Chapter 13

Stress-Induced Proteins in Recalcitrant Seeds During Deep Dormancy and Early Germination

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Additional information is available at the end of the chapter

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Abstract

The role and functions of dehydrins and heat shock proteins in seeds (especially in desiccation-sensitive recalcitrant seeds) are discussed.

During the periods of dehydration, a wide variety of plants can express dehydration proteins (dehydrins), which are also members of the plant late embryogenesis abundant (LEA) protein family. Dehydrins have been most extensively studied in relation to drought and cold stresses. Dehydrins are synthesized in orthodox seeds, and their development at the final stage is associated with genetically determined seeds drying. Dehydrins amount can reach 4% of total cell proteins. At the same time, dehydrins are found in desiccation-sensitive recalcitrant seeds. The following are the functions of dehydrins with experimental evidence: binding to water and ions, binding to phospholipids, radical scavenging, phosphorylation, binding to calcium, protection of enzymes, binding to cytoskeletons, and binding to nucleic acids.

It seems evident that, in the embryo cells, heat shock induced changes in gene expression and HSP synthesis but did not result in translational discrimination of mRNAs for non-heat shock proteins. Such specific feature has been observed earlier for orthodox seeds during their development and early stages of germination. It is suggested that such response to HS is characteristic just of embryo tissues; it could be considered an additional molecular mechanism improving embryo tolerance to unfavorable environmental conditions.

Keywords: Recalcitrant seeds, temperature stress, dehydrins, heat shock proteins

1. Introduction

Plant seeds are a unique object for studying the mechanisms of tolerance and adaptation to abiotic stresses. 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, that is, 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, that is, germination *per se*.

Different environmental stresses to a plant may result in similar responses at the cellular and molecular level. This is due to the fact that the impacts of the stressors trigger similar strains and downstream signal transduction chains. Stressors such as drought (lack of environmental water), salinity (high osmolarity), and cold, especially frost (lack of liquid water), induce water deficiency [1]. All three forms of abiotic stress affect the water relations of a plant on the cellular as well as whole plant level, causing specific as well as unspecific reactions, damages and adaptation reactions. The stabilizing effect of liquid water on the membrane can be supported by compatible solutes and special proteins. At the metabolic level, osmotic adjustment by synthesis of low-molecular osmolytes (carbohydrates, betains, proline) can counteract cellular dehydration and turgor loss [1].

During the periods of dehydration, a wide variety of plants can express dehydration proteins (dehydrins), which are also members of the plant late embryogenesis abundant (LEA) protein family. For the first time, LEA proteins were characterized in ripening seeds [2]. But they are widely investigated now [3–5]. However, their precise role has not been clear yet. These proteins are supposed to protect cells from water loss; they can behave as molecular chaperones [2]. Now the expression of LEA proteins has been shown not only in ripen seeds but also in other plant (or animal) tissues. LEA proteins are induced by osmotic or cold stress, by exogenous ABA (abscisic acid) [6]. It is believed that LEA proteins are non-catalytic proteins. Practically, all LEA proteins are water-soluble hydrophilic heat-stable and unfolded proteins. LEA proteins are synthesized in orthodox seeds, and the development of which at the final stage is associated with genetically determined drying. Their amount can reach 4% of total cell proteins [7]. At the same time, LEA proteins are found in desiccation-sensitive recalcitrant seeds. That is why the study of recalcitrant seeds is of great interest. On the one hand, these seeds produce LEA proteins, and on the other hand, they are sensitive to desiccation. Therefore, a characterization of LEA proteins in recalcitrant seeds can help understanding their role, for example when plant cells are challenged by abiotic stress as cold or salt stress.

LEA proteins are classified into more than seven distinct groups [8]. Among the induced LEA proteins, dehydrins (group II of late embryogenesis abundant proteins) have been most commonly studied, but our knowledge of their fundamental role in the cell is incomplete.

2. Dehydrins—stress-induced proteins

The accumulation of dehydrin transcripts and proteins during dehydration and a correlation between the level of drought tolerance and the amount of dehydrin present strongly suggest

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that they are involved in protecting the plant from the negative effects of dehydration [9–11]. In recent years, dehydrins are studied very intensively, which resulted in the appearance of numerous reviews and research papers [12–18].

