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The heat-shock response of developing barley aleurone layers

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Abstract

Aleurone layers of mature germinating barley (*Hordeum vulgare*, cv. Himalaya) grains respond to heat shock by synthesizing heat-shock proteins (HSPs) and by selectively suppressing the synthesis of proteins normally translated by endoplasmic reticulum (ER)-bound ribosomes. To determine if this also was the case during seed development, we investigated the synthesis of proteins translated by ER-bound ribosomes in heat-shocked aleurone layers isolated from developing barley grains. The optimal induction temperature for the heat shock response in developing aleurone layers was 37.5°C, and temperatures above 42°C inhibited translation. HSPs with apparent molecular masses of 71.1, 66.2, 57.8, 19.1 and 18.8 kDa were induced. Other studies have shown that, in gibberellic acid (GA)-induced aleurone layers from mature barley grains, these temperatures were 40°C and 45°C, respectively. Furthermore, in developing aleurone layers, mRNAs encoding proteins translated by ER-bound ribosomes (mRNAs for a lipid transfer protein and a putative amylase/protease inhibitor) remained stable during heat shock. The ER membranes themselves remained in stacks, but the lumen became distended with electron-dense material. Heat shock prevented the movement of proteins from the ER into the rest of the endomembrane pathway. In contrast, other studies show that in mature, GA-induced aleurone layers, heat shock dissociates ER stacks and blocks translation, but the processing of secretory proteins in the endomembrane pathway is not inhibited. The observation that the same tissue at different developmental stages may respond differently to heat shock indicates that components of the heat-shock response are developmentally regulated. This system provides an opportunity to better understand the nuances of the heat-shock response, especially the post-transcriptional gene regulatory mechanisms that occur.

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Introduction

The stress responses of plants are critically important to their survival, for, unlike animals, they do not have the option to move to a location with better growing conditions. Heat shock is among the best understood of the various stress responses. Like most stresses, it induces the synthesis of stress proteins, in this case heat-shock proteins (HSPs), and it suppresses the synthesis of the proteins (all, or usually at least a subset) made under normal conditions (Nover, 1994).

The induction of HSP synthesis involves the heat-catalysed conversion of monomers of the heat-shock transcription factor (HSF) into homotrimeric forms, which are capable of binding to the promoters of heat-shock genes and inducing their expression (Lis and Wu, 1993). This induction mechanism is so well conserved that vectors containing heat-shock promoters, from any foreign organism and fused to reporter genes, can be regulated in a tightly-controlled, heat-dependent manner when transformed into an apparently limitless range of host cells (Morimoto *et al*., 1994). The HSPs themselves function as molecular chaperones, refolding misfolded or heat-denatured proteins, or they are components of the protein turnover machinery (for example, ubiquitin and proteases are HSPs) (Spiess *et al*., 1999).

In contrast to the high degree of conservation in the mechanism for the induction of HSP synthesis, the mechanism for the suppression of normal-temperature protein synthesis during the heat-shock response varies widely among different organisms (Fig. 1). Fruit flies (Storti *et al*., 1980) and soybean (Nagao *et al*., 1986), tomato (Nover *et al*., 1989) and pea (Nagao *et al*., 1986) seedlings regulate this part of the heat-shock response at the translational level. The mRNAs encoding normal temperature proteins persist in the cytoplasm; however, their ribosomes translate only HSP mRNAs at heat-shock temperatures. The details of this mechanism are not well understood, although it has been observed that

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heat shock induces the phosphorylation of the S-6 subunit of the ribosome (Glover, 1982; Olsen *et al*., 1983), and that the 5′ untranslated regions of HSP mRNAs are unusually long, adenine-rich, and lack strong secondary structure (McGarry and Lindquist, 1985). In contrast, yeast uses transcriptional control to suppress the expression of normal-temperature protein synthesis. Transcript turnover is generally very rapid in yeast and, during heat shock, only genes encoding HSPs are transcribed, resulting in a shift to HSP expression (Lindquist, 1981). However, there are systems in which the synthesis of normal-temperature proteins is not affected during heat shock, such as in the roots of maize seedlings (Cooper and Ho, 1983).

