concentrating gel at 70 V during 1 h, the protein fractionation in a separating gel was run during 6 h at 160 V. Following separation the gel strips containing protein fractions were fixed by a mixture containing 30 % isopropanol and 20 % acetic acid and stained by a Coomassie brilliant blue solution. The stain excess was washed out by a mixture containing 5 % methanol and 7 % acetic acid. The gels stained were then photographed on a glass plate lit from the opposite side.

The isolation of total RNA preparations was realized after embryonic axis tissue destruction using a buffer solution (buffer I) containing 0.05 M tris-HCl, pH 7.6, 0.01 M $MgCl_2$, 0.06 M KCl, 1 % SDS, and 4 M guanidine isothiocyanate. The lysate obtained was twice treated by a mixture of hot water-saturated phenol and chloroform; the RNA was precipitated from a water phase by ethanol, treated by proteinase K, deproteinized again by the same phenol-chloroform mixture and precipitated by ethanol. Some polysaccharide contaminants present in RNA preparations (preventing fractionation molecular RNA and manifestation biological activity mRNA *in vitro*) were extracted by methoxyethanol; the RNA molecules were then precipitated by cetylthrimethylammonium bromide; this last reagent was eliminated by multiple re-dissolving of RNA preparations in a

0.01 M sodium acetate solution followed by sodium acetate saturated ethanol precipitation.

Poly(A)$^+$RNA separation from poly(A)$^-$RNA molecules was made using total RNA chromatography on the oligo(dT)-cellulose columns [7]. Total RNA preparations were analysed using electrophoresis in a 1.5 % agarose gel containing 7 M urea according to Locker [8]; the gels obtained had been saturated by ethidium bromide solution before RNA fractions photographing using an UV lamp. The poly(A)$^+$RNA was analysed using a Northern-blot approach [9]. cDNA was synthesized on poly(A)$^+$RNA template according to the protocol described by Buell et al. [10] using reverse transcriptase (revertase) and [α-^{32}P]-containing deoxy-CTP as a label. A poly(A)$^+$RNA preparation was fractionated by electrophoresis in an agarose gel in the presence of formalyn; electrophoretically pure poly(A)$^+$RNA fractions were transferred on nitrocellulose filters and hybridized with cDNA. The hybridization mixture contained 50 % formamide, 5X Upper buffer (pH 7.0), 5X Denhardt's solution [11], denatured calf thymus DNA (100 μg/ml), poly(A)$^+$RNA (1 μg/ml), 0.1 % SDS, and [α-^{32}P]-cDNA ($1.5 \cdot 10^8$ cpm). Following hybridization, filters were carefully washed from exogenous label and exposed during 24 h to a film PM-1 with an accelerating screen (at −70 °C). The limits of poly(A)$^+$RNA sizes were evaluated according to radioautograph distribution of labelled poly(A)$^+$RNA-cDNA hybrids along the gel lanes (with regard to marker polynucleotides).

The experiments with poly(A)$^-$RNA translation were carried out in cell-free systems derived from wheat embryos [12] for quantitative determination incorporation of radioactive label into polypeptide material and rabbit reticulocytes [9] for definition fractional composition of full-length new synthesizing polypeptides, using [^{35}S]-methionine as a labelling compound. The polypeptides synthesized *de novo* in this last *in vitro* system were analyzed using the one-dimensional PAGE approach as mentioned above. The gels obtained were dried using a heating vacuum-dryer («LKB», Sweden). The gel fluorography was made using a protocol described in [13], the gels having been saturated by a fluorescent reagent, 2,5-diphenyloxazole (PPO) [14].

Radioactive labelling of RNP-particles. The embryonic axis samples (100 axes) were thoroughly washed by distilled water and incubated in a [^{3}H]-uridine solution ($3.7 \cdot 10^6$ Bq/ml) containing also penicillin and streptomycin (50 μg/ml) to prevent the label incorporation by contaminating bacterial cells present in incubation mixture. The material was incubated during 1 h in a thermostat at 28 °C in a Petri dish and then washed by distilled water to remove all the non-incorporated label.

Isolation of labelled cytoplasmic RNP-particles. A sample of embryonic axis tissue was homogenized in the buffer II (containing 20 mM TEA-HCl, pH 7.6, 25 mM KCl, 3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 250 mM sucrose, heparin (100 μg/ml) as an RNAse inhibitor, phenylmethylsulfonyl fluoride (50 μg/ml) as a trypsin-like protease inhibitor, and cycloheximide (100 μg/ml) as an inhibitor of mRNA translocation on cytoplasmic ribosomes, the corellation weighed tissue sample to volume of this buffer being 1:10. The homogenate obtained was then filtered and run at 18.000 *g* (20 min). A cytoplasmic post-mitochondrial fraction was incubated with a 0.5 % Triton X-100 solution (20 min), layered on 0.5 ml 0.5 M sucrose, prepared on the buffer II solution containing no sucrose and centrifugated in a SW 60 Ti rotor (Beckman centrifuge L5-50) (3 h at 49.000 rpm).