The dehydrins are very hydrophilic proteins and exhibit an unusually low level of recognizable structure [10, 19]. The main characteristic of the dehydrins is the presence of one or more lysine-rich stretches of 15 amino acids (the sequence EKKGIMKIKEKLPG), called the K motifs (or K-segments), that are predicted to form class A amphipathic alpha-helices [20, 21]. All dehydrins investigated have K-segment; it is usually used for preparing anti-dehydrin antibodies [12, 13]. The K-segment occurs 1–12 times, with 1 or 2 repeats being the most common. Since the K-segments can form amphipathic alfa-helices, they may stabilize membranes against dehydration. Dehydrins can also contain two other motifs: an N-terminal Y segment (consensus V/TDE/QYGNP) and a serine-rich S segment [11]. The Y-segment is similar to the nucleotide-binding domain found in bacteria. Typically, 1–3 Y-segments are present at the N-terminus of a dehydrin [11]. The S-segment contains a tract of Ser residues and is present in one or no copies in a dehydrin. Dehydrins extracted from drought-stressed plants are phosphorylated on these serines [22]. The role of phosphorylation is not clear but may be correlated with translocation of dehydrins to the nucleus [23] or the increased negative charge could enhance the ability of the protein to bind divalent cations such as zinc. It has been proposed that the short amphipathic K segments of dehydrin polypeptides interact with solvent-exposed hydrophobic patches on proteins undergoing partial denaturation and thereby interfere with protein aggregate formation [10]. Amphipathic K helixes could also be involved in binding membrane lipids and thus could play a more specific role in protecting lipoproteins, proteins located in membranes, and/or the membrane structure itself [10, 24].

According to the presence of the K-, S-, and Y-segments, dehydrins can be divided into five structural subgroups: Kn, SKn, KnS, YnKn, and YnSKn [10, 11]. Although not specifically included in the YSK naming system, dehydrins also contain Φ-segments, which are rich in Gly, Thr, and many other polar amino acids. This poorly conserved segment tends to be located between the Y-, S-, and K-segments.

Dehydrins are evolutionarily conserved among photosynthetic organisms including angiosperms, gymnosperms, ferns, mosses, liverworts, algae and cyanobacteria, as well as in some non-photosynthetic organisms such as yeast [17, 25]. Dehydrins seem to be very ancient proteins—a 40-kD protein was observed in Calothrix sp. strain PCC 7601, and in *Nostoc* sp. strain Mac-R2, an osmotic-induced doublet at 39 and 40 kD was observed. It appears that cyanobacteria produce a dehydrin-like protein under osmotic stress [25].

The expression of many dehydrins increases by the phytohormone abscisic acid (ABA), they are also referred as RAB proteins (Responsive to ABA) [26–28]. On the other hand, protein level of some dehydrins is regulated by low temperature only [22]. In particular, studies of stress-induced accumulation of five dehydrins in *Arabidopsis* revealed that two of them (LTI30 and COR47) accumulated primarily in response to low temperature. The level of another two proteins (ERD14 and LTI29) was upregulated by ABA and low temperatures, whereas RAB18 was only found in ABA-treated plants [22]. Borovsky and coworkers [15] have found that not only cold but also drought, freezing, and exogenous ABA treatment also result in accumulation

of heat-stable dehydrin-like proteins in plant mitochondria. The most tolerant winter wheat and rye accumulate more of the heat-stable dehydrins than maize. Cold-induced accumulation of the heat-stable mitochondrial dehydrin-like proteins in all species studied was accompanied by increasing of plant cryotolerance [15].

Dehydrins have been most extensively studied in relation to drought and cold stresses [29–35]. Some experimental studies provide evidence that dehydrins contribute to freezing stress tolerance in plants and suggest that this could be partly due to their protective effect on membranes [36]. Dehydrins stabilize plant plasma and organellar membranes in conditions of stress, and further zinc may be an important co-factor in stabilization [37, 38].

The hypothesis that dehydrins have detergent and chaperone-like properties and may interact with compatible solutes to serve as structural stabilizers of macromolecules under conditions of water deficit [10] is now experimentally evident [17, 39].

