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Response of Dormant Recalcitrant Horse Chestnut (*Aesculus hippocastanum* L.) Seeds to Heat Shock

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ABSTRACT

The effects of heat shock on the *in vivo* protein synthesis in the embryo tissues of dormant recalcitrant horse chestnut (*Aesculus hippocastanum* L.) seeds in the course of their stratification were studied. Embryo axes, cotyledon pieces, and cotyledon petioles were excised from seeds in different times after the start of cold stratification and incubated at 28 or 40°C on the medium containing ^{35}S-methionine for 4 h. It was established that, in all embryo parts, especially in axes, heat shock markedly activated protein synthesis in the beginning of stratification and to a lesser degree after ten weeks of stratification; heat shock suppressed protein synthesis at radicle emersion and especially during axial organ growth. None of dominating heat-stable proteins was synthesized either at 28 or 40°C. The synthesis of heat shock proteins did not depend on transcription and occurred on pre-existing mRNAs. First data were obtained concerning functioning molecular mechanisms providing for perception and transduction of heat signal and heat shock proteins synthesis in embryo cells of mature dormant horse chestnut seeds, which were in metabolically active state but could not germinate. The bulk of proteins synthesized by embryo parts at 28°C continued to be synthesized under conditions of heat shock simultaneously with heat shock proteins. It seems evident that, in the embryo cells, heat shock induced changes in gene expression and heat shock proteins synthesis but did not result in translational discrimination of mRNAs for non-heat-shock proteins. It is suggested that such response to heat shock is characteristic just of embryo tissues; it could be considered an additional molecular mechanism improving embryo tolerance to unfavorable environmental conditions.

Keywords: heat shock proteins, seed dormancy, stress tolerance

INTRODUCTION

Heat shock i.e., a short-term increase in temperature by 8-10°C above the optimum one, is well known to induce rapid transient and reversible changes in gene expression in all living organisms. These changes result in the synthesis of specific group of polypeptides called heat shock proteins and suppression (complete or partial) the synthesis of "normal" cell proteins synthesized by the cells before heat shock. This general biological phenomenon was qualified as a response to heat shock (Kimpel and Key 1985; Nagao *et al.* 1986; Vierling 1991). The universality and conserve character of this response indicate its importance in cell physiology. Since heat shock proteins accumulation at heat shock in the cells of plant vegetative organs and seedlings was correlated with the development of plant tolerance to subsequent action of lethal temperatures, it was suggested that the response to heat shock is a manifestation of molecular mechanisms providing for cell heat tolerance (Kulaeva 1997). During two recent decades occurred after heat shock proteins discovery, the notions concerning their properties and role gradually widened and became much more complex. Now, we know that many heat shock proteins are molecular chaperons and facilitate protein-protein interactions in the cell, that heat shock proteins can be present in normal cells not subjected to stress, that they can be expressed at some developmental stages in the absence of heat shock, and that their synthesis can be induced by other stress types (Sabehat *et al.* 1996; Walters *et al.* 1996; Wehmeyer *et al.* 1996; Wehmeyer and Vierling 2000). Changes in gene expression resulting in heat shock proteins accumulation in the cells are evidently play an important physiological role and somehow protect cell structures and separate protein components against injuries induced by various stressors and increase cell tolerance and their adaptation to unfavorable environmental conditions (Wang *et al.* 2004; Kosakovskaya 2008). Nevertheless, so far we did not decipher completely heat shock proteins functions and molecular mechanisms of their action. The possible heat shock effects on the synthesis of normal non-heat-shock proteins in various plant tissues are still less studied; it is not clear whether these effects are universal to the same degree as those of heat shock proteins gene expression. In many cases, heat shock suppressed the total protein synthesis and especially that of non-heat-shock proteins. This effect was evidently controlled on the level of translation because the normal pattern of protein synthesis was rapidly restored after the change in the temperature and transcription suppression with α-amanitin. It is known that heat shock inhibited total protein synthesis in the vegetative organs of seedlings and plant cell cultures. This was related not to the inhibition of non-heat-shock mRNA synthesis but to incapability of these mRNAs to be translated under heat shock conditions. However, this specific response to heat shock was not evidently universal because it was not observed in seed embryos during seed development and germination. Thus, it has been shown for soybean and common bean seeds that the synthesis of storage proteins and many other non-heat-shock proteins and their mRNAs was not reduced and even was activated under heat shock and occurred along with the synthesis of heat shock proteins (Mascarenhas and Altshuler 1985; Chrispeels and Greenwood 1987). In embryos of wheat (*Triticum aectivum* L.) (Helm *et al.* 1989), sorghum (*Sorghum bicolor* L.) (Howarth 1991), maize (*Zea mays* L.) (Riley 1981), and pea (*Pisum sativum* L.) (Gumilevskaya *et al.* 1996), heat shock activated markedly protein synthesis