Investigation of RNP-particles in a CsCl density gradient. A pellet of cytoplasmic RNP-particles was suspended in the sucrose-free buffer II and divided by two; a half of this suspension was a control sample, its second portion was treated by a 15 mM EDTA-Na_2 solution to cause polyribosome dissociation. Both preparations containing RNP-particles were then fixed (during 24 h) in a 4 % formaldehyde solution, all the aggregates formed being removed by pelleting at 10.000 *g* (10 min). The soluble material was then centrifuged in a preformed CsCl density gradient (1.33—1.65 g/cm^3) (12 h, 45.000 rpm, 2 °C) using the same SW 60 Ti rotor.

The fractions of this soluble material were then obtained using a siphon device; each fraction refractive index was determined and compared to density values of a calibration curve. The fraction optical density was taken in a spectrophotometer at 260 nm. Buoyant density (ρ) of RNP-particles in CsCl was calculated according to an equation given in [15]:

$$\rho = D \cdot 10.8601 - 13.4979;$$

D is a refractive index, the values 10.8601 and 13.4979 are experimentally found corrections necessary because of polarizing buffer properties.

Radioactivity of proteins and RNP fractions was determined in a LS 100C scintillation counter («Beckman») using Millipore AP-15 fibrous glass filters and scintillator dissolved in toluene.

Results and Discussion. While elaborating our approaches concerning mechanisms of action some synthetic compounds regulating the plant growth we took into account a lot of data suggesting the gene expression changes caused by phytohormones to

belong to the principal mechanisms of plant growth and development [16—18] (however, the question concerning regulation expression of concrete genes by each from phytohormones are not yet correctly answerable). We proposed that the synthetic stimulators of seed germination (similarly to natural plant hormones) increasing drastically the embryonic organism size and its organ development, cannot mediate their action without some expression changes of genes coding synthesis of structural and functional proteins; the increasing of the plant mass (by cell enlargement and cell division) being impossible without intensive protein synthesis switching on a lot of reactions leading to cell and organ differentiation and specialization.

According to [19], the protein «spectrum» in any cell is to be changed during cell differentiation and specialisation, the protein synthesis process being a stage- and organospecific one. We proposed the protein assortment caused by any stimulated embryo growth (in view of accelerated development stages) is to be different from the assortment appeared in normally developed embryos during the same period; this difference was proposed to be nearly the same as between embryo protein «spectrum» determined at an early and at a later postembryonic stages during natural plant development.

The main protein mass in eucaryotic cells is known to be presented by structural proteins and by enzymes of the main metabolic pathway (named also multicopied, constitutive, abundant or major proteins), these compounds being present at all developmental stages and detected without any difficulty using any one-dimensional gel electrophoresis approach. However, this approach does not permit to find «rare» (so-called minor) stage- and organospecific enzymes and regulatory proteins, presented in some few copies and «hidden» on «one-dimensional» electrophoregrams by major protein bands. So we fractionated a total protein preparation isolated from embryonic axes using a two-dimensional polyacrylamide gel electrophoresis (TD-PAGE) protocol.

The stained gel photographs obtained after our TD-PAGE experiment are present in the Fig. 1 (*a*, *b*, *c*), the total protein preparations having been previously focused following the first one-dimensional separation according to their isoelectric point (pI) in a pH-gradient gel.

Our results demonstrate the absence of any differences in embryonic axis protein preparations of haricot seeds after 12 h of postembryonic development, both normal and LNO-stimulated (Fig. 1, *a* and *b*). Simultaneously, the 6-MTU-stimulated haricot bean germination causes the appearing of a

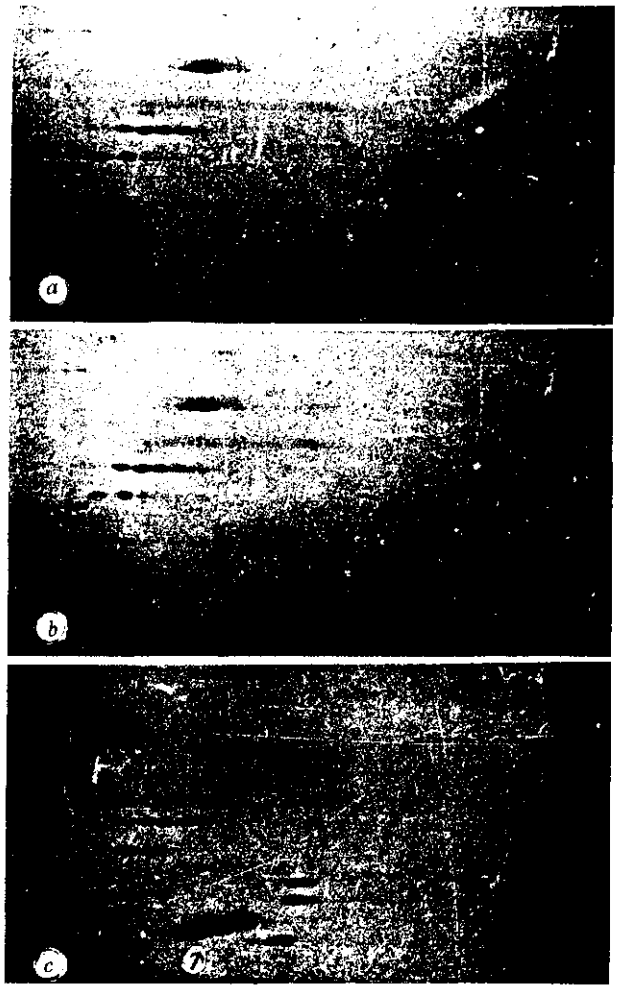

Fig. 1. Two-dimensional polyacrylamide gel electrophoresis of toplasmic proteins isolated from embryonic axis cells in 12 h post beginning of haricot bean seed germination; the proteins investiga are taken from: *a* — control (non-stimulated) seeds; *b* — LN stimulated seeds; *c* — 6-MTU-stimulated seeds

