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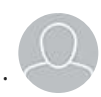

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Significant role for polysomes associated with the cytoskeleton in the control of protein synthesis during germination of triticale caryopses in the presence of abscisic acid

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Key words: triticale caryopses, germination, protein synthesis, cytoskeleton-bound polysomes

Abstract

The influence of abscisic acid (ABA) on the process of polysome formation and synthesis of newly-formed proteins by different polysome populations was studied. Triticale caryopses were germinated in water or various ABA concentrations for 48 hrs, and afterwards they were transferred to a solution of ^{14}C-amino acids and germinated for an additional 30 min. Embryos were separated from caryopses, and four polysome populations were isolated: the FP (free polysomes), MBP (membrane-bound polysomes), CBP (cytoskeleton-bound polysomes) and CMBP (cytoskeleton-membrane-bound polysomes). ABA retarded both the process of polysome formation and their activity in forming new proteins *in vivo* in all studied fractions. Participation of polysomes in total ribosomal materials (sub-units, monosomes and polysomes) of each polysome population in the control sample was as follows: FP – 77; MBP – 72; CBP – 70 and CMBP – 66 %, whereas in sample treated by ABA (100 μM) it was accordingly: 17; 23; 27 and 28 %. The largest population made up FP (in control sample 69 %), participation of MBP was always lower and ranged from about 19 to 30 %. Participation of polysome populations bound with the cytoskeleton CBP and CMBP, both in control sample as well as in samples treated with 1 and 10 μM ABA solution, was only a few per cent. It should be noted that when the ABA concentration was higher (100 μM) (process of germination was strongly inhibited), participation of those two populations (CBP and CMBP) was much increased in embryos, respectively to about 18 and 20 %. In both the control group and in embryonal tissue treated with ABA increasing incorporation of radioactive precursors to newly-formed proteins *in vivo* in fractions of polysomes isolated by following buffers: C (FP), C + PTE (MBP), C + Tris (CBP) and buf. U (CMBP) was observed. It should be noted, that the biggest incorporation of ^{14}C-amino acids into nascent polypeptide chains was found in the last polysome population (CMBP). In the sample treated with ABA (100 μM) the activity of this fraction (CMBP) in forming new proteins is several times, and in the case of FP dozens of times, more intense. Increased participation of CBP and CMBP in embryos of triticale caryopses treated with ABA (100 μM) and the largest incorporation of ^{14}C-amino acids into nascent polypeptide chains synthesised by CMBP, may indicate the important role of proteins formed by polysomes associated with cytoskeleton in inhibition of germination and seedling growth by ABA.

List of abbreviations: ABA - abscisic acid, CBP - cytoskeleton-bound polysomes, CMBP - cytoskeleton-membrane-bound polysomes, EGTA - ethylene glycol-bis (ß-aminoethyl ether) N,N,N',N'-tetraacetic acid, FP - free polysomes, GA - gibberellic acid, HEPES - *N*-2-hydroxyethylpiperazine-N'-2-

ethanesulfonic acid, KOAc - potassium acetate, MBP - membrane-bound polysomes, $Mg(OAc)_2$ - magnesium acetate, MT_s – microtubules, PMSF - phenylmethylsulfonyl fluoride, PTE - polyoxyethylene-10-tridecyl ether, Tris - tris-(hydroxymethyl) aminomethane.

Introduction

Cereal dormancy is an important agronomic and physiological problem (Bewley and Black, 1994). Abscisic acid (ABA) plays a decisive role in the induction and maintenance of seed dormancy (Weidner 1987, Karssen *et al.* 1989, Walker-Simmons and Sessing 1990, Wang *et al.* 1995). In barley aleurone layers ABA suppresses the expression of genes encoding GA-induced proteins (Higgins *et al.* 1982) and increases the levels of the ABA-induced proteins (Lin and Ho 1986; Jacobsen and Chandler 1987). It is also known that both ABA and GA have the ability to change the orientation of microtubules (MTs) by a mechanism that does not involve changes in the rate of the cell elongation (Sakiyama and Shibaoka 1990, Sakiyama-Sogo and Shibaoka 1993). The present outlook on the genetic and molecular basis for seed dormancy was presented recently (Bewley 1997, Li and Foley, 1997, Foley and Fennimore 1998).

Gene expression is a complex multiple-step process that can be controlled at many levels. Most available information relates to regulation at the transcriptional level. However, more and more attention is being given to post-transcriptional control, *e.g.* to determine if the cytoskeleton is involved in the regulation of protein synthesis by the plant hormone ABA.