In some systems, regulation of normal-temperature protein synthesis is more complex. For example, in mature barley aleurone layers stimulated with gibberellic acid (GA_3) (in this paper referred to as mature, GA-induced aleurone layers), heat shock selectively destabilizes mRNAs encoding secretory proteins, suppressing their expression, but leaving the expression of other normal-temperature proteins unaffected (Belanger *et al*., 1986; Brodl and Ho, 1991). This is also the case in wounded carrot root tissue (Brodl and Ho, 1992), oestrogen-stimulated *Xenopus* hepatocytes (Wolffe *et al*., 1984) and ripening tomato fruit (Picton and Grierson, 1988). In mature, GA-induced barley aleurone layers (Belanger *et al*., 1986) and wounded carrot root tissues (Brodl and Ho, 1992), selective secretory protein mRNA destabilization is concurrent with the dissociation of stacked endoplasmic reticulum (ER) lamellae. This has led to the hypothesis that heat-shock-induced

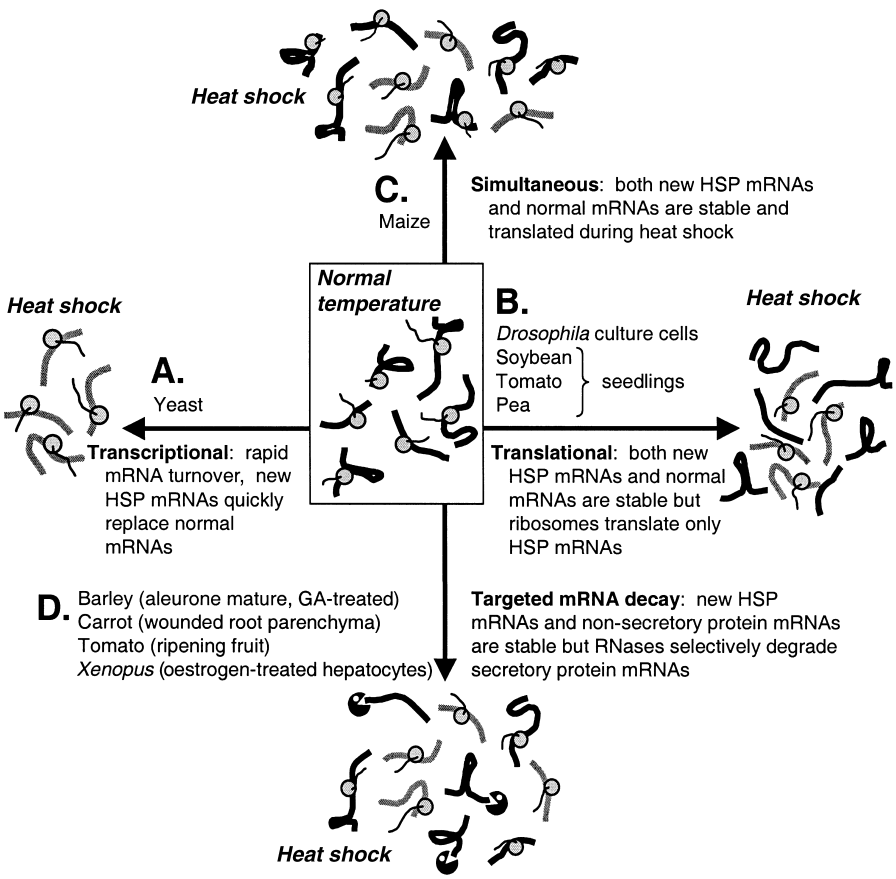


Figure 1. The variety of mechanisms used by organisms for down-regulating normal protein synthesis during heat shock. (A) Transcriptional mechanisms, exemplified by yeast, operate in cells where mRNA half-lives are measured in minutes rather than hours, where stopping the synthesis of normal protein mRNAs and inducing the synthesis of HSP mRNAs rapidly switches cells to a heat-shock phenotype. (B) Many plants and other organisms use a translational mechanism, where both normal protein mRNAs and HSP mRNAs are present in the cytoplasm, but only HSP mRNAs are translated by ribosomes. (C) In some organisms expression of normal proteins and HSPs is simultaneous, and both classes of mRNAs are translated by ribosomes during heat shock. (D) In some systems, mRNA species that are abundant and long-lived may be selectively targeted for degradation by RNases, leaving some normal mRNAs to be translated along with HSP mRNAs. Circles, ribosomes; 'pac man' figures, RNases; thick dark lines, normal mRNAs; thick grey lines, HSP mRNAs; thin lines, nascent polypeptide chains.

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changes at the ER may be important in targeting secretory protein mRNA for destabilization, for these mRNAs are translated by ER-bound ribosomes (Belanger *et al*., 1986; Chu *et al*., 1997). In mature, GA-induced barley aleurone layers, heat shock inhibits the release of the signal recognition particle (SRP) from the ER (Chu *et al*., 1997) and increases the fatty acid saturation of ER membrane phospholipids (Grindstaff *et al*., 1996; Campbell *et al*., 1997; Johnston *et al*., 2002). Since the SRP is responsible for bringing secretory protein mRNA-translating ribosomes to the ER, the inhibition of its release could 'pin' the mRNA at the ER in an arrested state of translation, leading to its destabilization (Chu *et al*., 1997). Heat-shock-induced changes in fatty acid saturation are hypothesized to be adaptive, maintaining a proper level of membrane fluidity to sustain the functioning of ER integral membrane proteins (Grindstaff *et al*., 1996; Campbell *et al*., 1997).