«blurred-bordered» spot formed by an about 30 kl protein in a zone containing positively charged pr teins (Fig. 1, *c*). This spot (although of decreas intensity) is also seen in photographs obtained fr TD-PAGE after minimal quantities of total prot (4 μg per lane) having been loaded into gel pocket

To determine this protein belonging to major minor ones, we realized in addition an experime with a one-dimensional PAGE approach in the pr sence of the SDS, total proteins of embryonic ax after 12 h of postembryonic development having be loaded on the gel. However, in this experiment failed to detect any separate band of the 30 kl

protein or to find any staining band differences between control and 6-MTU-stimulated protein samples. So we concluded this protein to be a minor one, its blurred spot in the TD-PAGE permits to suppose it to be also a short-lived compound. This 30 kDa protein was not detected in embryonic axis preparations in 24 h after the beginning of non-stimulated germination process, the embryonic axis sizes being the same as their sizes in 6-MTU-stimulated seeds in 12 h of postembryonic development (no photograph is presented).

The 30 kDa protein detected by the TD-PAGE approach appears only as a result of the 6-MTU-stimulated seed germination, no its traces having been found both in control and LNO-stimulated samples; our aim was to gain some data explaining such results. Some physiological consequences of seed treatment by LNO and 6-MTU are similar, the germination periods becoming twice shorter (and finishing in 2 days) with both stimulators. However, a lot of intracellular events followed by these substances treatment are to be quite different. There are some facts confirming this point of view. The LNO-treatment stimulates the total cellular protein synthesis proved in our one-dimensional electrophoresis experiment using a [^{35}S]-methionine labelled *in vivo* total proteins isolated from embryonic axes and also following a gel fluorography approach [20]. At the same time, the 6-MTU, according to our data, stimulates the only unusual 30 kDa protein synthesis.

To explain such a marked difference concerning the effect of two growth stimulating compounds, we suppose they action through different mechanisms influencing on certain stages of gene expression (transcription, formation of synthetically active RNP complexes, mRNA translation, etc.). The LNO was already proved to possess no action specificity on the level of gene expression regulation [20—22] activating both transcription and active RNP formation and increasing the synthesis of all the cellular proteins without changing their assortment.

We thought the appearance of the 30 kDa protein «non-typical» for a given developmental stage of embryonic axis cells might have been a result of some simultaneous changes at the translation level or at any precursor stage of the gene expression regulation (during transcription and/or transcript maturation levels). To answer this question, we realized some experiments concerning:

1) the comparative investigation of the LNO and 6-MTU effect on the translation process in an *in vitro* system of protein synthesis using a template of poly(A)$^+$RNA as standard isolated from non-stimulated embryonic axis cells;

2) the study of biological activities of poly(A)$^+$RNA preparations isolated from control embryonic axes as well as from those ones stimulated and non-stimulated by LNO and 6-MTU using the same *in vitro* system of protein synthesis;

3) the evaluation of activity protein-synthesizing apparatus (polyribosomes) *in vivo*, i. a. proportion of free and incorporated into polyribosomes (^{3}H-uridine labelling *in vivo*) of mRNP and rRNP-particles in embryo axis haricot bean seeds germinated without any treatment and following LNO and 6-MTU stimulation.

In the Fig. 2 (*a*, *b*) the results of summary embryonic axes RNA preparations (using agarose gel electrophoresis) obtained with control and 6-MTU-stimulated material; these data demonstrate the RNA preparations to contain non-degraded discrete fractions of both high and low molecular masses (hnRNA including preRNAs, preRNAs «waste products» originated due to processing rRNAs, mRNAs and tRNAs,

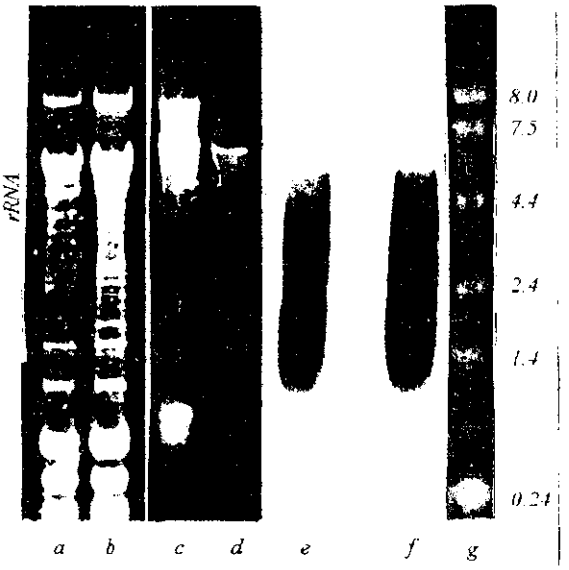

Fig. 2. Agarose gel electrophoresis of RNA preparations isolated from embryonic cell axes in 12 h post the beginning of haricot bean seed germination; *a* and *b* — total RNA preparations from control and 6-MTU-stimulated seeds, respectively; *c* and *d* — poly(A)$^-$RNA and poly(A)$^+$RNA preparations, respectively, separated on an oligo(dT)-cellulose column; *e* and *f* — radioautographs of hybrid molecules containing [^{32}P]-cDNA and poly(A)$^+$RNA fractions immobilized on nitrocellulose filter: poly(A)$^+$RNA and [^{32}P] cDNA isolated from embryonic axis cells of non-stimulated (*e*) and 6-MTU stimulated (*f*) haricot bean seeds, respectively; *g* — electrophoretic distribution of marker polynucleotides on a parallel gel lane