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during early stages of germination, and heat shock proteins synthesis was induced simultaneously with the synthesis of the bulk of proteins produced by embryo tissues before heat shock. On the basis of these facts, it was concluded that such a specific response of protein synthesis to heat shock observed in seed embryos of many grasses during seed development and germination could have a definite physiological significance; this could be a manifestation of additional molecular mechanisms improving embryo tolerance to unfavorable environmental conditions and, as a consequence, their viability (Gumilevskaya *et al.* 1996). However, at presence the response of embryos to heat shock is studied predominantly on orthodox seeds (tolerant to desiccation) characterized by exogenous dormancy. Any data concerning the response to heat shock of embryos in tree seeds belonging to the recalcitrant type (sensitive to desiccation) and being in the state of deep physiological dormancy are essentially absent. To evaluate whether common features are characteristic of all embryo responses to heat shock, we tried to elucidate how the cells of axial organs excised from recalcitrant seeds at different stages of their dormancy release responded to heat shock; we compared seeds in deep physiological dormancy and those subjected to cold moist stratification to release dormancy, i.e., seeds from the moment of their falling to the start of radicle emergence. The reasons and mechanisms providing for dormancy state, dormancy release, and sensitivity to desiccation are unclear so far. We worked with the seeds of horse chestnut, which have been studied by us earlier (Gumilevskaya and Azarkovich 2007). These seeds entry dormancy at a high content of water and maintain metabolic activity but cannot germinate without preliminary long stratification. As we have observed earlier, embryo axes excised from mature and stratified horse chestnut seeds could synthesize proteins and RNAs and grow *in vitro*. Their growth was inhibited by ABA treatment; these axes were enriched in heat-stable albumins but deficient in globulins; they contained a single heat-stable dehydrin with a mol wt of about 50 kD reacting with the antibody to one of heat shock proteins, ubiquitin, and a single heat-sensitive dehydrin-like protein with a mol wt of 80 kD (Gumilevskaya and Azarkovich 2007, 2010).

The objective of this work was to characterize the response to heat shock of embryo axes and other embryo parts excised from horse chestnut seeds. To this end, heat shock action on protein synthesis in embryo tissues, the pattern of synthesized polypeptides, their intracellular distribution, their relation to heat denaturing, and relation to the seed physiological state were analyzed.

MATERIALS AND METHODS

Materials

Experiments were performed on the seeds of horse chestnut (*Aesculus hippocastanum* L.) collected immediately after their falling in the Moscow parks in 2002-2007 and subjected to moist cold storage (stratification) at 4°C for 19-20 weeks until visible germination, i.e., radicle emersion.

Treatments

Seeds taken in different times after the start of stratification were surface-sterilized, washed with run tap water and then distilled water for 1 h, and embryo axes were excised manually. Portions of axes (five axes) were incubated in darkness for 4 h at 28 or 40°C in double distilled water containing ^{35}S-methionine (100 μCu/ml) (^{35}S-methionine was obtained from the Institute of Physics and Power Ingineering, Obninsk, Russia) and 50 μg/ml of chloramphenicol (Serva, Germany) with the addition of 7 μg/ml of α-amanitin (Sigma, Unated States) or without it. Thereafter, axes were washed with a great volume of distilled water and frozen. Frozen axes were homogenized in 0.25 M sucrose prepared in 0.05 M Tris-HCl (pH 7.2) containing 0.01 M Mg-acetate, 0.025 M KCl, 7 μg/ml of pepstatine, 5 μg/ml of leupeptine, and 1 mM PSMF (Serva, Germany). The homogenate was subjected to differential centrifugation. As a results, we obtained the following fractions: total cell extract (1000 *g* supernatant), cell structures (20,000 × *g* pellet), and postmitochondrial cell extract (20,000 *g* supernatant). Postmitochondrial cell extract was subjected to heat treatment (5-10 min at 70-80°C) to separate heat-stable and heat-sensitive proteins. In the total cell extract (1000 × *g* supernatant), TCA-soluble and TCA-insoluble radioactivity was measured, i.e, ^{35}S-methionine uptake and incorporation into proteins. In aliquots of each fractions, proteins precipitated with TCA and subjected to SDS-electrophoresis. Electrophoresis was performed under reducing dissociating conditions on the gel plates with 10-20% acrylamide gradient. Gels were stained with Coomassie R-250 (Sigma, United States), dried, and exposed to the Retina Kodak roentgen film (Kodak, Germany). The images obtained were transferred to the phototechnical FT11 film (Phomos, Russia) by a contact mode. All experiments were run in at least three replicates with three recordings each. All above described procedures, material treatment, and also the volumes and concentrations of used solutions and quality of used reagents have been described earlier in detail (Gumilevskaya *et al.* 2001, 2003; Azarkovich and Gumilevskaya 2006).

