Effect of osmotic stress on in vitro translational capacity of polysomes and on the composition of polysome-associated proteins in germinating seeds of pea (*Pisum sativum* L.)

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Abstract

Plant growth throughout the world is often limited by unfavourable environmental conditions. This paper reports results of a study on long- and short-term osmotic stress (−0.5 MPa) followed by a recovery on in vitro translational capacity of polysomes and on the composition of polysome-associated proteins in germinating pea (*Pisum sativum* L.) seeds. Here we show that, under osmotic stress, cytoskeleton-bound polysomes were charaterized by the highest translation activity, which may be indicative of an important role that this population of polysomes plays in the synthesis of the so-called "stress proteins". We also find out that in response to osmotic stress, new proteins (22.01, 96.47 and 105.3 kDa), absent in the unstressed sample, associated with the total pool of polysomes, whereas the protein of 22.95 kDa, which was present in the embryonic tissue of seeds germinating under unstressed conditions, disappeared. These changes may have affected both the stability and the translational capacity of polysomes.

Keywords: germinating, osmotic stress, pea seeds, polyethylene glycol, polysomes, ribonuclease, translation in vitro

Introduction

Changes in the environment, such as drought, salinity, high or low temperature, are an important factor which affects the growth of crops and the volume of crop yields [1–3]. Very important legume grown and consumed extensively worldwide is pea [4]. As a rich source of proteins, carbohydrates, fibre, vitamins and minerals, peas are important in human nutrition [5]. Pea is the fourth leading legume in terms of consumption in the world after soybean, peanuts and bean [6]. One of the major abiotic stress often occurs in Europe is osmotic stresses induced with polyethylene glycol (within −0.5 MPa). The earliest metabolic change caused by water stress is a decreased amount of polysomes [7]. Once the polysomes have been disaggregated, the plant growth is inhibited due to a slower protein synthesis rate [7–9]. Considerable reduction in the amount of polysomes in plant tissues is observable after osmotic stress lasting for just 20–30 minutes [10]. Decrease of the content of polysomes in response to abiotic stresses is connected with the process of "switching" the expression of genes from those participating in the growth and development of plants under unstressed conditions to the ones active in response to stress [11].

In plant tissues, polysomes can occur as free polysomes (FP), endoplasmic reticulum membrane-bound polysomes (MBP) [12], cytoskeleton-bound polysomes (CBP) [13,14] and cytoskeleton-membrane-bound polysomes (CMBP) [15]. Changes in the distribution of polysomes between the particular fractions reflect changes in the complement of proteins, as each population of polysomes is engaged in the synthesis of specific proteins [16]. Thus, the FP population is mainly involved in the synthesis of soluble proteins of the cytoplasm, cellular nucleus, mitochondria and peroxisomes [17]; the MBP population is engaged in the production of secretory proteins, lysosome proteins and the proteins which are an integral component of the plasmatic membrane and intracellular membranes, including the endoplasmic reticulum [16,17]; finally, the CBP and CMBP populations are responsible for the synthesis of cytoskeleton and stress proteins [16,18–21]. The largest share in the total polysome pool consists of the CBP population, which sometimes reaches 70% of the total polysome content [22]. Polysomes contain mRNAs, which may undergo selective translation, result in modification of protein synthesis in response to stress conditions in plants [23,24].

The objective of this study was to indicate differences in the composition of polysome-bound proteins and in the products of in vitro translation in pea (*Pisum sativum* L.) seeds germinating under unstressed conditions and under long- and short-term osmotic stress (−0.5 MPa) followed by post-stress recovery.