Numerous *in vitro* functions have been described and proposed for dehydrins, including cryoprotection of lactate dehydrogenase (LDH), cryoprotection of purified protoplasts and chloroplasts, prevention of water loss, binding of excess ions, binding of nucleic acids, prevention of protein aggregation at elevated temperatures, and prevention of ice crystal growth. RAB18 (Y_2SK_2) accumulates in response to the phytohormone abscisic acid (ABA), drought, and low temperature [22]. LTI29 and COR47 (SK_3) accumulate primarily in response to low temperature but also to ABA and salt stress [22]. LTI30 (K_6) accumulates mainly under cold stress [22]. ERD14 (SK_2) is present in non-stressed plants although the protein level is upregulated by stress, particularly drought stress [22]. Dehydrin XERO (YSK_2) mRNA has been found to be constitutively expressed [39].

Of these functions, the most extensively studied has been the cryoprotection of LDH, where it has been shown that dehydrins are more effective than small molecules such as sucrose at protecting LDH activity from freeze-thaw damage [39].

The following are the functions of dehydrins with experimental evidence: binding to water and ions, binding to phospholipids, radical scavenging, phosphorylation, binding to calcium, protection of enzymes, binding to cytoskeletons, and binding to nucleic acids [17].

DHN genes are also expressed significantly in seeds toward the end of maturation, a period when the seed undergoes a developmentally programmed reduction in water content [22, 40]. The LEA/dehydrin proteins have been estimated to comprise up to 4% of the total seed protein [7].

3. Dehydrins in orthodox and recalcitrant seeds

The categories “orthodox” and “recalcitrant” seeds are used to describe the storage behavior of seeds. Orthodox seeds undergo maturation drying and are shed from the parent plant at low moisture contents. During maturation, they acquire desiccation tolerance, allowing them to be dried without irreversible damage. Because of this ability, seeds can be stored for long

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periods in cold and dry vaults. On the other hand, recalcitrant seeds do not undergo maturation drying and are shed at relatively high moisture contents.

It is believed that, in orthodox seeds, dehydrins favor the development of tolerance to osmotic stress at seed dehydration during their maturation [2, 6].

The lack of resistance of recalcitrant seeds to drying was thought to be the result of the absence of dehydrins [9, 41]. Subsequent studies, however, demonstrated that dehydrins are present in the fraction of heat-stable proteins in recalcitrant seeds of many woody species of the temperate climatic zone [41–43], including those of horse chestnut, but they were not found in species inhabiting humid tropics. This raises the question of the function performed by dehydrins in recalcitrant seeds.

In this connection, the investigation of dehydrin functions, properties, and distribution in recalcitrant seeds becomes actual. The data available so far indicate that dehydrins are present in some but not all recalcitrant seed species. They appear in response to low-temperature stress, an increase in the ABA content, and natural or artificial limited dehydration [42, 44–46].

Two tropical recalcitrant species exhibited a differential capacity to produce dehydrin-related proteins during seed maturation [43]. Dehydrins were present in axes and cotyledons of *Castanospermum australe* seeds during mid-maturation and at maturity. However, in *Trichilia dregeana*, no dehydrin-related polypeptides were detected in the mature seed. During the development of *C. australe* seeds, the nature of the dehydrin-related polypeptides accumulated in the cotyledons and axis changed and new polypeptides were detected in the mature seeds. The dehydrins present in cotyledons of mature seeds (31, 37, and 40 kDa) were still detectable after germination (i.e., in untreated seedlings) [43].

Kalemba and Pukacka [47] have compared mature and dried seeds from three species of the Acer genus, which differed in desiccation tolerance. Seeds of three Acer species—*Acer platanoides* L. (Norway maple, orthodox), *Acer pseudoplatanus* L. (sycamore, recalcitrant), and *Acer saccharinum* (silver maple, recalcitrant)—harvested during various cropping years were compared and analyzed to determine whether a genetic or an environmental influence dominated the regulation of dehydrin protein expression. The authors compared the appearance of dehydrins and small heat shock proteins in seedlots originating from cropping years that differed in weather conditions, which were monitored in detail during seed development. The experiments showed that three main dehydrins with approximate molecular weights of 46, 35, and 23 kDa were characteristic of all examined Acer species seeds. The three proteins were present in two seedlots of the orthodox Norway maple seeds and were noted either individually or together in all seedlots of recalcitrant Acer seeds. The modulation of dehydrin expression by environmental factors such as developmental heat sum and rainfall is supposed [47].