Since polysomes are the site of translation and mediate the final step in gene expression, their subcellular location is important for the ultimate density of their products. In a wide variety of animal cells, mRNA localization is dependent on the cytoskeleton (St. Johnson 1995). Both the microtubule and microfilament networks are known to function together or independently in the localization of mRNAs (Sundel and Singer 1990, St. Johnson 1995). In plants, the cytoskeleton has also been implicated in the localization of actin mRNA during *Fucus* embryo development (Bouget *et al.* 1996) and in the intercellular transport of plant viral RNAs (McLean *et al.* 1996). The cytoskeleton may also play a role in translation, as the bulk of the polysomes in the cytosol appears to be associated with the microfilament component of the detergent resistant cytoskeleton scaffolding network (Patcher 1992, Hesketh, 1994). Evidence for cytoskeleton-bound polysomes (CBP) in pea stems and roots, and corn endosperm was obtained (Davies and Abe 1989, Davies *et al.* 1993, Davies *et al.* 1998). There is also evidence for the association of polysomes with both the cytoskeleton and membranes in plants – CMBP (cytoskeleton-membrane-bound polysomes) (Davies *et al.* 1993, Stankovic *et al.* 1993, Ito *et al.* 1994).

Polysome isolation protocols from plant tissue generally employ high ionic strength Tris buffers (Davies *et al.* 1972), modified in various ways to furnish free polysomes (FP) and membrane-bound polysomes (MBP) (Larkins and Davies 1975, Abe and Davies 1985). These buffers were designed specifically to prevent RNase activity and thus maximize polysome integrity, but no attempt was made to preserve polysome adherence to subcellular structures such as the cytoskeleton. Some years ago, however, it was shown that the use of low ionic strength buffers designed specifically to maintain the integrity of the cytoskeleton (Abe and Davies 1991) reveals that about 70 % of the total polysomes are in the cytoskeletal pellet and that high ionic strength buffers cause the release of cytoskeleton-bound polysomes (CBP) from this pellet (Davies *et al.* 1991).

In this work a new set of cytoskeleton stabilizing buffers was used, which enabled the isolation of four polysome populations from plant tissue (Davies and Abe 1995): the FP (free polysomes), MBP (membrane-bound polysomes), CBP (cytoskeleton-bound polysomes) and CMBP (cytoskeleton-membrane-bound polysomes). The aim of the work was to study the influence of ABA on the process of germination, polysome formation and also intensity of production of a newly-formed proteins, synthesised by various populations of polysomes. Special attention, however, was paid to participation and activity of polysome populations bound with the cytoskeleton during triticale germi-

nation in the presence of various concentrations of ABA.

Materials and methods

Plant material, germination conditions and conditions of labelling

The experiments were conducted on non-dormant triticale (*Triticosecale*) cv. Grado, supplied by the Plant Cultivation Station in Wrocikowo. The grains were washed with tap water, placed for 3 min in a 1 % solution of sodium hypochlorite, and washed with sterilized water. The materials were then germinated in water or in solutions of abscisic acid (1, 10 and 100 μM) on Petri dishes in darkness at 21 °C. Twenty grains were placed on two layers of Whatman paper No.1 (Whatman, Maid Stone, Kent, UK) in a Petri dish (9 cm) containing 3 ml distilled water or solution of ABA (mixed isomers – Sigma, USA). During incubation, the plates were sealed with parafilm to prevent evaporation. Grains were scored as germinated when the roots were ≥ 1 mm.

In part of research, after 48 hrs of incubation in water or in ABA solutions, caryopses were transferred to a solution of ^{14}C-amino acid hydrolysate (3.7 M Bq·ml - supplied by the Chemapol UVVVR, Czech Rep.) and incubated for 30 min. at 21 °C. At specific times, embryos (or germs) were isolated from grains, the unincorporated precursor was carefully rinsed off with water, the embryo surfaces dried and then stored in liquid nitrogen until further study.