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Material and methods

Plant material and germination under normal and osmotic stress conditions

The experiments were conducted on *Pisum sativum* L. cv. six-week TOR seeds, supplied by Torseed S.A. (Toruń, Poland). Seeds were surfaced disinfected in 1% sodium hypochloride for 3 minutes and washed with tap water and sterile water. Disinfected and intact seeds were placed on Petri dishes with two layers of Whatman 2 filter paper No. 1 (Whatman, Maidstone, Kent, the UK) wetted with 40 ml distilled water and germinated in the dark at 20°C for 48 (C48), 72 (C72) and 96 hours (C96). After 48 hours, some seeds germinated under unstressed conditions (distilled water), whose roots were ≥1 mm, were transferred for next 24 h to osmotic stress conditions with polyethylene glycol (PEG), which caused decrease of water potential to −0.5 MPa (S72; 48 h distilled water + 24 h osmotic stress). After short-term osmotic stress, early seedlings of pea were transferred again to unstressed germination conditions for another 24 hours – recovery (SR96; 48 h distilled water + 24 h osmotic stress + 24 h distilled water). At the same time, other pea seeds were germinated under long-term osmotic stress conditions (−0.5 MPa) at 20°C for 96 hours (S96). Having fixed the time of germination, sprouts or early seedlings (according to Gong et al. [25]) were isolated from seeds and used for further tests. All determinations were repeated three times.

Polysome isolation

The tissue (400 mg) was homogenized in 3 ml of cytoskeleton-stabilizing buffer C [22], filtered and centrifuged at $27000 \times g$ for 10 min. Buffer C consist of 5 mM HEPES (N-2-hydroxyethylpiperazine-N″-2-ethanesulfonic acid), 10 mM Mg(OAc)$_2$ (magnesium acetate), 2 mM EGTA [ethylene glycol-bis (aminoethyl ether) N,N,N″,N″-tetraacetic acid], 1 mM PMSF (phenylmethylsulfonyl fluoride) adjusted to pH 7.5 with 9.8 mM KOH. This buffer allows sequential isolation of four polysome populations [26]: FP, MBP, CBP and CMBP. The supernatant polysomes included 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 (potassium acetate), 25 mM Mg(OAc)$_2$, 2 mM EGTA, 100 mg/ml heparin, 2% PTE (polyoxyethylene-10-tridecyl ether, a non-ionic detergent) and 1% DOC (sodium deoxycholate) [27] by adding ¼ vol. of 4× concentrated of buffer U and held on ice prior to further processing. The remaining polysomes were in the pellet and were sequentially solubilized as followed. The pellet was resuspended in 3 ml of buffer C + 0.5% PTE to disrupt membranes, centrifuged for 10 min at $27000 \times g$ to leave MBP in the supernatant (again converted to buffer U to maintain polysome integrity). All the other polysomes were still in the pellet. This pellet was next resuspended in 3 ml of buffer C + 200 mM Tris-HCl, pH 8.5 to disrupt the polysome-cytoskeleton interaction and centrifuged for 10 min at $27000 \times g$ to leave CBP in the supernatant (again converted to buffer U). The final pellet was resuspended in 4 ml of 1× concentrated of buffer U and re-centrifuged to release CMBP. All supernatant fractions (FP, MBP, CBP, CMBP) were layered on a 0.5 ml "pad" of 50% (w/v) sucrose in buffer B [50 mM Tris-HCl, pH 7.5, 20 mM KOAc, 10 mM Mg(OAc)$_2$] and centrifuged for 90 min at $300000 \times g$ in Beckman 65 Ti rotor. Sprouts or early seedlings of pea were also homogenized in buffer U [27] to solubilize total polysomes.

Polysomes protein separation with the Laemmli method

The content of protein was determined using Bradford's method [28]. The polysome pellets were resuspended in 0.15 ml of lysis buffer, consisting of 0.125 M Tris-HCl pH 6.8, 4% SDS, 10% 2-merkaptoethanol, 20% glycerol and 0.05% bromophenol blue. The resuspended polysomes were boiled at 95°C for 4 min and centrifuged at $27000 \times g$ for 3 min, after which they were cooled to 0–4°C.