Heat shock does not always suppress the synthesis of proteins translated on ER membranes. In developing soybean cotyledons, heat shock enhanced the synthesis of the storage proteins conglycinin and glycinin, and the stacks of ER lamellae remained intact (Altschuler and Mascarenhas, 1985). This is also the case for the expression of phytohaemagglutinin in developing cotyledons of common beans (Chrispeels and Greenwood, 1987) and the expression of zein in developing maize kernels (F. Belanger, personal communication). Given these observations, we were curious as to whether developing barley aleurone layers responded to heat shock in a manner similar to other developing seeds, or whether they responded in a manner similar to their mature, GA-induced counterparts. In the present paper we examined the effect of heat shock on the synthesis of hordeins, on the amounts of transcripts encoding proteins translated on ER-bound ribosomes, and on ER ultrastructure.

Materials and methods

Chemicals and plant material

Unless otherwise noted, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Barley (*Hordeum vulgare*, cv. Himalaya, obtained from the Agronomy and Soils Club, Washington State University, Pullman, WA, USA; 1988 harvest) plants were grown to maturity in Forma Scientific (Marietta, OH, USA) walk-in growth chambers, maintained at constant conditions (photon flux density of 250 µmol m^{-2} s^{-1}; 22°C, 12 h day and 18°C, 12 h night; 75% RH). Spikes were tagged on the day of anthesis and harvested 18 days post anthesis (dpa; at the milk stage; these grains require 32 days to maturity). Developing grains were immediately sterilized after harvest with 8% hypochlorite for 10 min and then rinsed in at least seven changes of sterile water. The aleurone layers were peeled from the soft endosperm under aseptic conditions and then incubated in buffer [20 mM sodium succinate (pH 5.0), 20 mM $CaCl_2$, 1 µg ml^{-1} chloramphenicol] in a reciprocating water bath (120 strokes min^{-1}) during temperature treatments.

In vivo *labelling and analysis of proteins*

Ten aleurone layers per sample were incubated in sterile 25-ml flasks containing 2 ml of incubation buffer. One hour prior to the end of incubation, the samples were labelled with 50 µCi [^{35}S]methionine (specific activity > 650 Ci $mmol^{-1}$, New England Nuclear, Boston, MA, USA). Following incubation, the samples were homogenized and electrophoresed into 11% SDS-containing polyacrylamide gels (Grindstaff *et al*., 1996). Equal amounts of protein were electrophoresed for each sample, as determined by the Bradford assay (Bradford, 1976). For Western blots, proteins were electroblotted to Hybond® ECL nitrocellulose (Amersham International, Buckinghamshire, England), and the resulting blots were probed with rabbit anti-hordein primary antibodies (Rastogi and Oaks, 1986) and mouse anti-rabbit IgG–horseradish peroxidase-linked secondary antibodies (Amersham). The Amersham ECL kit was used for chemiluminescence detection. For tissue prints, cross-sections of developing barley grains were firmly pressed on to Hybond® ECL and probed with the rabbit anti-hordein primary antibodies and mouse anti-rabbit colloidal gold-linked secondary antibodies (BioRad, Hercules, CA, USA).

Northern hybridizations

Fifty aleurone layers per sample were incubated in 10 ml of incubation buffer in sterile 100-ml flasks. RNA was isolated from the samples using the procedure of Chirgwin *et al.* (1979). Twenty µg of total RNA was electrophoresed, blotted and visualized according to the procedure of Campbell *et al.* (1997). The blots were probed with cDNA clones for a putative amylase/protease inhibitor (Mundy and Rogers, 1986) and a putative lipid transfer protein (Jakobsen *et al*., 1989), which were nick-translated with α-[^{32}P]dCTP (specific activity > 500 Ci $mmol^{-1}$, obtained from New England Nuclear).

Electron microscopy

Tissue samples approximately 0.5 mm^2 in size were fixed, postfixed, dehydrated and embedded according to the procedure of Campbell *et al*. (1997).