«mature» forms these RNAs of nuclear and cytoplasm, regulatory RNA molecules). The ratios E_{260}/E_{280} and E_{260}/E_{230} for our preparations were ≥ 2.0 and ≥ 2.3, respectively, confirming a good degree of isolated RNA purification being practically free from protein and polysaccharide contaminations. We present also our electrophoregrams of a poly(A)$^-$RNA preparation (Fig. 2, *c*) and of a poly(A)$^+$RNA one (Fig. 2, *d*) demonstrating the rRNA to be practically absent.

In the Fig. 2 (*e, f*) our radioautographs are given obtained as a result of a Northern blot-hybridization procedure of poly(A)$^-$RNA fractions with a [^{32}P]-cDNA preparation; our results demonstrate these fraction to contain highly heterogeneous poly(A)$^+$RNA molecules of different molecular masses (due to differences the lengths of mRNA coding regions as well as of its regulatory ones, apparently); so there is no contradiction to other data [23] concerning the existence of a marked discretion (in the same size ranges) for eucaryotic mRNAs (from 8.0 up to 0.24 kbs); a series of spots fused along gel lanes can be seen on our photographs because of radioactive track autographs being overlapped on the X-ray film due to labelled highly heterogeneous hybrid mRNA-cDNA molecules localized on the filter too near from each other. An electrophoregram presenting marker polynucleotides is also shown (see Fig. 2, *g*). So it is clear the poly(A)$^+$RNA preparations studied here to be non-degraded ones and to keep their high molecular components.

However, the principal nativity criterion for any poly(A)$^+$RNA molecules is their messenger activity directing the polypeptide synthesis process in *in vitro* (cell-free systems); the evaluation of RNA messenger activity was a principal moment in our investigation.

In the Fig. 3 (*a, b*) the kinetics of [^{35}S]-methionine incorporation into the TCA-insoluble material is presented; these data were obtained in a well-known wheat embryo cell-free system using a template poly(A)$^+$RNA as standard isolated from embryonic axes of non-stimulated haricot bean seeds. Our control data concerning TCA-insoluble fraction radioactivity were obtained with the same *in vitro* system containing no growth activator (see Fig. 3, *a*). We evaluated also the effect of the LNO and 6-MTU added to the incubated mixture (Fig. 3, *b* and *c*, respectively). We would like first of all to note the label incorporation increase during incubation in all the experiment materials (including also control ones) suggesting the nativity of poly(A)$^+$RNA preparations used. Our second finding is that the 6-MTU inhibits slightly the level of protein label incorporation during all the incubation period comparing to the control

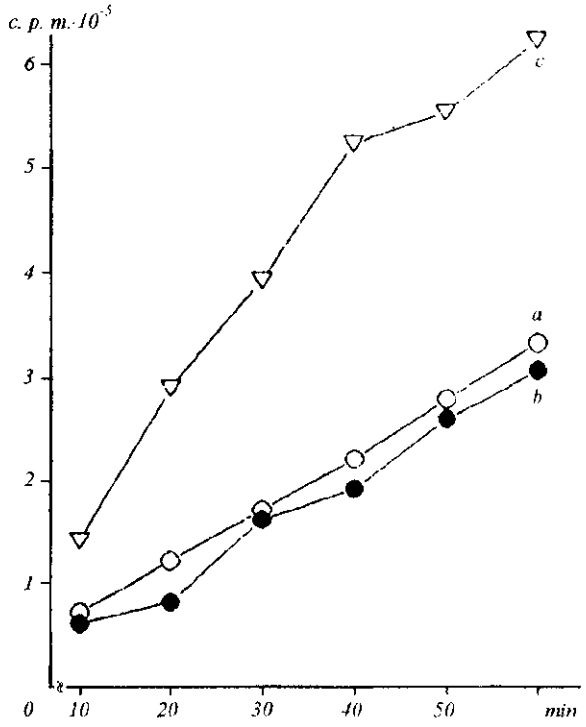

Fig. 3. Kinetics of [^{35}S]-methionine incorporation into the TCA-insoluble material in a wheat embryo *in vitro* system with using as template poly(A)$^+$RNA: *a* — a control sample (no growth stimulators were used); *b* and *c* — label incorporation into peptides in the presence of the 6-MTU and LNO, respectively

incorporation level. On the contrary, the LNO stimulates the poly(A)$^+$RNA directed polypeptide synthesis in our *in vitro* system, the synthesis level becoming almost twice higher.

Thus our results prove directly that the LNO activates the translation processes as well as the transcription one [20, 21]; while the 6-MTU have no regulatory effect on this crucial stage of gene expression.