The presence of dehydrins alone in recalcitrant seeds is not sufficient to prevent desiccation injury [42, 9].

In two papers published recently [48, 49], the authors suggest an interesting point of view and "paradigm change": LEA proteins are synthesized as response to drought stress, which takes place at the end of maturation (of orthodox seeds). But it is known that dehydrins/LEA proteins

are synthesized before maturation drying [6]. And maturation drying is genetically determined in orthodox seeds. During embryogenesis and maturation drying of orthodox seeds, dehydrins are synthesized and seeds may survive dry storage. "Typical" recalcitrant seeds [48, 49] such as *Avicennia* and *Brunguiera* have no dehydrins and high water content and they do not survive water loss during storage. Intermediate seeds (*Coffea, Barringtinia*) have no dehydrins during embryogenesis but have genes of dehydrins and may synthesize dehydrins after partial water loss in storage, so these seeds may survive [48, 49]. And "atypical" recalcitrant seeds (*Camellia, Castanea, Euterpe, Quercus*) accumulate some dehydrins at the end of embryogenesis and undergo a weak maturation drying and can be stored for a limited time [48, 49]. So, the role of dehydrins in recalcitrant seeds is not clear, and further comprehensive studies are required.

4. Dehydrins in horse chestnut seeds during dormancy and germination

The mature horse chestnut (*Aesculus hippocastanum* L.) seeds are not tolerant to dehydration (i.e., belong to the recalcitrant seed type), but they are resistant to long cold stress, for instance, during winter in the central Russia when seeds are under the snow cover. The second biological peculiarity of horse chestnut seeds is that they are in the state of deep dormancy, that is, they are incapable of germinating under favorable conditions without prior prolonged incubation at low above-zero temperatures and high water content, known as stratification.

The analysis of horse chestnut seed proteins made it possible to reveal a number of unique characteristics of their proteome, which distinguish recalcitrant horse chestnut seeds from the majority of orthodox seeds and is evidently related to the specific features of their physiological behavior. These characteristics include extremely low content of globulins, predominance of water-soluble proteins located in the cytosol, and the high level of non-compartmentalized heat-stable proteins [50, 52].

The presence of heat-stable proteins (i.e., proteins resistant to high-temperature denaturation) in horse chestnut seeds is the most interesting feature of the object of our study. The results obtained demonstrated that heat-stable proteins accumulate during maturation of horse chestnut seeds and are present in freshly picked seeds in considerable amounts. These proteins account for more than 30% of soluble cytosolic proteins of the axial organs and most of soluble proteins of cotyledons (more than 80%). It is possible that in some seeds certain heat-stable proteins may function as storage deposits, being a source of nitrogen for the seedling. The involvement of heat-stable proteins in some other specific functions related to their extreme temperature resistance, characteristic amino acid composition, and high cellular content cannot be ruled out.

In order to identify and characterize stress-induced dehydrin-like polypeptides in mature recalcitrant horse chestnut seeds, we analyzed the fraction of cytosolic heat-stable proteins isolated in the period of seed dormancy and germination [52]. In our experiments, in tissues of dormant seeds, dehydrin was identified by immunoblotting as a single bright band of the polypeptide with a mol wt of about 50 kD. During radicle emergence, not only the fraction of

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heat-stable proteins was reduced but also the proportion of dehydrins in it decreased. Apparently, recalcitrant seed germination is accompanied by dehydrin disappearance, like this occurs during orthodox seed germination.