RESULTS

Effect of heat shock on the protein-synthesizing capacity of embryo axes excised from dormant recalcitrant horse chestnut seeds in the course of stratification

We have demonstrated earlier that, in dormant horse chestnut seeds, embryo axes do not manifest their own dormancy, and, under *in vitro* conditions, they become adequate to axes of *in vivo* protruded intact seeds in many characteristics. It has been established that, the cells of axial organs and cotyledon storage parenchyma of fallen mature seeds retain an active translational machinery; when these embryo tissues were separated from each other, they could synthesize *in vivo* a great number of diverse polypeptides (Gumilevskaya *et al.* 2001, 2003; Gumilevskaya and Azarkovich 2004; Azarkovich and Gumilevskaya 2006). It has been also noted that the total content of proteins in axes does not change during stratification until radicle emersion. Therefore, the level of specific radioactivity of total proteins in axes isolated and pulse-labeled in different times after the start of stratification can serve as a measure of the rate of protein synthesis. To assess heat shock effect on protein-synthesizing capacity of the cells in embryo axes in the course of stratification and to improve our understanding a possible relation between the response to heat shock and the state of deep dormancy of intact seeds, a great number of experiments was performed with the incorporation of ^{35}S-methionine into proteins of excised axes at 28 and 40°C. Experiments were performed with freshly collected seeds during 2002-2007 years and stratified for 17-19 weeks until visible germination, i.e., radicle emersion. The results obtained are presented in **Fig. 1** where the ratios between radioactivities of total axial proteins at 40 and 28°C in different times after the start of stratification are shown. In the axes of freshly collected and briefly stratified (2-3 weeks) seeds, heat shock usually activated protein synthesis. Thus, specific protein radioactivities of the coarse cell extract (1000 *g* supernatant) at 40 and 28°C in different years were 1.7×10^6 and 1.35×10^6; 3.7×10^6 and 3.3×10^6; and 8.7×10^6 and 8.2×10^6, respectively. The levels of label uptake at 40 and 28°C differed insignificantly. In some cases, label incorporation at 40 and 28°C was similar. Some variability of protein synthesis response to heat shock could be related to heterogeneity of seeds selected for experiment because the process of entry in dormancy is not synchronous one for seed population and occurring physiological and biochemical changes and tests for their detection are not elucidated so far. In spite of this variability in the response to heat shock, it seems evident that axes excised from freshly fallen mature horse chestnut seeds, being in the state of deep dormancy, responded to heat shock by some activation of the

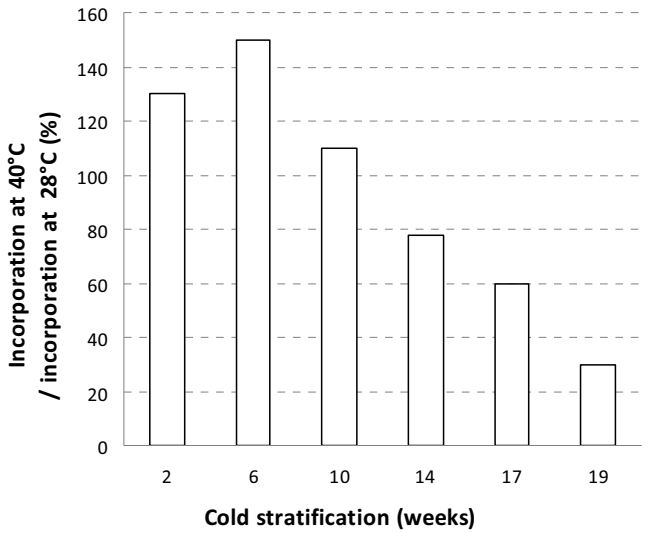

Fig. 1 Effect of heat shock on protein synthesis in axes isolated from dormant horse chestnut seeds.