So it became of great interest to compare some functional properties of embryonic axes poly(A)$^+$RNA preparations isolated from 6-MTU-stimulated and control haricot bean seeds. In the Fig. 4 (*a, b*) there are fluorograms demonstrating the PAGE distribution of polypeptide fractions. They had been previously labelled by [^{35}S]-methionine in cell-free system from rabbit reticulocytes using of poly(A)$^+$RNA as template RNAs isolated from embryonic axes of haricot been

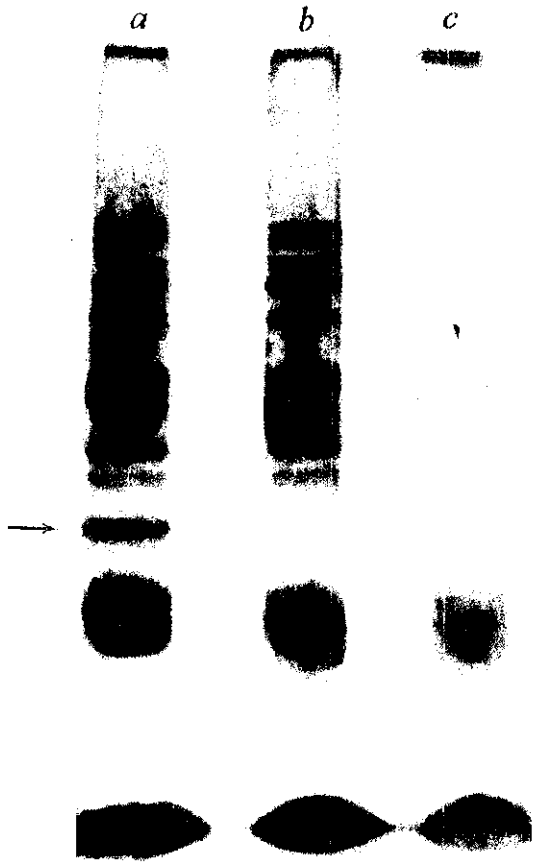

Fig. 4. Fluorographic analysis of electrophoretic polypeptide «spectrum» in the course of poly(A)$^+$RNA-directed *in vitro* synthesis (rabbit reticulocyte cell-free system): polypeptide synthesis in the presence of poly(A)$^+$RNA preparations from 6-MTU stimulated (*a*) and non-stimulated (*b*) embryonic axis cells, respectively; *c* — label incorporation *in vitro* synthesized peptides in the absence of any added poly(A)$^+$RNA preparation (control sample)

seeds following germination stimulated and non-stimulated by 6-MTU. The *in vitro* synthesized polypeptide fractions are very similar with both RNA preparations used with the only exception: an additional polypeptide was detected in a gel zone of low molecular weight proteins while analyzing the labelled material from the incubation mixture containing the poly(A)$^+$RNA preparation isolated from 6-MTU-stimulated embryonic axes. Our data do not irreproachably prove this additional peptide to be identical to the one detected in our previous *in vivo* studies. However, we have a fact of some 6-MTU-induced changes of an active poly(A)$^+$RNA pool, i. e. of the 6-MTU participation in the regulatory transcription mechanisms.

However, contrary to *in vitro* experiments, any regulative events having taken place at the transcription level *in vivo* cannot be realized at translation level because of several following circumstances: mRNA molecules exist and function in any plant cell as informosomes, i. e. mRNP-particles [24], no «naked» RNA is there present; at any stage of plant development there are both a reserved pool of functionally inactive mRNP being activated at following stages and a pool of «working» mRNP taking part in the translation process.

The isolation of any poly(A)$^+$RNA preparation using phenol deproteinization of mRNP abolishes these mentioned above differences of mRNA pools, both types of them becoming equally able to be translated. So we needed to answer the question concerning the correlation between the appearance of the 30 kDa minor protein *in vivo* and the changes at the translation level due to the 6-MTU-induced regulatory changes of transcription process. So we studied the correlation of active (incorporated into polyribosome complexes) and free, inactive in protein synthesis mRNPs in embryonic axis cells following stimulated and non-stimulated haricot bean seed germination.

The data concerning the CsCl gradient fractionation of formaldehyde-fixed RNP-particles isolated from stimulated and control seeds are given in the Fig. 5 (*a*—*d*). In both cases the obtained radioactive profiles of RNP-particles in the CsCl gradient are presented by two radioactivity maxima, their densities being 1.39 and 1.42 g/cm^3, respectively (small «peaks» of free informosomes), and two large peaks with their densities 1.52 g/cm^3 (for the 6-MTU-stimulated material) and 1.54 g/cm^3 (for the control sample), respectively, presenting *de novo* synthesized mRNP and rRNP-particles incorporated into polyribosomes. While EDTA treating of RNP preparations only a small part of radioactivity (following pulse RNP labelling during 1 h) remains in the region of ribosome subparticles localization, the main radioactive pool in both control and experimental samples being transported to the region of free informosomes; its peak in control is localized at 1.45 g/cm^3; however, the pulse-labelled RNPs of stimulated material form two peaks, the lesser one being concentrated in a region of the buoyant density 1.39 g/cm^3, the last main labelled material being found in a narrow *de novo* appeared peak at 1.46 g/cm^3.