Polysome isolation and quantification

The newly-developed, cytoskeleton-stabilizng buffer C (Abe and Davies, 1991), consisting of 5 mM HEPES, 10 mM MgOAc, 2 mM EGTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), adjusted to pH 7.5 with 9.8 mM KOH, was used for isolation of cytoskeleton fractions from cells of triticale embryos. This buffer allows for the sequential isolation of four polysome populations (Davies and Abe, 1995) the FP (free polysomes), MBP (membrane-bound polysomes), CBP (cytoskeleton-bound polysomes) and CMBP (cytoskeleton-membrane-bound polysomes). All operations were conducted at 0-4 °C. The tissue (1 g) was homogenized in at least 5 vol. buffer C (10 vol is preferred, since buffer C resists RNase poorly), filtered and centrifuged at 27,000 x g for 10 min. The supernatant polysomes are the FP and to prevent their degradation by RNase, the samples were adjusted to buffer U, consisting of 200 mM Tris-HCl, pH 8.5, 50 mM KOAc, 25 mM MgOAc, 2 mM EGTA, 100 mg·ml^{-1} heparin, 2 % PTE (polyoxyethylene-10-tridecyl ether, a non-ionic detergent) and 1 % sodium deoxycholate (Abe *et al.*, 1992) by adding 1/4 vol of 4 x U and held on ice prior to further processing. All other polysomes were in the pellet and were sequentially solubilized as follows. The pellet was resuspended in buffer C + PTE to disrupt membranes, centrifuged 5 min at 27,000 x g to leave MBP in the supernatant (again, converted to buffer U to maintain polysome integrity). All other polysomes were still in the pellet. This pellet was next resuspended in buffer C plus 200 mM Tris-HCl, pH 8.5 to disrupt the polysome-cytoskeleton interaction and centrifuged for 5 min at 27,000 x g to leave CBP in the supernatant (again converted to buffer U). The final pellet was resuspended in buffer U and re-centrifuged to release the CMBP. All supernatant fractions were layered on a 4 ml "pad" of 50 % (w/v) sucrose in buffer B (50 mM Tris-HCl, pH 7.5; 20 mM KOAc, 10 mM MgOAc) and centrifuged for 3 h at 300,000 x g in a Beckman 65 Ti rotor. The polysome pellets were rinsed in water and then resuspended in 1 ml of buffer U. The resuspended polysomes were centrifuged at top speed (approx. 18,000 x g) for 2 min in a microfuge prior to layering 0.2 ml aliquots on linear 15 % to 60 % (w/v) sucrose gradients in buffer B and centrifuged at 122,000 x g in an SW-41 Beckman rotor for 75 min. Gradients were next scanned at 254 nm on a UA-5 flow recorder (ISCO, Lincoln, NE, USA) to display subunits, monosomes and polysomes. The percentage of polysomes in the total ribosomal preparation was calculated by xeroxing the A_{254} profile, drawing the base line (obtained by scanning a blank gradient) under the profile, and then cutting out and weighing the paper (uniform in thickness) representing the areas of monosomes and polysomes of the profiles. Quantitation of the ribosomes was done assuming that the absorbance of a 1 % solution of ribosomes (measured in a cuvette with a 1 cm optical path at 260 nm) equals = 13.5 (Gualerzi and Cammarano, 1969). Radioactivity was assayed

Table. The percentage of polysomes in the total ribosomal materials (sub-units monosomes and polysomes), of each polysome population and the incorporation of ^{14}C-amino acids into polysome fractions of triticale embryos after germination of caryopses. After initial 48 h of germination in water or in ABA solutions, caryopses were transfered to a solution of radioactive precursors and incubated for 30 min at 21 °C. Embryos were then isolated from grains after 48.5 h of germination and four polysome populations were extracted from this plant material (FP – free polysomes, MBP – membrane-bound polysomes, CBP – cytoskeleton-bound polysomes and CMBP – cytoskeleton-membrane-bound polysomes). Initially the cereal tissue was homogenized in buffer C to yield FP. The pellet was extracted sequentially in buffer C + PTE to release MBP, buffer C + Tris to release CBP and buffer U to release CMBP.

Treatment	Fraction							
	FP		MBP		CBP		CMBP	
	polysomes %	Bq × mg^{-1} polysomes	polysomes %	Bq × mg^{-1} polysomes	polysomes %	Bq × mg^{-1} polysomes	polysomes %	Bq × mg^{-1} polysomes
Control	77	236.40	72	503.79	70	857.68	66	1255.50
1 µM ABA	70	–	69	–	68	–	66	–
10 µM ABA	70	–	60	–	67	–	53	–
100 µM ABA	17	15.21	23	177.47	27	165.39	28	934.62

using a Beckman LS-1801 liquid scintillation counter with Tritosol as the scintillator (Fricke, 1973). All experiments were conducted at least in triplicate.