Proteins connected with the total polysome pool and with particular polysome fractions were separated in 12% SDS-PAGE on an electrophoretic apparatus Mini-PROTEAN GEL II (Bio-Rad). Each time, 40 μg of protein was applied to each lane. Electrophoresis was run for 45 min at the temperature of 0–4°C and 200 V, using electrode buffer containing: 0.025 M Tris-HCl pH 3, 0.192 M glicyne and 1% SDS. The gels were fixed in 5% solution of trichloroacetic acid (TCA) for 30 min, then stained for 90 min with Coomassie Blue according to Laemmli [29], consisting of 0.1% Coomassie Blue R-250, 40% methanole and 10% acetic acid. When the protein had been stained, the gels were destained using a solution containing 25% of methanol and 10% of acetic acid. The gels were scanned with the Labscan 5.0, using an Image Scanner (Amersham). For the analysis of the gels, ScanGel 1.0 (Kucharczyk) was used.

Electrophoresis and Western Blotting of in vitro synthesized proteins

Sediments of the total ribosomal fraction and of particular populations of polysomes, i.e. FP, MBP, CBP and CMBP were dissolved in 0.05 ml of 0.5% PTE solution and centrifuged. For each sample, optical density (*OD*) was determined at the wavelengths of 260 and 330 nm. The difference between the result at 330 nm and the one at 260 nm was the value of the *OD* of a given sample. For an in vitro translation reaction, 2.0 *OD* of polysomes were needed.

Polysomes (2.0 *OD*) were mixed with the components of a translation mixture according to the instruction attached to the "Rabbit Reticulocyte Lysate System" (Promega), incubated at 30°C for 90 min, and then mixtures were placed on ice to stop the reaction. The content of a protein was established with Bradford's method [28]. The reagent mixture was dissolved in a lysing reagent (the contents as above) in a 1:4 ratio, heated at the temperature of 95°C for 4 min, centrifuged at $27000 \times g$ for 3 minutes and chilled to 0–4°C. The biotinylated proteins were separated in 12% SDS-PAGE according to Laemmli [29]. 20 μg of the protein was applied to each lane. The control sample, with which it was possible to eliminate the so-called background effect, contained all the components of the translation mixture (except polysomes) as well as the luciferase RNA standard.

The biotinylated proteins separated by electrophoresis transferred to polyvinylidene difluoride membrane (PVDF; Immobilon-P of the pore diameter equal 0.45 μm; Millipore). The electrotransfer was conducted at fixed voltage of 100 V for 60 min at 15–20°C. The buffer for the transfer contained 25 mM Tris, 192 mM glicyne, 20% methanole, 0.1% SDS. Afterwards, biotinylated proteins were visualized by binding Streptavidin-HRP, followed by chemiluminescent detection (Transcend™ Chemiluminescent Translation Detection System; Promega) and scanned using Labscan 5.0 software on an ImageScanner (Amersham). To determine the apparent weight of the translated biotinylated proteins, biotinylated protein standards (Bio-Rad) were used. For analysis, ScanGel 1.0 (Kucharczyk) was run.

Results

SDS-PAGE showed that small-molecule proteins – less than 40 kDa (Fig. 1) – dominated among the ribosomal proteins bound with the total population of polysomes as well as with their particular fractions, such as FP, MBP, CBP and CMBP. In the profile of polysomal proteins isolated from early pea seedlings grown under unstressed conditions and under long-term osmotic stress (−0.5 MPa), obtained after 96 hours of seed germination, qualitative and quantitative changes were observed. It was found out that in response to the osmotic stress the 22.0, 96.5 and 105.3 kDa proteins, absent in the control samples, bound with the total population of polysomes. It is noteworthy that these proteins (22.0 and 105.3 kDa) also bound to all fractions of polysomes (FP, MBP, CBP and CMBP) isolated from seeds germinated under stress conditions. Besides, in response to stress conditions, plants produced such proteins that bound to specific polysomal fractions, e.g. the 50.3 kDa protein which bound to the FP population, 58.9 kDa – to CMBP, and 101.4 kDa – to FP, CBP and CMBP. In turn the 23.0 kDa protein, which was present in pea seeds germinating under unstressed conditions, under osmotic stress was absent, both from the total polysomal population and from any of the polysomal fractions (Fig. 1). Furthermore, it was also observed that under the effect of water stress, the amounts of 23.5, 24.9, 50.3, 58.9 and 110.0 kDa proteins bound with the total population of polysomes increased in comparison with the control (Fig. 1). Some other proteins of the particular polysomal populations formed during the water stress of −0.5 MPa also occurred in either larger or smaller quantities than in the control. The proteins bound with MBP were observed in smaller amounts while the ones bound with FP increased (Tab. 1).