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Sections approximately 60 nm thick were cut on a diamond knife, stained with 1% methanolic uranyl acetate and Reynold's lead citrate (Hayat, 1989), and viewed at magnifications of 8000–20,000× with a JEOL JEM 100SK transmission electron microscope (JEOL Ltd., Tokyo, Japan). Ribosome densities were assessed by counting ER-bound ribosomes along ER membranes that were cut in clear cross-section in a minimum of 20 different cells from three independent trials. A total of 50 µm of ER were counted for each cell. The counts were done 'blind' and independently verified.

Cell fractionation

Fifty aleurone layers per sample were incubated in 10 ml of incubation buffer in sterile 100-ml flasks. During the final 20 min of incubation, the samples were labelled with 100 µCi [^{35}S]methionine (specific activity > 650 Ci mmol^{-1}, New England Nuclear). The aleurone layers were lysed and fractionated by continuous sucrose gradient ultracentrifugation according to the procedure of Grindstaff *et al.* (1996), using the ethylene-diaminetrienoic acid (EDTA) and Mg^{2+} modifications of Cooper and Ho (1987). The fractions were assayed for reduced nicotine adenine dinucleotide (NADH) cytochrome *c* reductase activity, to determine the position of ER-containing fractions, and for inosine diphosphatase (IDPase) activity, to determine the position of Golgi-containing fractions (Jones, 1980).

Results

To observe the effect of sudden temperature shifts on protein synthesis in developing barley aleurone layers, samples were harvested, sterilized, incubated at 25°C for 2 h, then shifted to temperatures from 35 to 45°C in the presence of [^{35}S]methionine. Such treatment induced the synthesis of a new set of proteins, HSPs, with apparent molecular masses of 71.1, 66.2, 57.8, 19.1 and 18.8 kDa (Fig. 2). Their synthesis was maximal at 37.5°C. At higher temperatures, the synthesis of all proteins began to diminish, and at 42.5°C and higher the aleurone layers no longer incorporated [^{35}S]methionine into proteins, indicating that translation was inhibited. In subsequent experiments, temperatures intermediate to those shown in Fig. 2 were used, and the results from those experiments confirmed that 37.5°C was the optimal temperature for the induction of HSP synthesis, and that temperatures above 42°C inhibited translation (data not shown). The shift in temperature had only a minor effect on the synthesis of proteins expressed prior to the temperature shift. The synthesis of one protein, approximately 43 kDa in size, and another, approximately 39 kDa in size, was very reduced. The synthesis of most of the other proteins was not markedly affected by the heat shock.

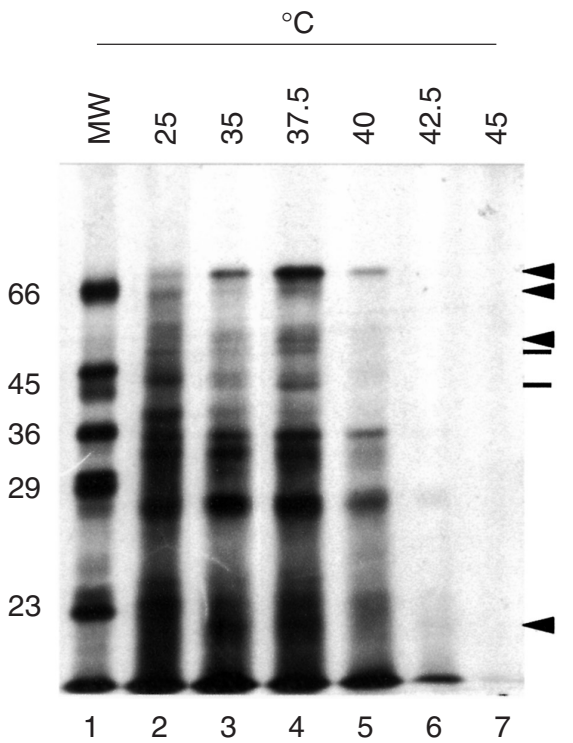


Figure 2. Effect of temperature on the pattern of protein synthesis in developing barley aleurone layers (18 dpa). Samples were incubated at 25°C for 2 h and then for 3 h at the temperatures indicated above each lane. They were labelled with [^{35}S]methionine during the final 1 h of incubation. Proteins in the homogenates were separated by 11% SDS-PAGE and visualized by fluorography. Equal amounts of protein were electrophoresed for each sample. Arrowheads indicate the positions of heat-inducible proteins. MW indicates the positions of molecular weight markers in kDa. Dashes indicate the position of hordeins (see Fig. 3).