Results

Triticale grains were germinated in water (control) and in solutions of 1, 10 and 100 µM abscisic acid. ABA retarded the ability of caryopses to germinate proportionally to its concentration (Fig. 1). ABA also influenced accumulation of dry and fresh weight in embryos during germination. The most intensive inhibition of germination processes was observed in embryos of triticale grains germinating in 100 µM ABA. The average dry weight of one embryo within 60 hrs of germination increased by only 0.4 mg. In contrast, the average dry weight increase of an embryo isolated from a grain germinated in water (control) was about 3.64 mg (Fig. 1). At the same time, ABA inhibited the fresh weight increase of embryos in a similar manner.

Next, the percentages of polysomes in particular populations (FP, MBP, CBP and CMBP) extracted from the embryo tissue after 48 hrs germination of triticale caryopses were quantified. The proportion of ribosomes existing as polysomes ranged from 66-77 % in the four fractions (Table, Fig. 2). At 100 µM ABA, the percentage of ribosomes as polysomes was greatly reduced (17 to 28 %).

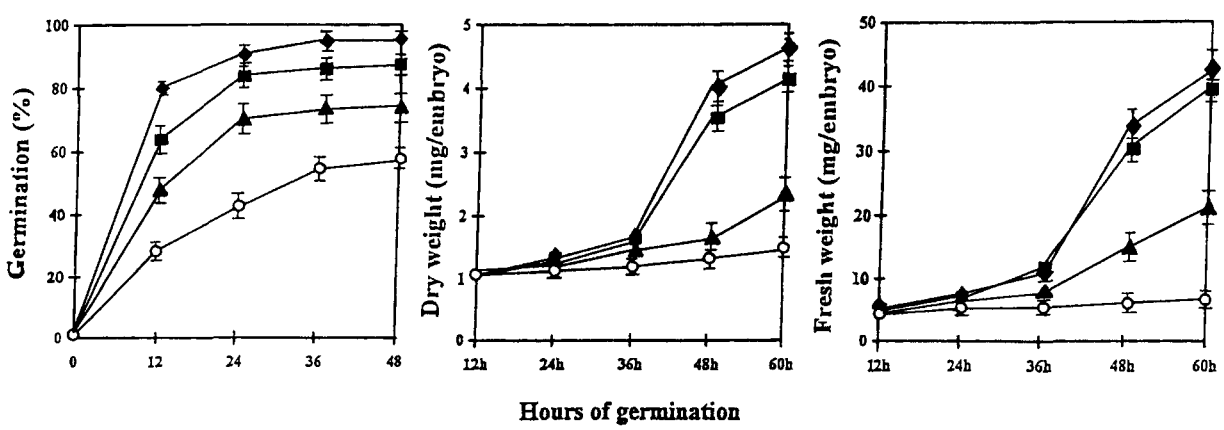

Fig. 1. Germination percentages of caryopses and changes in dry and fresh weight of embryos during germination in water ◆ (control) and in the presence of abscisic acid: ■ 1 µM ABA, ▲ 10 µM ABA and ○ 100 µM ABA. Vertical bars represent ±SE from 3 independent experiments.

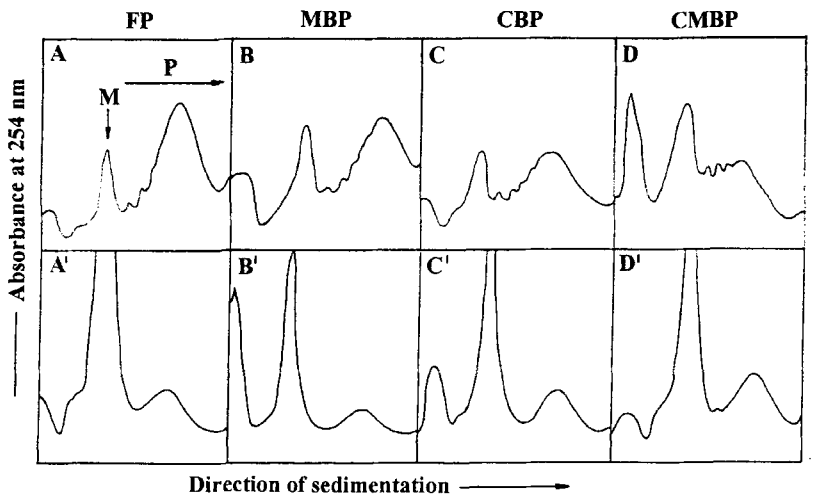

Fig. 2. Sedimentation in sucrose gradients of polysomes from embryos of germinating (48 hrs) caryopses in water (A, B, C, D) and in the presence of 100 μM abscisic acid (A', B', C', D'). FP – free polysomes (A, A'), MBP – membrane-bound polysomes (B, B'), CBP – cytoskeleton-bound polysomes (C, C'), CMBP – cytoskeleton-membrane-bound polysomes (D, D'). M - monosome fraction, P - region of polysomes.