In vitro translation primed by total polysomes and their particular fractions (FP, MBP, CBP and CMBP) indicated that there were differences in the newly synthesised proteins, both during the seed germination under the unstressed conditions and between the control sample versus the samples treated with osmotic stress. These differnces were both quantitative and qualitative. During the germination under the unstressed conditions, the in vitro synthesis of 108.2 and 158.3 kDa proteins decreased, while that of 20.7 and 21.9 kDa proteins increased (Fig. 2). Under the effect of both long- and short-term osmotic stress (S96, S72), the 20.7, 21.9, 26.6, 108.2 and 158.3 kDa proteins were observed to be synthesized more effectively, and therefore appeared in larger quantities than under the unstressed conditions (C96, C72, respectively). However, during the recovery after the short-term osmotic stress (SR96), the synthsis of these proteins decreased in comparison with the sample exposed to the stress (S72).

With respect to in vitro translation which involved particular populations of polysomes isolated from 96-hour pea seedlings grown under the unstressed conditions and under long-term water stress of the intensity of −0.5 MPa, differences were mainly observed between the 108.2 and 158.3 kDa proteins. In pea seedlings grown under the unstressed conditions, the 108.2 kDa protein was synthesized mainly by the MBP as well as CBP and CMBP fractions, while the 158.3 kDa protein – by the FP, MBP, CBP populations and, only weakly, by CMBP. For comparison, in pea seedlings growing under long-term osmotic stress, the 108.2 kDa protein was synthesized in vitro mainly by the MBP and CBP populations and, to a smaller extent, by the FP and CMBP fractions, while the 158.3 kDa protein – by the MBP and CBP populations. It was also demonstrated that the most important differences between the osmotic stress and control samples appeared in the proteins which were synthesized on the CBP population. In response to the stress conditions, these proteins (>66 kDa) were more intensively synthesized than under the unstressed conditions (Fig. 3).
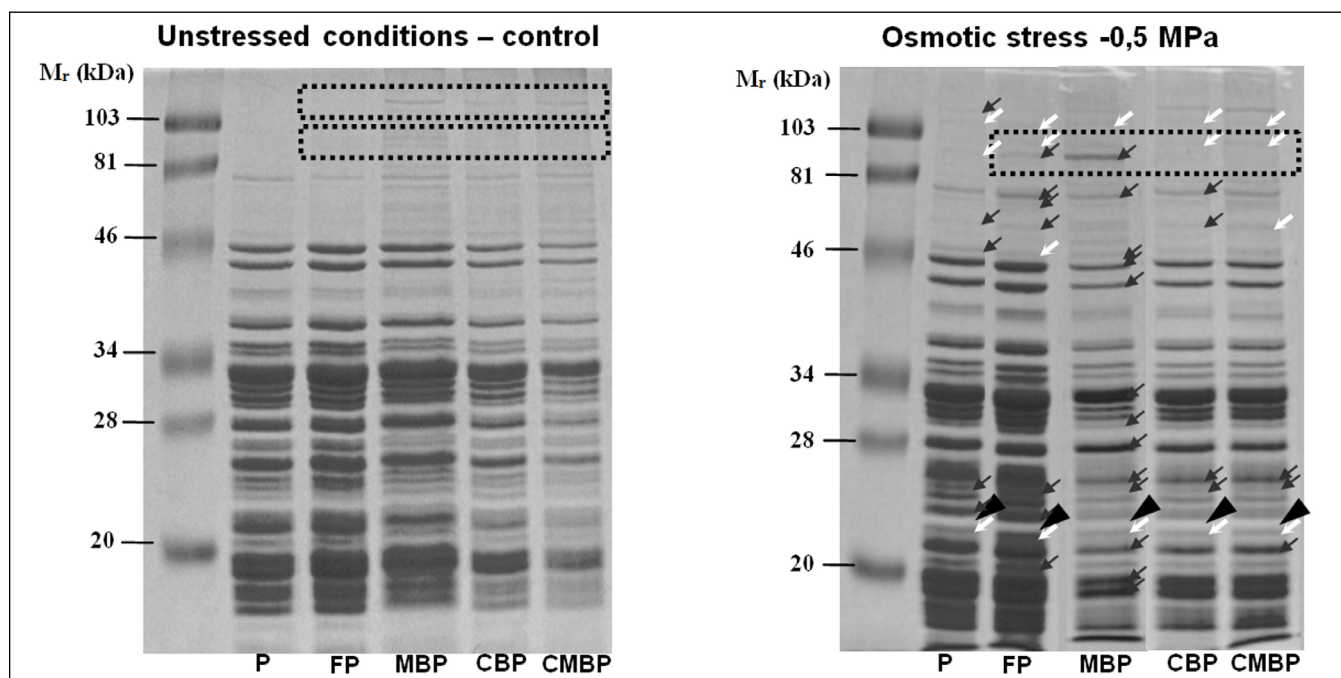
Fig. 1 SDS-PAGE of proteins bound to the total polysomal population and to particular polysomal fractions: FP, MBP, CBP and CMBP, isolated from embryonic tissue after 96 h of pea seed germination under unstressed (control) and long-term osmotic stress (−0.5 MPa) conditions. Molecular weight (kDa) of protein markers are given to the left. Black arrowheads – proteins which were absent under stress conditions; black frames – differences between proteins within a given sample; grey arrows – proteins different in quantities between control and stress samples; white arrowheads – proteins which were joined to polysomes during stress. P – total polysomal population.