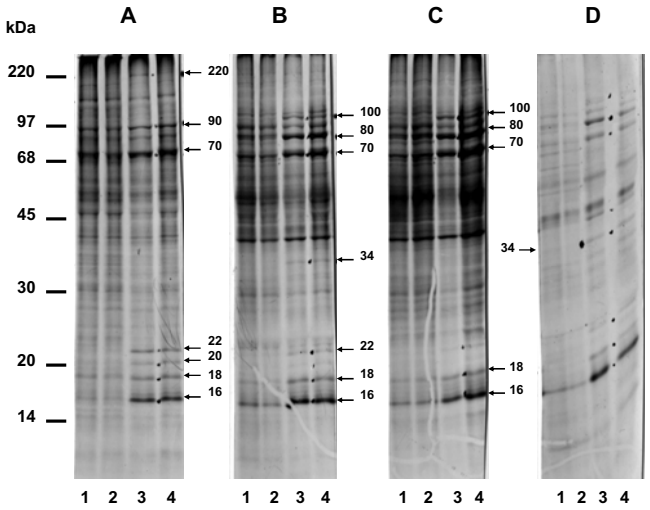

Fig. 2 Synthesis of heat shock proteins in isolated axes of dormant horse chestnut seeds. (**A**) ^{35}S-proteins of 20 000 *g* pellet; (**B**) total cytosolic ^{35}S-proteins; (**C**) cytosolic heat-sensitive ^{35}S-proteins; (**D**) cytosolic heat-stable ^{35}S-proteins; lines 1 and 2 - 28°C, lines 3 and 4 - 40°C; lines 1 and 3 – control; lines 2 and 4 – with α-amanitin.

translational machinery. As evident from **Fig. 1**, heat shock effect on translational activity of the cells in isolated embryo axes depended on the time of stratification. In the beginning of stratification, specific radioactivity of total protein in heat shock-treated axes was by 50-70% higher than in control axes. After 10 weeks of stratification, it was only by 10% higher, and then, in 14 and 17 weeks of stratification, it became by 20-40% lower than in control axes. In axes excised from protruded seeds, heat shock suppressed protein synthesis substantially, and the level of total protein radioactivity in such axes was by 2-3 times lower than in control axes. Thus, it is evident that heat shock activated markedly translation in the cells of axial organ excised from seeds during the first half of stratification and suppressed markedly translation before and especially after radicle emersion. The presented material indicates that axes excised from dormant seeds are capable of protein synthesis at both 28 and 40°C during the entire period of stratification, but they manifest different sensitivity to heating. This can indicate indirectly some changes in the axis physiological state occurring in the course of stratification of intact dormant seeds. Additional confirmation of this suggestion was obtained in experiments on the effect of various physiologically active compounds (abscisic acid, indol-3-acethyc acid, 6-benzylaminopurine) on *in vitro* growth of isolated axes (Gumilevskaya and Azarkovich 2004). These compounds exerted the stronger effects in the first than in the second half of stratification. So far, the reasons of these changes are not clear. However, it might be that these changes could be related to protein synthesis occurring although at the low rate during stratification, i.e., at 5°C. For detection of this process, long exposure to the label is required (Gumilevskaya and Azarkovich 2007). Since stratification lasts 19-22 weeks, it might be that some polypeptides are synthesized for this long period and they change the balance between proteins inhibiting germination (if there are such proteins) and proteins required for germination, and as a result, the physiological state of embryos changes. In its turn, this can reflect on the response to heat shock and action of physiologically active compounds. On the other hand, the effect of cotyledons on the state of axial organs cannot be excluded. The interrelation and interaction between these embryo organs in dormant recalcitrant horse chestnut seeds is poorly studied so far.