Figure 3 presents the percentage of the polysomal populations (FP, MBP, CBP and CMBP) in the total ribosomal material, isolated from embryo tissue of caryospes. In all batches of germinating triticale caryopses (control and ABA treated samples), the largest populations were made up of free polysomes (FP) in control embryo tissue 69 % and after 1 μM ABA treatment 64 %, still lower in a sample treated with 10 μM ABA solution (54 %), and the lowest in a sample treated with 100 μM ABA solution (37 %). Participation of membrane-bound polysomes (MBP) was always lower and ranged from about 19 to 30 %. As for the particiption of cytoskeleton-bound polysomes (CBP) and cytoskeleton-membrane-bound polysomes (CMBP), both in control and ABA-treated samples was only a few per cent. It should be emphasized that when 100 μM ABA was used, the participation of those two fractions – CBP and CMBP, increased significantly, respectively to 18 and 20 % (Fig. 3).

The influence of 100 μM ABA on protein synthesis *in vivo* with particular populations of polysomes isolated from embryos of triticale caryopses was also studied (Table). The grains were germinated in water or in ABA solutions for 48 hrs, and then they were transfered to a solution with radioactive amino acids and germinated for another 30 min. Embryos were then isolated from caryopses and the four polysome populations were obtained. In the control group the incorporation of radioactive precursors into nascent polypeptide chains was observed in all populations of polysomes. The least active were FP, more active MBP, still more active CMBP and the most active polysome population (in the synthesis of nascent proteins) was CMBP frac-

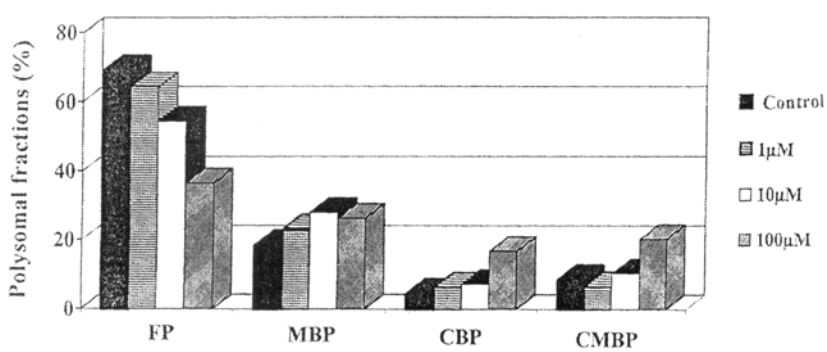

Fig. 3. The percentage of the polysomal populations (FP – free polysomes, MBP – membrane-bound polysomes, CBP – cytoskeleton-bound polysomes and CMBP – cytoskeleton-membrane-bound polysomes) in the total ribosomal material, after extraction of triticale embryos in different buffers. Embryos were isolated from germinated caryopses after 48 hrs of incubation. Caryopses were germinated in water (control) and in the presence of abscisic acid at the following concentrations: 1 μM, 10 μM and 100 μM. Four polysome populations were extracted from this plant material (FP, MBP, CBP and CMBP).

tion (Table). In studies on embryo tissue treated with ABA, similar trends were observed, with the only difference that incorporation of ^{14}C-amino acids into proteins on MBP and CBP was similar. However, in tissue treated with ABA the synthesis of newly-formed proteins in all polysome populations was much lower (Table). In both treatments the most intensive incorporation of precursors into nascent polypeptide chains was in the CMBP fraction. In samples treated with ABA (100 μM) the activity of the CMBP fraction in synthesizing new proteins is several, but in the case of FP, dozens of times higher (Table).

Discussion

The effects of ABA on fresh and dry weight of embryos isolated from intact caryopses (Fig.1) are similar to our earlier results with wheat caryopses (Weidner *et al.*, 1991). Further research concerned the analysis and activity of different populations of polysomes from triticale caryopses. Animal cells have been shown to have three classes of cytoplasmic (non-organellar) polysomes: (**1**) free polysomes (FP) are thought to be involved primarily in the synthesis of cytosolic proteins, (**2**) membrane-bound polysomes (MBP) involved in the synthesis of secreted and sequestered proteins (Elder and Morré 1978) and (**3**) cytoskeleton-bound polysomes (CBP) possibly involved in the synthesis of cytoskeletal, viral or stress proteins (Adams *et al.* 1983, Hesketh and Pryme 1991). Based on the results of Ito *et al.* (1994) a cytoskeleton-membrane-bound polysome complex, which might be described as cytoskeleton-membrane-bound polysomes or cytomatrix-bound polysomes (CMBP), also exists in plant material.