Tab. 1 Differences in proteins associated with the total population of polysomes and with their particular fractions: FP, MBP, CBP, CMBP, isolated from embryonic tissue after 96 h of germination under unstressed (control) and long-term osmotic stress (−0.5 MPa) conditions.

| Protein (kDa)	Control					Osmotic stress −0.5 MPa				
	P	FP	MBP	CBP	CMBP	P	FP	MBP	CBP	CMBP
110.0	+	+	+	+	+	↗	+	+	+	+
105.3	−	−	−	−	−	+	+	+	+	+
101.4	+	−	+	−	−	+	+	+	+	+
96.5	−	+	+	+	+	+	↗	↗	+	+
77.8	+	+	+	+	+	+	↗	↗	↗	+
67.4	+	+	+	+	+	+	↙	+	+	+
58.9	+	+	+	+	−	↗	↗	+	↗	+
50.3	+	−	+	+	+	↗	+	↙	+	+
46.1	+	+	+	+	+	+	+	↙	+	+
32.0	+	+	+	+	+	+	+	↙	+	+
30.5	+	+	+	+	+	+	+	↙	+	+
28.0	+	+	+	+	+	+	+	↙	+	+
25.0	+	+	+	+	+	+	+	↙	↗	↗
24.9	+	+	+	+	+	↗	↗	↙	↙	↙
23.5	+	+	+	+	+	↗	↗	+	+	+
22.9	+	+	+	+	+	−	−	−	−	−
22.0	−	−	−	−	−	+	+	+	+	+
20.2	+	+	+	+	+	+	+	↙	+	↗
19.9	+	+	+	+	+	+	↗	+	+	+
18.3	+	+	+	+	+	+	+	↙	+	+
17.3	+	+	+	+	+	+	+	↙	+	+

"+" – presence; "−" – absence; "↗" – increased; "↙" – decreased of amount of a protein fraction joined with the total polysomal population and/or particular polysomal fractions in an experimental sample.

Discussion

The PEG used in our experiments is a neutral polymer which binds water, thus simulating soil drought stress [30,31]. Scientific experiments which simulate naturally ocurring conditions enable us to expand our knowledge of a given environmental stress factor and plant reactions to this stress, thus making it possible to obtain crops which are more tolerant to a given stress factor.

Osmotic stress inhibits the process of seed germination and subsequent plant growth [32–37] as well as the process of polysome formation [36,38,39]. Changes in the profiles of polysomes, which contain mRNAs undergoing translation, enable us to follow very precisely the rate of in vivo synthesis of proteins in cells [40].