Heat shock effect on the pattern of protein synthesis

To evaluate the effect of heat shock on gene expression on the level of protein synthesis, it was necessary to learn which polypeptides are synthesized at heat shock, which of them could be referred to heat shock proteins, what is their localization, a dependence of their synthesis on transcription level and time of stratification. To this end, isolated axes were *in vivo* labeled at 28 and 40°C for 4 h, separated by one-dimensional electrophoresis, and radioautograms were obtained. Earlier, we have shown that not only total protein content but also their distribution between subcellular fractions was not changed during stratification (Gumilevskaya *et al.* 2003). The fraction of cellular structures (20,000 *g* pellet) comprised only small portion of cell extract proteins (about 15%). The bulk of protein was present in the postmitochondrial extract; 70% of theses proteins were heat-sensitive and 30% were heat-stable proteins (Gumilevskaya *et al.* 2001). Proteins of cell structures were labeled most intensely; specific radioactivity of heat-sensitive cytosolic proteins was by 2.0-2.5 times higher than that of heat-stable proteins. As was mentioned above, heat shock activated protein synthesis during the first half of stratification period and later reduced it markedly (**Fig. 1**). Substantial differences in the levels of specific radioactivity of total protein in control axes and those subjected to heat shock and also of proteins in different sub-cellular fractions complicated markedly their comparative analysis by the methods of one-dimensional SDS-electrophoresis and radioautography. Nevertheless, in spite of limitation of the methods used, numerous experiments performed in several years with variations in the protein content, radioactivity, and the duration of gel exposure to roentgen film, some well reproducible and specific features of heat shock influence on gene expression on the level of protein synthesis in the cells of embryo axes excised from dormant recalcitrant horse chestnut seeds in the course of stratification were established. Electrophoretic and radioautographic analyses of labeled axial proteins showed that, independently on the duration of stratification gradually releasing horse chestnut seed deep dormancy, excised axes synthesized *in vivo* a great number of polypeptides at 28 or 40°C (**Fig. 2**). The set of these proteins changed little during stratification. Newly synthesized polypeptides were present in the fraction of cell structures and in the cytosol and differed in molecular weights, the labeling intensity (especially under heat shock), and a degree of their tolerance to heat denaturing. A comparison of stained gels and radioautoradiograms showed that none of dominating polypeptides initially present in the axes and belonging to heat-stable cytosolic proteins was synthesized at normal temperature or heat shock. This means that proteins synthesized in response to heat shock

were not characterized by any specificity in their intracellular localization. This was confirmed by the analysis of specific radioactivity of proteins in different subcellular fractions obtained from labeled control axes and those subjected to heat shock. It was established that heat shock did not induce changes in the pattern of labeled polypeptides inside the cell. Like in control axes, proteins of subcellular structures were labeled most intensely and radioactivity of cytosolic heat-sensitive proteins was by 2.0-2.5 times higher than that of heat-stable proteins.

One of characteristic features of the protein synthesis pattern is its likeness with that in control axes. Most of proteins synthesized by axes at 28°C continued to be synthesized under heat shock conditions. However, some polypeptides started to be synthesized at 40°C or their synthesis was greatly enhanced. These polypeptides we referred to heat shock proteins. The two groups of heat shock proteins with high (above 65-82 kD) and low (16-22 kD) molecular weights were labeled most intensely. Weakly labeled and minor components were present in different zones of the gel and sometimes were not detected in the total protein of the cell extract. To detect most heat shock proteins and characterize their intracellular distribution, total proteins of the cell extract (1000 × *g* supernatant) and proteins of the subcellular structures (20,000 × *g* pellet) and cytosol (20,000 × *g* supernatant), and also heat-sensitive and heat-stable cytosolic proteins were analyzed (**Fig. 2**). As a result, at least 10 heat shock proteins with mol wts of about 220, 100, 90, 82, 74, 34, 22, 20, 18, and 16 kD were detected. All heat shock proteins, except for 220 and 34 kD heat shock proteins, were labeled most intensely at heat shock. Some of them were characteristic of cell structures (220, 90, 20, and 18 kD), others were detected only in the cytosol (100, 76-82, and 34 kD). Heat shock proteins with mol wts of 68-72 and 82-86 kD and also 16-18 kD were present in both cell structures and cytosol. All cytosolic heat shock proteins, except for 34 kD and partially 16 kD protein, belonged to heat-sensitive proteins: they coagulated rapidly at heat treatment (5 min at 75°C). Low-molecular (16 kD) heat shock proteins and traces of high-molecular heat shock proteins could be detected also among heat-stable proteins. Our results concerning the number and intracellular distribution of heat shock proteins is not absolutely precise because mutual fraction contaminations are not excluded and the presence of several electrophoretically similar heat shock proteins in the zone of gel blackening on the radioautograms is also possible. Nevertheless, the results obtained demonstrated that the cells of embryo axes excised from dormant recalcitrant horse chestnut seeds responded to heat shock by the intense synthesis of heat shock proteins with mol wts above 60 kD characteristic of all living organisms and heat shock proteins with mol wts of 16-30 kD characteristic of plants, and that predominant heat shock proteins belonged to heat-sensitive soluble cytosolic proteins.