In these studies ABA inhibited both the capacity of triticale caryopses for germination as well as forming of polysomes and their activity in synthesizing new proteins *in vivo* in all studied fractions (FP, MBP, CBP and CMBP) during seedling growth. The results are in agreement with our earlier findings concerning translational activity of polysomes (FP + MBP) from embryos during germination of wheat caryopses in the presence of abscisic acid (Weidner *et al.* 1991). In those studies ^{3}H-leucine incorporation into protein *in vitro* by polysomes from embryos incubated with ABA, was lower in all stages of germination investigated. The synthesis of one polypeptide (28 KDa) was strongly inhibited by ABA, whereas the appearance of others was delayed.

In both plant and animal cells, changes in the polysome profiles may be correlated with changes in the rate of protein synthesis (e.g. Goustin and Wilt 1981; Laroche and Hopkins 1987). In our studies we applied a short 30 min incubation of seedling with labelled amino acids. In such conditions the vast majority of polysome-associated label is in nascent protein (Dommes and Van de Walle 1990; You *et al.* 1992).

Compared to the free polysomes, the membrane-bound polysomes were more efficient in incorporation of ^{14}C-amino acids into protein *in vivo*. The same dependence was observed earlier in studies on germinating barley caryopses (Weidner and Łukaszewicz 1997b) and in studies *in vitro* on developing lupin seeds (Gwóźdź and Deckert 1989). Gwóźdź and Deckert (1989) also found that membrane-bound polysomes produced peptides of high molecular weight, and a correlation was demonstrated between cotyledon growth, polysome formation and their capacity for protein synthesis *in vitro*. In our experiments two other polysome populations, CBP (cytoskeleton-bound polysomes) and CMBP (cytoskeleton-membrane-bound polysomes), appeared to be even more active in protein synthesis (in control sample) than FP and MBP. The highest incorporation of ^{3}H-uridine and ^{14}C-amino acids *in vivo* was also observed in CMBP during germination of barley caryopses (Weidner and Łukaszewicz, 1997b) and in TBP (tightly-bound polysomes - putative CBP) during precocious germination of triticale caryopses (Weidner and Łukaszewicz 1997a).

Since the function of polysomes is to translate mRNA into proteins, their attachment to the cytoskeleton might enhance this process. Perhaps factors supportive of protein synthesis (in addition to the polysomes themselves) are present in the cytoskeleton pellets. Abe *et al.* (1995) found a 40-kD protein that may act to attach the ribosomes to the cytoskeleton. There is also a different protein, which is more abundant in actively translating

polysomes (Garcia-Hernandez *et al.*, 1994, 1996). The latest data presented by Davies *et al.* (1998) indicate, that the role of the cytoskeleton would probably relate more to translational efficiency, than to segregation of mRNAs to different subpopulations of polysomes.

Relatively high participation of two fractions – CBP and CMBP in embryos of triticale caryopses during germination and seedling growth in the presence of abscisic acid (100 μM), as well as the most intensive incorporation of ^{14}C-amino acids into nascent polypeptide chains synthesized by CMBP population, may indicate the significant role of newly-formed proteins created by polysomes associated with cytoskeleton in the mechanisms of inhibiting germination by ABA. Promotion of synthesis of some proteins in cotyledons or in cultured embryos by ABA (which at the same time inhibits germination) is well documented (for example, Black 1991). It was shown that ABA-responsive genes are expressed more readily in embryos of dormant grains, raising the possibility that a set of "dormancy proteins" exists. So far there is no information available as to the possible functions and physiological actions of such proteins. It should be added that in embryos of barley caryopses in natural dormancy was observed the mechanism (Weidner and Łukaszewicz 1997b) similar to that in artificially induced by ABA (100 μM) dormancy. In both cases, cytoskeleton-membrane-bound polysomes (CMBP) appeared to be the most active in the synthesis of newly-formed proteins, and embryos of dormant imbibed caryopses were more abundant in polysome populations bound with the cytoskeleton (CBP + CMBP).

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