Time-course experiments indicated that the induction of HSPs was maximal after 2 h of heat shock, and that new synthesis of HSPs gradually diminished after that (Fig. 3A, lanes 2–5). Throughout the 3-h time course, the synthesis of most non-HSPs continued, as had been observed in Fig. 2. This was true for the synthesis of the storage protein, hordein, whose position in the SDS-polyacrylamide gels was established by Western blotting (Fig. 3A, lane 5). This protein, although synthesized chiefly by endosperm cells, is also synthesized by the aleurone layer of developing barley grains, as indicated by tissue prints of cross-sectioned developing barley grains using anti-hordein antibodies (Fig. 3B).

Heat shock did not suppress the synthesis of hordein in developing barley aleurone layers, which

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suggested that the mRNAs encoding proteins translated on ER-bound ribosomes are stable during heat shock. To investigate whether this was the case, Northern hybridizations for the mRNAs encoding a putative amylase/protease inhibitor (PAPI) and a putative lipid transfer protein (LTP) were performed. Sequence data obtained from the cDNAs encoding these genes shows the presence of classical signal peptides, which would direct the translation of these transcripts to the ER (Mundy and Rogers, 1986; Jakobsen *et al.*, 1989). In both cases, the transcript amounts for these proteins were as high in heat-shocked cells as they were in cells incubated continuously at 25°C (Fig. 4).

The stability of PAPI and LTP transcripts in developing barley aleurone layers indicated that the ER membranes of these layers may be stable during heat shock, and transmission electron microscopy was used to determine whether this was the case. Isolated developing barley aleurone layers, incubated continuously at 25°C, contained ER lamellae arranged in parallel stacks, chiefly in the cortical cytoplasm (Fig. 5A). These ER membranes had attached ribosomes, often clustered in regions of high density. Heat-shocked developing barley aleurone layers also had ER membranes with attached ribosomes; however, the lumen of these ER membranes was highly distended and usually filled with an electron-dense flocculant (Fig. 5B and C). In some extreme cases, the ER appeared to have vesiculated and anastomosed into larger vesicles (Fig. 5D). Membrane distention and vesiculation were also apparent at the nuclear envelope (Fig. 5E), a membrane that is continuous with the endoplasmic reticulum (Staehelin, 1997). The overall density of ribosomes on the ER of heat-shocked developing aleurone layers was very comparable to the density of ribosomes on the ER of samples incubated at normal temperature (31.6 ± 4.8 versus 33.2 ± 5.4 ribosomes μm^{-1} of ER membrane for heat-shocked and normal-temperature samples, respectively).

The observation that secretory protein synthesis continued during heat shock, but that ER membranes became distended with apparently proteinaceous material, suggested that heat shock caused a block in the movement of proteins from the ER to the rest of the endomembrane pathway. To investigate whether