So we note a clear correlation between the 6-MTU-induced changes of transcription process as well as of poly(A)$^+$RNA messenger activity (inducing the additional protein synthesis) influencing the

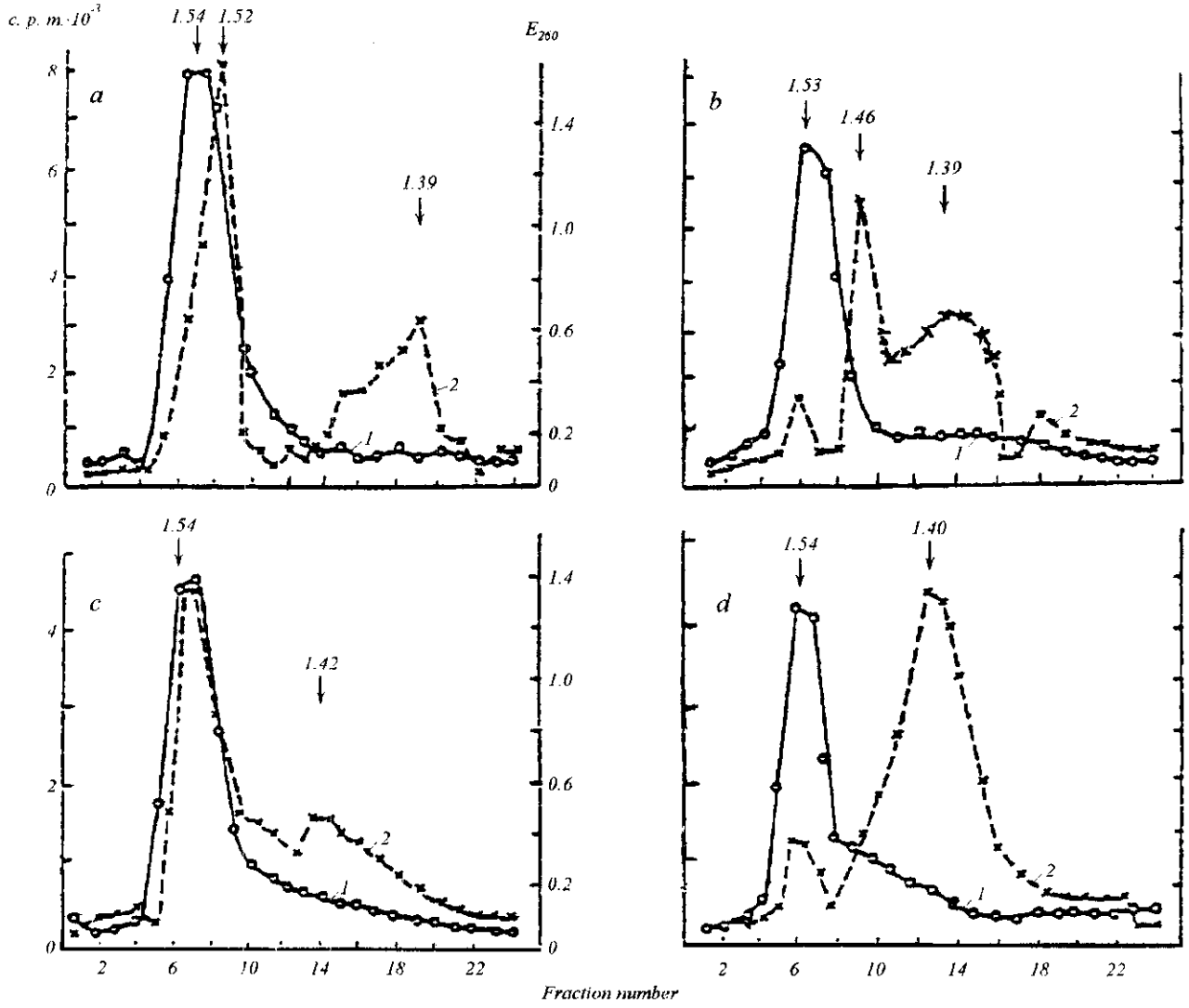

Fig. 5. Fractionation of RNP-particles in a CsCl density gradient: *a* and *b* — RNP-particle preparations from embryonic axes of MTL-stimulated seeds, untreated by EDTA and treated ones, respectively; *c* and *d* — RNP-particle preparations from embryonic axes of non-stimulated seeds, untreated by EDTA and treated ones, respectively

qualitative changes of acting mRNPs (included into polyribosomes) and the appearance of the minor protein in embryonic axis cells *in vivo*; however, to prove the identity of protein molecules produced *in vitro* and *in vivo*, immunological approaches are necessary as well as the study of *in vitro* obtained protein fractions whose synthesis is directed by mRNAs isolated from polyribosomes.

It is also evident the changes being realized by synthetic plant growth activators on the gene expression level and triggering most probably the acceleration of plant growth and development are to be a result of natural developmental process modifications due to the activator effect.