Earlier, we have demonstrated that low concentrations of α-amanitin (7 μg/ml), usually applied for inhibition of mRNA synthesis, did not affect the protein-synthesizing capacity of embryo axes excised in the course of stratification but inhibited it in the axes of germinated seeds (Gumilevskaya *et al.* 2003). Experiments with α-amanitin performed in this study confirmed this observation and showed that the synthesis of heat shock proteins, like non-heat-shock proteins, displayed low sensitivity toward α-amanitin. This allows us to believe that the stores of mRNAs are present in the embryo cells of mature dormant recalcitrant horse chestnut seeds, which were synthesized during seed development and retained in the embryo axis cells after seed falling in the state ready for translation and could direct the synthesis of diverse cellular polypeptides and heat shock proteins at both 28°C and heat shock under favorable conditions. Similar pattern was observed also in the axes from non-stratified seeds. This means that heat shock proteins genes were activated still during seed development and their expression on the level of protein synthesis could be induced in any time independently of the time of seed stratification and their capability of germination. This does not exclude a possibility of α-amanitin action on the synthesis of minor protein components, which contribution to protein synthesis is insignificant and for which detection other methods of analysis are required.

In this work, first data are obtained about functioning of molecular mechanisms providing for perception and transduction of heat signal and inducing heat shock proteins synthesis in the cells of embryo axes of dormant recalcitrant seeds, which are in metabolically active state but could not germinate. The analysis of heat shock effect on gene expression on the level of protein synthesis in embryo axes excised from dormant recalcitrant horse chestnut seeds in the course of stratification demonstrated that heat shock changed gene expression and induced heat shock proteins synthesis but did not discriminate translation of non-heat-shock mRNAs.

DISCUSSION

Plant seeds are a unique object for studying the mechanisms of tolerance and adaptation to unfavorable environmental conditions. The reasons are as follows. The seeds could not escape unfavorable environmental conditions but must adapt to overcome them, to retain a capability of germination, and to fulfill their physiological destination, i.e., species preservation and distribution. Furthermore, in seeds the developmental program of the individual plant is switched over from embryogenesis to germination; in the periods of seed development and germination, seed embryos, being subjected to the action of unfavorable conditions, must change cell activity on the level of gene expression and induce the synthesis of anti-stress proteins to protect themselves and overcome stress effects. On the other hand, embryos have to provide expression of genes for proteins required for further development, i.e., germination *per se*. According to current knowledge, accumulation of heat shock proteins in the cell is a universal factor of defense against stress (Wehmeyer and Vierling 2000; Burrke and O'Mahony 2001; Jaya *et al.* 2009); the synthesis of these proteins is readily detected under short-term temperature increase, i.e., at heat shock. In the cells of vegetative organs, this is accompanied by a transient reduction or complete cessation of the synthesis of the bulk of cell proteins. However, studies performed with embryos of orthodox seeds of many plant species during seed development and germination (Mascarenhas and Altshuler 1985; Gumilevskaya *et al.* 1996) showed that induction of the heat shock proteins synthesis occurred in them on the background of continuation of the synthesis of non-heat-shock proteins and was accompanied by enhanced protein-synthesizing activity. It remained unclear whether such response to heat shock was characteristic for just embryo cells or it was determined by the seed type and the depth of their dormancy. The results of this work showed that embryo tissues isolated from dormant recalcitrant horse chestnut seeds responded to heat shock similarly as embryos of orthodox seeds with exogenous dormancy. At heat shock, the synthesis of high- and low-molecular heat shock proteins occurred simultaneously with the synthesis of the bulk of cellular proteins produced by axes before heat shock and was accompanied by activation of protein synthesis during the first half of the stratification period. However, in the axes excised from germinated seeds, heat shock induced the heat shock proteins synthesis but reduced total protein synthesis, like in the cells of vegetative organs.