Since horse chestnut seeds contain along with heat-stable proteins numerous heat-sensitive proteins, it was of importance to elucidate whether dehydrin-like proteins are present among the latter. Nobody analyzed this protein fraction for the presence of dehydrin-like proteins. In the fraction of heat-sensitive proteins of horse chestnut seeds, we detected a component with a mol wt of 80 kD, which cross-reacted with anti-dehydrin antibody, that is, it was immunologically revealed as dehydrin-like protein. The analysis of different subcellular protein fractions of axial organs, cotyledons, and cotyledon petioles showed that both 50-kD dehydrin and 80-kD dehydrin-like protein could be detected in the total homogenate protein. It seems important that one of the axis heat-sensitive polypeptides cross-reacted with the anti-dehydrin antibody but differed from heat-stable dehydrin by a higher molecular weight (about 80 kD). This is the first indication on the possible presence of dehydrin-like proteins among heat-sensitive polypeptides of horse chestnut seeds [52].

During stratification, there were no substantial changes in the content of 50-kD dehydrin. However, this protein disappeared rapidly during seed germination. Since a small heat shock protein ubiquitin plays an important role in cell protein degradation, it was of interest to elucidate whether there is any connection between dehydrin, which should disappear during seed germination, and ubiquitin, which marks specifically proteins destined for degradation. It turned out that 50-kD dehydrin cross-reacted with anti-ubiquitin antibody. This means that dehydrin ubiquitination might provide for dehydrin rapid disappearance after radicle protrusion. This fact seems very interesting because just 50-kD dehydrin disappeared firstly during horse chestnut seed germination.

Earlier we have established that embryo axes of dormant seeds are not in the dormant state and could germinate *in vitro* (72 h on water at 28 °C) in each period of stratification of dormant seeds [53]. Like during seed germination *in vivo*, 50-kD dehydrin was not detected in such axes germinated *in vitro*. As we have demonstrated [53], treatment of excised axes with ABA (10^{-5} M), cycloheximide, or α-amanitin suppressed their germination. Under these conditions of suppressed growth, 50-kD dehydrin remained in the axes and was easily detected by immunoblotting. The mechanism of ABA inhibitory action on excised axis germination remains unknown. It is also unknown whether ABA induces 50-kD dehydrin synthesis in axes or simply prevents its degradation, resulting in its presence in axes on the level detected before germination. However, at the comparison of ABA-treated axes with those treated with cycloheximide, or α-amanitin, the substantially stronger signal may be noted in the case of ABA treatment. This may indicate indirectly on the induction of dehydrin synthesis at germination inhibition by ABA [52].

The molecular weight of the single dehydrin we detected in the horse chestnut seeds was slightly above 50 kD. At the same time, other researchers reported other values [41, 42], for example, 12, 14, and 18 kD or 30–55 kD. So far it is difficult to explain such differences in dehydrin sizes. It is not excluded that this is related to some specific feature of horse chestnut plants or their populations in different countries. It seems more likely that these differences

are related to the influence of different growth conditions. Thus, seeds collected in different years differed in the size of dehydrins: 12, 14, and 18 kD in seeds collected in 1992; 14 kD in seeds collected in 1993, and 23, 30, and 35–55 kD in seeds collected in 1994 [41, 42].

However, dehydrins comprise only a small part of heat-stable proteins of recalcitrant seeds. The functions of other heat-stable proteins accumulating in horse chestnut seeds in great amounts during dormancy and germination, the reasons for their extreme heat resistance, and their relation to the low-temperature action during stratification remain unclear. These questions are of great interest and require further study.

We believe that the presence of the proteome of horse chestnut seeds of hydrophilic proteins capable of holding moisture may be related to the recalcitrant character of these seeds. Heat-stable proteins also promote the resistance of highly watered seed to cold stress under the conditions of stratification, thereby keeping the embryo viable. From our point of view, the mere presence of a large amount of heat-stable proteins in dormant recalcitrant horse chestnut seeds is of great interest.

5. Heat shock proteins

Another interesting family of stress-induced proteins is the heat shock family [54, 55].

It is known that heat stress may inhibit growth and development of plants. This may be due to heat stress itself or due to formation of ROS and other oxidants induced by heat stress. Heating also changes the structure of proteins, up to complete denaturation, and alters the activity of many enzymes. Disturbances in membranes structure, in their permeability and fluidity may cause partial or total disintegration of cells. Heat shock, that is, 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) of 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 [55, 56]. The universality and conserved 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 cell heat tolerance [57–59].