Today two tightly interconnected forms of plant growth and regulation are known — genetic control

and phytohormonal regulation; the results of their interaction may be summarized as follows:

(*i*) the plant genome codes the synthesis of cellular constitutive (i. e. common for all the development stages) structural and functional compounds as well as of stage- and organospecific ones to assure all the successive events of cell differentiation and specialization accompanying the formation of new tissues and organs;

(*ii*) phytohormones being derivative of gene functions (through a series of protein-enzyme molecules) as well as some other factors according to the feed-back regulation realize the re-programming of the cell genome by switching on and off conditionally «early», «middle», and «late» genes [25] controlling the formation of stage-specific cell homeostasis and, besides, participating in the regulation of «peripheral» intracellular metabolic processes.

The question to be now answered is how the synthetic plant growth regulators are wedged (inscribed) in such a well-coordinated multi-step regulation hierarchy?

This problem was earlier discussed in detail in our previous paper [1]; we propose that the synthetic compounds with their unusual structures having been never found in any plant cell are hardly able to realize their effect through the cell receptor system specific for a lot of natural compounds. It is more probably they mediate their effect by changing the active endogenic phytohormonal pool. An alternative explanation may also be proposed — a non-specific effect of growth regulators due to, apparently, of their higher binding (affinity) with cellular effector systems (being their specific «binding sites» or «targets») realized more quickly comparing to natural phytohormones, the last ones being forced out from their own receptors. The combined action of growth regulators (observed in our experiments with the LNO) cannot be also ruled out; it may include a phytohormone-mediated effect at the transcription level and a direct regulator effect at the translation one (in addition to mentioned above the taking down of inhibitory action ABA by growth activators is possible).

Discussing a possible 30 kDa protein function one may suppose that the plants possess some genes coding some «obscure» protein products being normally absent; the promoters of these «silent», «cryptic», «hidden» genes («archaeological signs») are able [26], however, to be switched on by certain stress factors; in our experiment the 6-MTU was shown to be such a factor. We suppose that the 30 kDa protein is one from enzymes transforming the «unsuitable» («strange») for cells 6-MTU structure into a natural substance possessing a phytohormone activity; this phytohormone is able to accelerate drastically the plant cell growth by enlargement because in early postembryogenesis embryonic axis growth due to enlargement of hypocotyl. The «ephemeral» 30 kDa protein appears and disappears very quickly, according to entering into cells and disappearance from cells the 6-MTU. The data of work [27] witness in behalf of such possibility. Authors discovered the timing changes in enzymes synthesis in elicitor-treated cell suspension cultures of Parsley.

It should be noted that this 30 kDa polypeptide is highly similar to polypeptide, forming the base of hydroxyproline-rich glycoprotein extensin (the molecular mass of glycoprotein is 86 kDa, while its polypeptide without carbohydrate part forms precisely 30 kDa) [28—30], which is the major protein components of the cell wall of dicotyledon plants. Extensin comprises 5—10 % from all proteins of cell wall and executes plural functions for cell. According with these works 30 kDa polypeptide is positively charged (pI of 9.9) and soluble in water medium precursor of extensin. This polypeptide is synthesised and glycosylated by posttranslational modifications in cytoplasm and then integrated into the cell wall space. The expression one of the extensin gene family (SbHRGP3) increases with seedling maturation, and its expression is relatively high in the mature regions of the hypocotyl and in the root of soybean seedlings (it is possibly that the intensification of expression some from extensin genes under the of 6-MTU stimulated haricot bean seed germination is noted).

The finale step of our work consisted of experiments concerning the effects of the LNO and 6-MTU (possessing quite different mechanisms) on the following haricot bean ontogenesis. So the plants originated from germinated seeds (with or without growth stimulators) were cultivated further on the minimal nutrient media. It was shown the 6-MTU-stimulation of seed germination to cause a significant plant development acceleration comparing to control plant ontogenesis duration (it is about 40 days in laboratory conditions); a plant originated from a 6-MTU-stimulated seed is able to complete its development in 25 days having passed all the ontogenesis steps including also flowering, ovary and even pod formation, its root network being also well developed (see Fig. 6, *a, b*). At the same time the LNO-induced seed germination leads to the plant growth deformations — accelerated growth of stems carrying underdeveloped foliage, no reproductive organs having been formed (not shown).

It is also noteworthy the similar effect of vegetative organs predominance is usually seen in plants

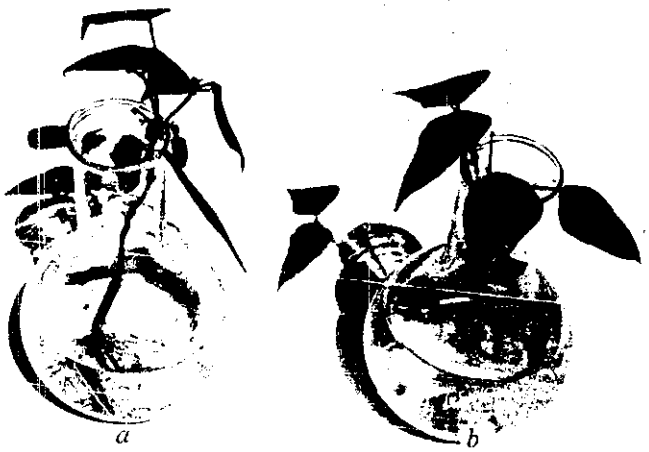

Fig. 6. Plants grown from haricot bean seeds germinated without any stimulation (*a*) and following the 6-MTU directed stimulation (*b*)

growing on soils containing superfluous organic fertilizers; the abundance of organic compounds is known to cause the nitrogen metabolism activation in plant cells, this process being somewhat analogous to the LNO-induced total protein synthesis.