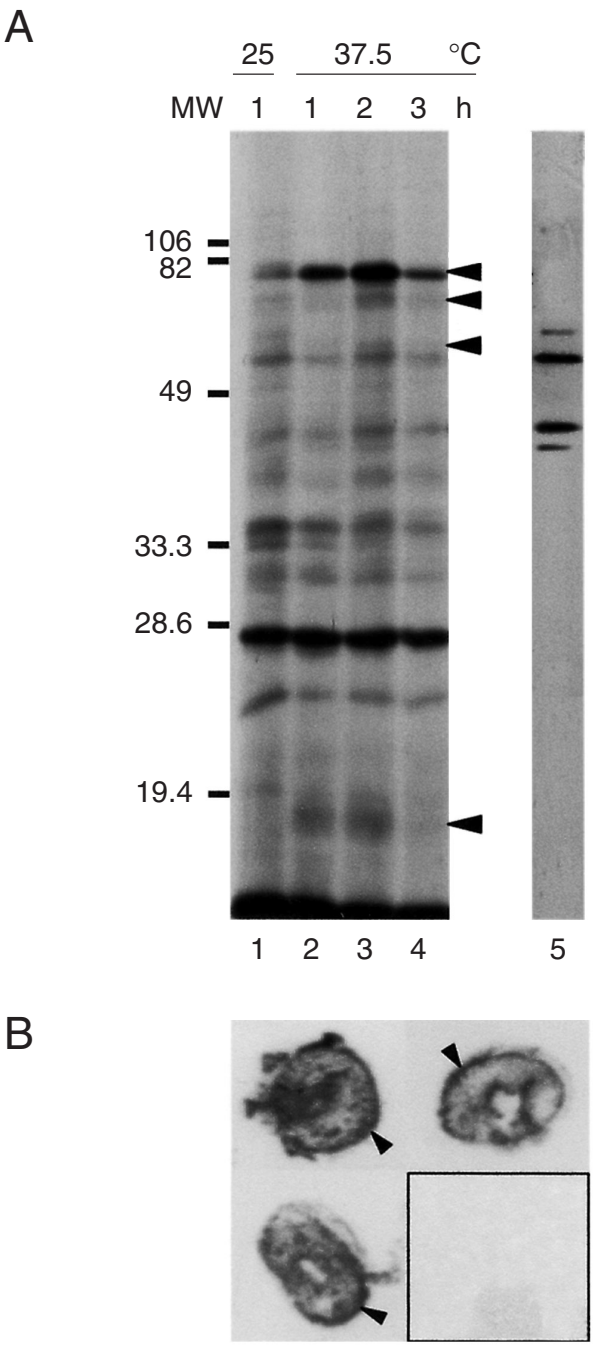


Figure 3. Time course of the heat-shock response of developing barley aleurone layers. (A) At the top of the figure, the numbers above the bars in lanes 1–4 indicate the temperature, and the numbers below the bars indicate the number of hours of incubation for each sample. All samples were labelled with [^{35}S]methionine during the final 1 h of incubation and then homogenized. Proteins in the homogenates were separated by 11% SDS-PAGE and visualized by fluorography (lanes 1–4) or by Western blotting (lane 5). Equal amounts of protein were electrophoresed for each sample. Arrowheads indicate the positions of heat-inducible proteins. MW indicates the positions of molecular weight markers in kDa. Lane 5 shows proteins recognized by anti-hordein antibodies. (B) Cross-sections of developing barley grains were tissue printed on to nitrocellulose and probed with anti-hordein antibodies. The outer dark ring of staining (arrowhead) corresponds to the aleurone layer. The boxed inset in the lower right shows tissue prints probed with pre-immune serum.

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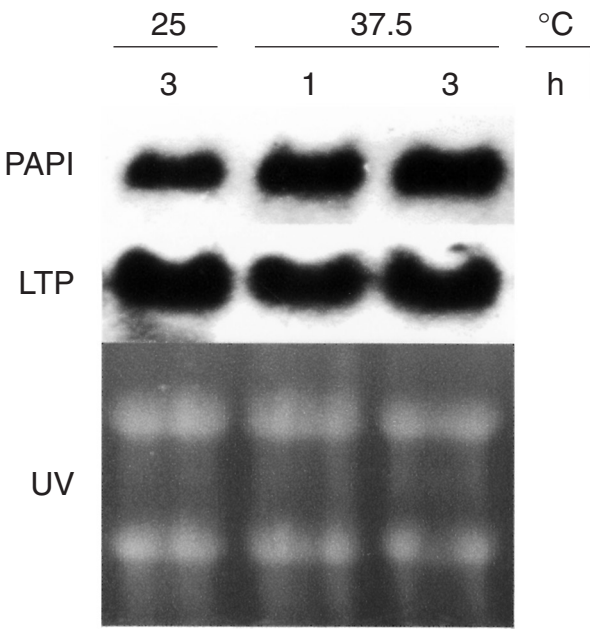


Figure 4. Amounts of mRNAs encoding proteins synthesized by ER-bound ribosomes in heat-shocked developing barley aleurone layers. At the top of the figure, the numbers above the bars in lanes 1–3 indicate the temperature and the numbers below the bars indicate the number of hours of incubation for each sample. Total RNA was isolated from each sample and analysed by Northern hybridization using [^{32}P]dCTP-labelled probes encoding a putative amylase/protease inhibitor (PAPI) and a putative lipid transfer protein (LTP). The bands shown represent the only radioactivity detectable on these two fluorograms. The bottom panel is a photograph of the ethidium bromide stained gel from which the Northern hybridizations were performed. It shows that equivalent amounts of RNA were loaded in all lanes.

this was the case, developing aleurone layers were pulse-labelled with [^{35}S]methionine, homogenized and fractionated on isopycnic sucrose gradients, to determine the density of the organelles with which the radioactive label was associated. Such analyses showed that, at 25°C, the bulk of the [^{35}S]methionine-labelled protein was associated with fractions that co-migrated with peak activities for the ER-marker enzyme, NADH cytochrome *c* reductase, at 30–33% sucrose (Fig. 6A). In heat-shocked samples, the density of the fractions containing [^{35}S]methionine-labelled protein increased to 32–36% sucrose, and the density of peak NADH cytochrome *c* reductase-associated fractions increased to 32–36% sucrose, as well. To confirm that the NADH cytochrome *c* reductase activities were ER-associated, the experiment was repeated using buffers with an increased concentration of EDTA and low Mg^{2+} – a 'Mg^{2+} shift' experiment. Such treatment strips ribosomes from the ER, decreasing the apparent density of this organelle on isopycnic sucrose gradients (Cooper and Ho, 1987). In the 25°C samples, the peak of NADH cytochrome *c* reductase activity was shifted to 28–31% sucrose (Fig. 6B), as was the bulk of the [^{35}S]methionine-labelled protein. A similar ER-associated Mg^{2+} shift was observed in heat-shocked samples, where the peak of NADH cytochrome *c* reductase activity and the fractions containing [^{35}S]methionine-labelled protein were shifted to 30–34% sucrose.