The changes in the composition of polysomal proteins presented in this paper may play an important role in the regulation of translation under stress conditions and can result in the differentiated translational activity of particular fractions of polysomes, which had been demonstrated earlier [19,20,39,41,42]. The end products from in vitro translation of polysomes also reflect the activity of mRNAs that respond to stress [43]. Synthesis of most proteins under environmental stresses is reduced but translation of individual mRNA species is differentially regulated [23,44–46]. Kawaguchi et al. [45] showed that less than 1% of the stress-induced mRNAs increased significantly in polysome association, what can suggest that proteins encoded by this mRNAs were synthesized at higher levels during stress conditions. In turn on Shenton et al. [47] suggested that under low intensity of oxidative stress
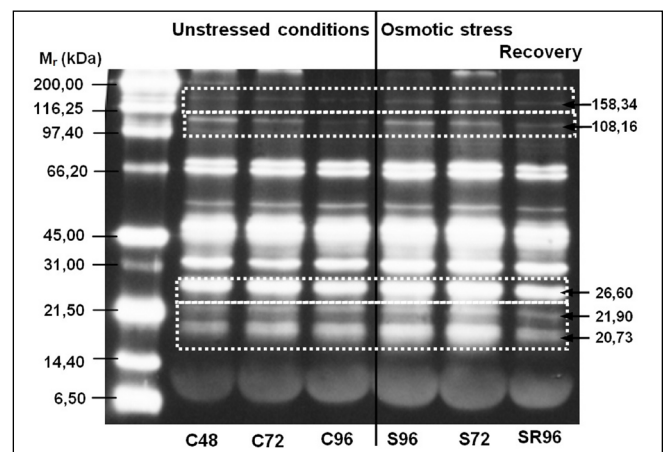
Fig. 2 Chemiluminescent detection of biotinylated proteins which were synthesized in vitro by total polysomal isolated from embryonic tissue of pea seeds germinated under unstressed conditions after 48 h (C48), 72 h (C72), 96 h (C96), under long-term (S96; 96 h osmotic stress) and short-term (S72; 48 h distilled water + 24 h osmotic stress) osmotic stress (−0.5 MPa) and during recovery after short-term osmotic stress (SR96; 48 h distilled water + 24 h osmotic stress + 24 h distilled water) separated by SDS-PAGE. Biotinylated proteins were visualized by binding Streptavidin-HRP, followed by chemiluminescent detection. Differences between the proteins are presented in the boxes and the arrows indicate precisely these protein fractions.
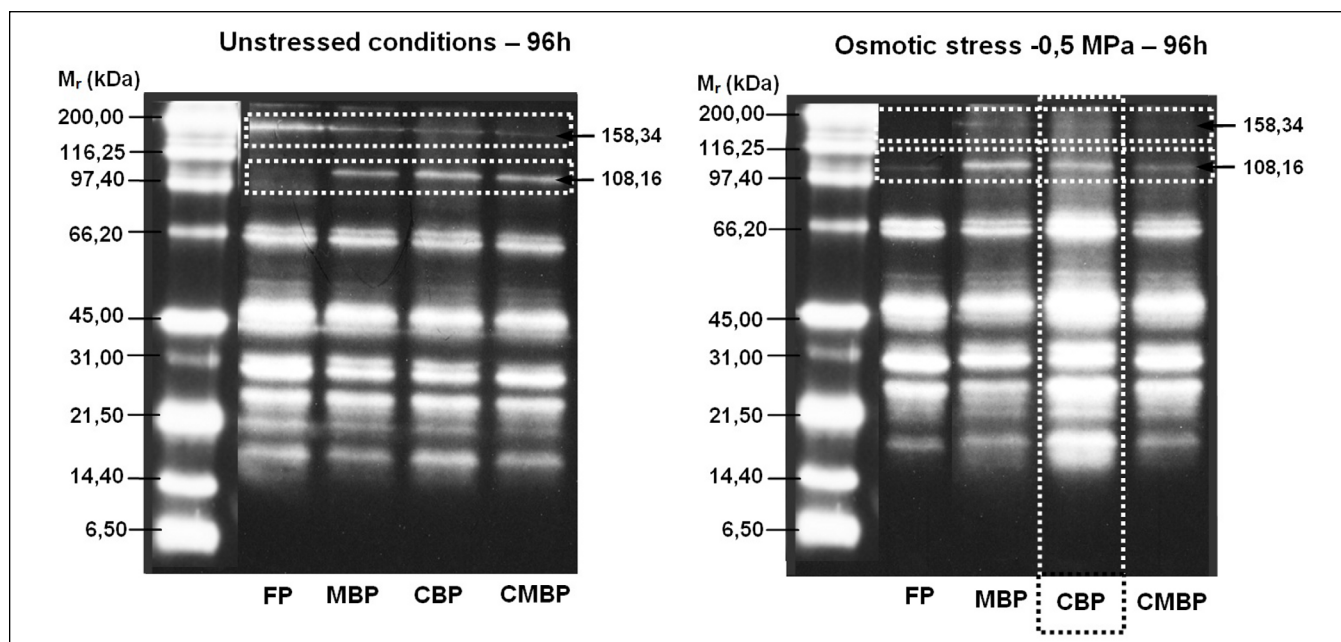
Fig. 3 Chemiluminescent detection of biotinylated proteins which were synthesized in vitro by particular polysomal fractions: FP, MBP, CBP and CMBP, isolated from embryonic tissue of pea seeds germinated under unstressed conditions (C96) and under long-term (S96) osmotic stress (−0.5 MPa) separated by SDS-PAGE. Biotinylated proteins were visualized by binding Streptavidin-HRP, followed by chemiluminescent detection. Differences between the proteins are presented in the boxes and the arrows indicate precisely these protein fractions.