The induction of transcription of heat shock proteins is a common phenomenon in all living organisms. These proteins are grouped in plants into five classes according to their approximate molecular weight: HSP100, HSP90, HSP70 (chaperones), HSP60 (chaperonins), small heat shock proteins (sHSPs), and ubiquitin (8.5 kD). Higher plants have at least 20 sHSPs and there might be 40 kinds of these sHSPs in one plant species. All of the major HSPs (that is, those expressed in very high amounts in response to heat and other stresses) have related functions: they ameliorate problems caused by protein misfolding and aggregation. However, each major

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HSP family has a unique mechanism of action. Some promote the degradation of misfolded proteins (ubiquitin, and various ubiquitin-conjugating enzymes); others bind to different types of folding intermediates and prevent them from aggregating (HSP70 and HSP60); and still another (HSP100) promotes the reactivation of proteins that have already aggregated [55].

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 [57, 60–62]. 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 [63]. 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 [64, 65]. In embryos of wheat [66], sorghum [67], maize [68], and pea [69], heat shock markedly activated protein synthesis 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 [69].

Small heat shock proteins function as intracellular chaperones for other proteins. They play an important role in protein–protein interactions such as folding. They assist in the establishment of proper protein conformation and prevent unwanted protein aggregation. By helping to stabilize partially unfolded proteins, HSPs aid in transporting proteins across membranes within the cell [57]. HSPs recognize and bind to other proteins when these other proteins are in non-native conformations. The non-native conformations of these proteins could be due to protein-denaturing stress or due to immature peptides folded, assembled, or localized to an

appropriate cellular compartment. In the presence of ATP at normal physiological concentrations, sHSPs change their conformation and releases denatured protein, allowing other molecular chaperones such as HSP70 to renature the protein and renew its biological activity. In the absence of ATP, sHSPs such as α-crystallin are more efficient than HSP70 in preventing stress-induced protein aggregation. *In vitro*, sHSPs selectively bind and stabilize proteins and prevent their aggregation at elevated temperatures in an ATP-independent way and protect enzymes against heat-induced inactivation.

In recalcitrant of chestnut (*Castanea sativa*) seeds, a 20-kD protein has been purified from cotyledons, where it accumulates at levels comparable to those of major seed storage proteins [58]. This protein, termed Cs sHSP 1, forms homododecameric complexes under non-denaturing conditions and appears to be homologous to cytosolic class I small heat shock proteins (sHSPs) from plant sources. *In vitro* evidence has been obtained that the isolated protein can function as a molecular chaperone; it not only increases, at stoichiometric levels, the renaturation yields of chemically denatured citrate synthase but also prevents the irreversible thermal inactivation of this enzyme. Although a role in desiccation tolerance has been hypothesized for seed sHSPs, this does not seem to be the case for Cs sHSP 1. The presence of immunologically related proteins in orthodox and recalcitrant seeds of 13 woody species has been investigated [58]. The results indicate that the presence of Cs sHSP 1- like proteins, even at high levels, is not enough to confer desiccation tolerance and that the amount of these proteins does not furnish a reliable criterion to identify desiccation-sensitive seeds. Additional proteins or mechanisms appear necessary to keep the viability of orthodox seeds upon shedding. The synthesis of small HSPs may be an important part of seed developmental program [61]. As detected in pea [70], and in other species, the class I sHSPs accumulate in Arabidopsis seeds at midmaturation and decline during germination [61]. The similarity of sHSP regulation in such diverse species supports the conclusion that there is a selective advantage to this pattern of sHSP accumulation. The correlation of sHSP expression with the development of desiccation tolerance and dormancy suggests a possible role for the sHSPs in either or both of these processes, as has been previously hypothesized [62, 71].

It was found that one major small heat shock protein existed with a molecular mass of 22 kDa and was detectable at high concentrations in seeds of three Acer species (orthodox and recalcitrant). After the seeds were dried, the content of this protein significantly increased. The largest content of this protein was observed in the oldest seeds, especially in embryonic axes. The proteins identified may play a protective role during water deficit and storage [47].