A pulse-chase experiment was conducted to determine whether the [^{35}S]methionine-labelled protein that had accumulated in the ER would move down the endomembrane pathway to the Golgi following a 6-h recovery from heat shock. In these experiments, the peak of radioactivity shifted to 36–40% sucrose on isopycnic sucrose gradients with EDTA (Fig. 6C). The peak of NADH cytochrome *c* reductase activity in these samples was approximately 28 and 33% sucrose, respectively, for normal-temperature and heat-shocked samples. This suggested that a recovery period following heat shock appeared to permit movement of radiolabelled proteins out of the ER. Since IDPase activity (a marker for dictyosomes) fractionated at approximately 28% sucrose (data not shown), this indicated that the radiolabelled proteins were probably packaged into storage vesicles.

Discussion

We have shown that heat shock did not suppress the expression of non-heat shock proteins synthesized by ER-bound ribosomes in developing barley aleurone layers, and did not affect the amounts of two mRNAs translated by ER-bound ribosomes in this tissue. Developing barley aleurone layers possessed intact ER membranes, and ER-bound ribosomes were present in quantities comparable to those observed in samples incubated at normal temperature. However, heat shock caused a dilation of the ER lumen, and cell fractionation showed that radiolabelled proteins accumulated in the ER, indicating that heat shock impaired normal protein trafficking along the endomembrane pathway. Cell fractionations of pulsed-chased samples indicated that if recovery followed heat shock, radiolabelled proteins moved from the ER to more dense sucrose fractions, likely corresponding to storage vacuoles.

The proteins induced in developing barley aleurone layers by heat shock had mobilities in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) that were similar to those of HSPs induced in mature, GA-induced barley aleurone layers (Belanger *et al.*, 1986) and other systems (Key *et al.*, 1981; Cooper and Ho, 1983; Brodl

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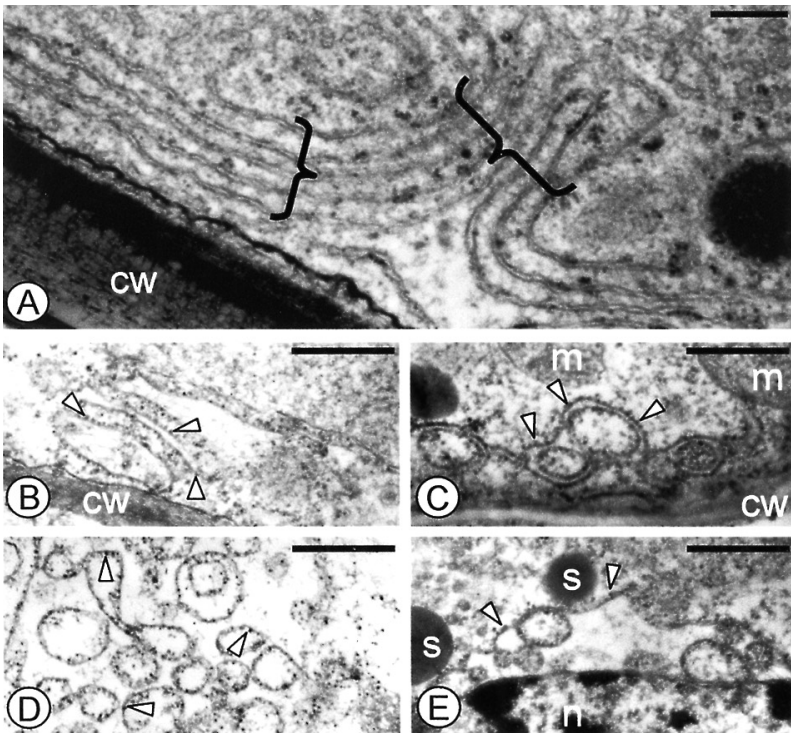


Figure 5. Ultrastructure of ER membranes in heat-shocked developing barley aleurone layers. Samples were incubated at 25 or 37°C for 3 h, then immediately processed for transmission electron microscopy. (A) At 25°C, ER lamellae have normal cisternae arrayed in stacks. (B, C) Heat shock at 37°C causes ER membranes to vesiculate. (D) Where the ER network is particularly complex, the vesicles formed during heat shock may become engorged and anastomose. (E) The outer membrane of the nuclear envelope, an ER-derived membrane, also vesiculates. Brackets, stacked ER cisternae; arrowheads, ribosomes on vesiculated ER membranes; cw, cell wall; m, mitochondrion; n, nucleus; s, spherosome. Bars in the upper right of each micrograph indicate 0.5 μm.