It should be also taken into account the LNO-induced increased total protein synthesis and the 6-MTU-induced accelerated minor protein synthesis (these phenomena are somewhat similar to synthesis with protective functions of heat and cold shock proteins in plants to response on critical or extremely high or low temperatures, respectively, for example [31]) may be genetic «markers» determining the following plant development (vegetative organs predominance or complete although shortened ontogenesis) already at the early postembryogenesis stage.

Concluding this paper we would like to note the selective 6-MTU-induced high level synthesis of the only minor protein suggests the gene coding this protein to belong to a family of unique genomic DNA sequences, its function being controlled by a strong promoter. The cloning of this markedly inducible promoter is to be perspective in the field of plant genetic engineering, namely, for recombinant gene constructions whose expression in transgenic plants is to be induced by 6-MTU.

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В. А. Циганкова, В. М. Заєць, Л. О. Галкіна,
Л. П. Приказчикова, Я. Б. Блюм

Поява незвичайного мінорного білка в клітинах зародкової осі при стимуляції проростання насіння квасолі 6-метилтіоурацилом

Резюме

За допомогою двомірного електрофорезу білків у поліакриламідному гелі виявлено незвичайний мінорний білок з молекулярною масою ≈ 30 кДа у клітинах зародкової осі при стимуляції проростання насіння квасолі (Phaseolus vulgaris L.) 6-метилтіоурацилом. Появу цього білка не зафіксовано в нормі та при проростанні, стимульованому N-оксидом лутидина. Синтез додаткового низькомолекулярного білка спостерігався також у безклітинній системі з ретикулоцитів кроля на матриці полі(А)⁺ РНК, одержаній з клітин зародкової осі квасолевого насіння, обробленого 6-метилтіоурацилом. У той же час у безклітинній системі з проростків пшениці при використанні «стандартного» препарату полі(А)⁺ РНК показано, що N-оксид лутидина різко стимулює синтез поліпептидів, а метилтіоурацил прямо не впливає на процес трансляції. Вивчаючи співвідношення рибосом, вільних та включених у полірибосоми in vivo, за допомогою фракціонування РНП-часточок у преформованому градієнті густини CsCl, встановлено присутність додаткового піка (1,46 г/см³) у фракції полірибосом з зародкових осей насіння, обробленого 6-метилтіоурацилом. Така стимуляція суттєво скорочує період онтогенезу, ніяк не пошкоджуючи фенотипу рослини. При цьому обробка пророщуваного насіння N-оксидом лутидина призводить до деформованого прискореного розвитку вегетативних органів без розвитку органів розмноження рослини. Обговорюється природа білка 30 кДа і взаємозалежність між змінами в експресії генів, що викликані ростовими стимуляторами у клітинах зародкової осі під час раннього постембріогенезу, та наступними різнонаправленими процесами росту та розвитку рослин квасолі. Розглянуто також деякі практичні напрямки застосування ростових стимуляторів, пов'язані з результатами проведених експериментів.

В. А. Цыганкова, В. Н. Заец, Л. А. Галкина,
Л. П. Приказчикова, Я. Б. Блюм

Появление в клетках зародышевой оси необычного белка при стимуляции прорастания семян фасоли 6-метилтиоурацилом

Резюме

С помощью двухмерного электрофореза белков в полиакриламидном геле показано появление в клетках зародышевой оси при стимулируемом 6-метилтиоурацилом прорастании семян фасоли (Phaseolus vulgaris L.) необычного минорного белка с молекулярной массой около 30 кДа. Этот белок не обнаруживался в норме и при стимулируемом N-окисью лутидина прорастании семян растений. Синтез дополнительного низкомолекулярного белка отмечен и в бесклеточной системе белкового синтеза из ретикулоцитов кролика на матрице поли(А)⁺ РНК из клеток зародышевых осей со стимулируемым 6-метилтиоурацилом прорастанием семян фасоли. В то же время с помощью бесклеточной системы белкового синтеза из проростков пшеницы с использованием в качестве матрицы стандартного препарата поли(А)⁺ РНК установлено, что N окись лутидина резко стимулирует синтез полипептидов, а 6-метилтиоурацил не оказывает прямого влияния на процесс трансляции. Изучение соотношения свободных и включенных в полирибосомы информосом in vivo методом фракционирования РНП-частиц в преформированном градиенте плотности CsCl

выявило присутствие во фракции полирибосом из зародышевых осей при стимулируемом 6-метилтиоурацилом прорастании семян дополнительного пика активных в белковом синтезе информосом с плавучей плотностью 1,46 г/см³. Показано, что стимуляция прорастания семян фасоли 6-метилтиоурацилом приводит к существенному сокращению сроков онтогенеза растения фасоли без каких-либо нарушений фенотипа растения, а стимуляция N-окисью лутидина — к деформированному ускоренному развитию вегетативных органов без развития репродуктивных органов растения. Обсуждается природа белка 30 кДа и связь между различиями в изменении ростстимуляторами экспрессии генов в клетках зародышевых осей в раннем постэмбриогенезе и последующими разнонаправленными процессами роста и развития растений фасоли, а также некоторые практические аспекты, вытекающие из этого.

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