translation of stress protective proteins increased, whereas under high intensity – mRNAs increased in polyribosome association but translation activity does not increased. Under the influence of osmotic stress, in this study, some proteins were synthesized in vitro more intensively by the total population of polysomes than in the optimal conditions too. Higher translational activity of polysomes during cold acclimation of wheat seedlings and wounding of potato tubers, compared to the control conditions, was also observed by Perras and Sarhan [48] and Morelli et al. [49], respectively. The most important change was the more effectively synthesis of five polypeptides under stress, suggesting that their higher expression was associated with tolerance capacity to this stress. The expression of specific proteins during stress conditions may be determined by presence of IRES (internal ribosome entry site) at mRNAs 5′ UTR region. Spriggs et al. [50] described that 10–15% of all mRNAs may be translated by IRES and there is coordinated translation of subsets IRESs during stress. This is probably alternative mechanisms of translation initiation selectively recruit mRNAs to polysomes during cell stress.

Davies et al. [19] described important role of the cytoskeleton in the translation process in plants. They suggested that initiation or elongation of the translation process, or else both of these stages, were faster on the cytoskeleton-bound polysomes (CBP and CMBP) than on polysomes not bound with the cytoskeleton (FP, MBP). Changes in translational activity may be determined by dynamics of the actin cytoskeleton. Depolimerization of actin filaments (e.g. under stress conditions) reduce translational activity, because components of the translational machinery (e.g. polysomes, initiation factors) are dispersed. In turn on, association of translational components with cytoskeleton may prevent diffusion of factors and thus increase translational activity [51]. The highest translational activity under water stress, in this study, was also demonstrated by the CBP. Higher translational activity of the CBP population than that of the FP or MBP fractions in bean tissues had

been shown previously by Klyachko et al. [41]. Their results confirmed some subsequent findings reported by Weidner et al. [20], who observed that most of exogenous 14C-amino acids were incorporated, in the embryonic tissue of pea, in the newly formed polypeptide chain, synthesized with the CMBP population. They claimed that the CBP population may play an important role in the synthesis of the so-called stress proteins [20,21]. According to Kosowska et al. [36], there must be some mechanism of regulating translation which is activated in seeds germinating under osmotic stress that enables synthesis of specific proteins despite the total protein synthesis being reduced.

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