6. Heat shock proteins in recalcitrant horse chestnut seeds

In the work [72], 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. Embryo axes, cotyledon pieces, and cotyledon petioles were excised from recalcitrant horse chestnut (*Aesculus hippocastanum* L.) seeds in different times after the

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start of cold stratification and incubated at 28 or 40 °C on the medium containing ^{35}S-methionine and 50 μg/ml chloramphenicol for 4 h. The total rate of protein synthesis, the pattern of synthesized polypeptides, their distribution between subcellular fractions, and their relation to heat denaturing (5°min at 75 °C) were assessed. 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. Independently of the duration of stratification, which gradually released seed deep dormancy, isolated axes, cotyledons, and cotyledon petioles synthesized *in vivo* numerous diverse polypeptides at both 28 and 40°C. Newly synthesized polypeptides were present in the fractions of cell structures and cytosol; they differed in molecular weights, the intensity of labeling, and tolerance to heat denaturing. None of the dominating polypeptides present initially in all embryo parts and belonging mainly to heat-stable proteins was synthesized either at 28°C or 40°C. Some proteins synthesized at 40°C could be considered heat shock proteins because they were not synthesized at 28°C or their synthesis was markedly activated by heat shock. No less than 10 proteins behaved as obvious heat shock proteins; they were predominantly heat-sensitive soluble cytosolic proteins. All heat shock proteins, except those with mol wts of 220 and 34 kD, were highly labeled proteins. Some of them were characteristic of cell structures (220, 90, 20, and 18 kD); others were detected only in the cytosol (100, 80, and 34 kD). The synthesis of heat shock proteins did not depend on transcription and occurred on pre-existing mRNAs. An embryo capability of responding to heat shock did not depend on the seed physiological state and their germinability; it was similarly manifested in stratified and non-stratified seeds [72].

The analysis of heat shock action on gene expression in embryo tissues in dormant horse chestnut seeds demonstrated translation activation leading to the induction of a wide set of heat shock protein synthesis at the maintenance of the bulk of normal (non-shock) cellular protein synthesis. 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 to embryo tissues and could be considered an additional mechanism facilitating seed adaptation to unfavorable environmental conditions and successful germination.

Horse chestnut seeds survive successfully (and even require for deep dormancy release) 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, heat shock proteins 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, that is, in response to heat shock, and they were preserved in the cells after seed entry into deep dormancy in the metabolically active state;

they could improve embryo tolerance to unfavorable environmental conditions during stratification and thus increase seed viability.

We believe that the absence of discrimination of non-heat shock mRNA translation during heat shock is specific to 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, that is, it 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 [53], some characteristics of the proteome changed [50], and 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, heat shock proteins 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, that is, 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

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that one of heat shock proteins, ubiquitin, was present in dormant horse chestnut seeds in functionally active state (i.e., in association with dehydrins) [52].

7. Conclusions

Plants cannot avoid the exposure to different abiotic factors but adapt morphologically and physiologically by some other mechanisms. Almost all stresses induce the production of groups of proteins called dehydrins and heat shock proteins (HSPs), which comprise several evolutionarily conserved protein families. Accumulation of dehydrins can be induced not only by drought but also by cold, salinity, and treatment with abscisic acid.

Since HSPs 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 cell heat tolerance.

Heat stress induces the known genes for HSPs and chaperones: *hspA, groES, groEL1, groEL2, dnaJ, htpG, dnaK2, clpB1*, and *htrA* for protease, *sigB* for the σ-factor of RNA polymerase, *hik34* for sensory histidine kinase, *sodB* for superoxide dismutase, and some other genes. Using DNA microarrays, it has been shown that none of the aforementioned genes is induced by heat stress specifically [73, 74]. Expression of these genes is induced by high osmolarity, NaCl, oxidative stress (H_2O_2), high light, and UV-B. This phenomenon has been observed earlier. However, before the application of DNA microarrays, the information on HSPs that respond to various stresses has been fragmented and limited to studies of individual genes. Now it is clear that the genes whose transcription is specifically induced by high temperatures are classified as unknown. The entire list of genes of the genuine HSPs is limited to following titles: *sll0441, sll0688, sll1106, sll1884, slr0852, slr0095*, and *slr1597* [75]. The remaining genes that are induced by heat shock belong rather to a group of the general-stress-responsive (GSR) genes [76], and HSP may be renamed as general stress protein (GSP) [76, 77].

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