Thus, heat shock proteins gene induction without discrimination of non-heat-shock mRNA translation is a characteristic feature of plant seed embryo response to heat shock. This specific feature does not depend on seed tolerance to desiccation (orthodox or recalcitrant seeds) and the type of their dormancy (deep or exogenous dormancy); it is manifested during seed development and early stages of germination. A definite physiological sense of such response could be suggested. Thus, at the final stages of seed deve-

lopment, when the active synthesis of storage deposits occurs, tolerance of non-heat-shock mRNA translation to transient action of unfavorable conditions would facilitate the formation of well-developed embryo and provide for its subsequent successful germination (Wehmeyer *et al.* 1996; Wehmeyer and Vierling 2000; Kotak *et al.* 2007). Tolerance of translation of the bulk normal non-shock mRNAs to transient action of unfavorable conditions (for example heat shock) during early stages of germination, which has been earlier observed in orthodox seeds with exogenous dormancy (Gumilevskaya *et al.* 1996) and now found in recalcitrant seeds being in deep dormancy, can also play an important physiological role. Due to this specific embryo response to heat shock, at early stages of germination and even under unfavorable conditions, embryo cells retain a capability of continuation or supporting on the sufficient level of the synthesis of proteins required for cell activity switching over to new developmental program, from embryogenesis to germination, and thus increase the reliability of germination. We believe that the absence of discrimination of non-heat-shock mRNA translation during heat shock is specific for embryo tissues and could be considered an additional mechanism facilitating seed adaptation to unfavorable environmental conditions and successful germination.

Induction of heat shock proteins synthesis is a universal feature of the response to heat shock. According to our data, all tissues isolated from the embryos of dormant recalcitrant horse chestnut seeds responded to heat shock not only by continuation of non-heat-shock protein synthesis but also by induction of similar sets of heat shock proteins. Two observations are of interest. We did not observe any dramatic changes in the set of heat shock proteins synthesized by isolated axes in the response to heat shock in the course of stratification, which evidently facilitate seed deep dormancy release. Moreover, heat shock proteins synthesis was readily detected in axes excised from non-stratified seeds, i.e., did not depend on seed capability of germination. This indicates independence of heat shock proteins synthesis at heat shock in the course of stratification of the embryo physiological state and its capability of germination. At the same time, some of our data indicate that stratification still somehow affected embryo physiological state. Thus, in the course of stratification, sensitivity of isolated axis growth to abscisic acid and indol-3-acethyc acid decreased (Gumilevskaya and Azarkovich 2004), some characteristics of the proteome changed (Gumilevskaya *et al.* 2001), sensitivity of isolated axis translation to heat shock changed as well. However, this did not affect embryo tissue capacity to respond to heat shock. It is likely that signals providing for dormancy state, its release, and seed germination do not interact with signals leading to heat shock proteins synthesis induction. Furthermore, heat shock proteins gene expression in isolated axes of dormant recalcitrant horse chestnut seeds was not dependent on transcription and was controlled predominantly on the level of translation. This means that all components required for the complex molecular mechanism of heat shock proteins gene expression were present in axis cells of mature seeds and were evidently produced still during seed development, may be under the influence of elevated temperatures. After mature seed falling, this mechanism is retained in the cells in the functionally active state and is capable of a rapid initiation of heat shock proteins synthesis in response to heat shock or another stress. However, the realization of this mechanism of heat shock proteins accumulation under natural conditions of stratification seems not very probable because the rate of protein synthesis under low temperature is low and heat signal is absent. Nevertheless, horse chestnut seeds survive successfully the period of long cooling (18-22 weeks), retaining the high water content in their cells and thus they are well adapted to overcome or correct damages arising under these conditions. However, according to current knowledge, just fulfill this protective function. Therefore, it might be that, in mature dormant horse chestnut seeds, some amounts of required heat shock proteins are already present. These heat shock proteins could be synthesized and accumulated under the influence of elevated temperatures in the embryo cells during seed development or after their falling, i.e., in response to heat shock, and they were preserved in the cells after seed entry into deep dormancy in metabolically active state; they could improve embryo tolerance to unfavorable environmental conditions during stratification and thus increase seed viability. This suggestion is supported by our observation that one of heat shock proteins, ubiquitin, was present in dormant horse chestnut seeds in functionally active state (i.e., in association with dehydrins) (Gumilevskaya and Azarkovich 2010).

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