and Ho, 1992). However, our other observations are very different from those of the heat-shock response of mature, GA-induced barley aleurone layers, where secretory protein synthesis is suppressed (Belanger *et al*., 1986), secretory protein mRNAs are selectively destabilized (Brodl and Ho, 1991) and stacked ER lamellae are dissociated during heat shock (Belanger *et al*., 1986). In contrast, the heat-shock response of developing barley aleurone layers closely paralleled that of other seed tissues engaged in the synthesis of storage proteins (Altschuler and Mascarenhas, 1985; Chrispeels and Greenwood, 1987). The differences between the heat-shock responses of developing and mature barley aleurone layers may well be an important evolutionary adaptation. Maintaining the synthesis of storage proteins during aleurone development would help to ensure the success of the next generation, but suppressing the synthesis of secreted hydrolases during germination would prevent a fragile seedling from emerging under potentially lethal conditions. Furthermore, crop plants have been subjected to intensive artificial selection to accomplish pod and grain fill at a time in the season when stressful growing conditions, such as high temperatures and low precipitation, are more common. The significant observation is that the characteristics of the aleurone-layer heat-shock response are determined by its developmental state.

In studies of isolated mature barley aleurone layers, GA_3 is applied exogenously. We have compared the heat-shock response of GA_3-stimulated and unstimulated mature aleurone layers and found that unstimulated layers maintain the synthesis and secretion of proteins during heat shock (Grindstaff *et al*., 1996). Addition of GA_3 promotes the formation of prominent stacks of ER lamellae, apparently through the building of new membranes (Jones, 1969a, b; Fernandez and Staehelin, 1987). The ER membranes present in mature aleurone layers incubated in the absence of GA_3 may be remaining ER that was synthesized during seed development. Many of the organelles present following seed dehydration are reactivated during imbibition and used in the early stages of germination (Gram, 1982). In support of this observation, mature aleurone layers that are not stimulated with GA_3 (like their developing barley aleurone layer counterparts) synthesize and secrete significantly more secretory protein than do mature,

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GA_3-stimulated aleurone layers (Grindstaff *et al.*, 1996). An explanation for this difference in heat-shock responses is that ER phospholipids from unstimulated layers have a higher degree of fatty acid saturation, which may enhance their stability at heat-shock temperature (Grindstaff *et al.*, 1996).

It was noted that heat shock increased the apparent buoyant density of ER membranes in developing barley aleurone layers. A similar increase in the buoyant density of ER membranes was observed in heat-shocked developing cotyledons of common beans (Chrispeels and Greenwood, 1987). Heat shock also induced a shift in the buoyant density in ER membranes isolated from the mature, GA-treated aleurone layers (Sticher *et al.*, 1990; Grindstaff *et al.*, 1996). These increases in buoyant density may be ascribed to heat-shock-induced accumulation of proteins in the ER. However, pulse-chase experiments showed that, unlike developing barley aleurone layers and common bean cotyledons, radiolabelled protein did not accumulate in ER membranes isolated from the aleurone layers of mature, GA-treated barley grains upon heat shock (Sticher *et al.*, 1990; Grindstaff *et al.*, 1996). Apparently something in ER membrane biochemistry changes during heat shock as well. We have found that heat shock affects the lipid to protein ratios of the ER from mature, GA-treated barley aleurone cells (M.K. Johnston and M.R. Brodl, unpublished results); these decreases in the relative amount of lipids in the ER are likely responsible for the change in their buoyancy.

The heat-shock responses of developing and mature, GA-induced barley aleurone layers also differed in the temperature required to induce the heat-shock response. In developing barley aleurone layers, the temperature that maximally induced HSP synthesis was 37.5°C. At temperatures above 37.5°C, protein synthesis was sharply reduced, and at temperatures just above 40°C, samples no longer incorporated [^{35}S]methionine into proteins. In contrast, HSP synthesis is maximally induced at 40°C in mature, GA-induced barley aleurone layers (Belanger *et al.*, 1986), and temperatures of 45°C and higher are required to inhibit the incorporation of [^{35}S]methionine into proteins (Lanciloti *et al.*, 1996). It is not clear what particular attributes of the developmental state of aleurone layers are key factors in their heat-shock response, for the history of the aleurone layer from early development through germination is highly complex. Several factors may be

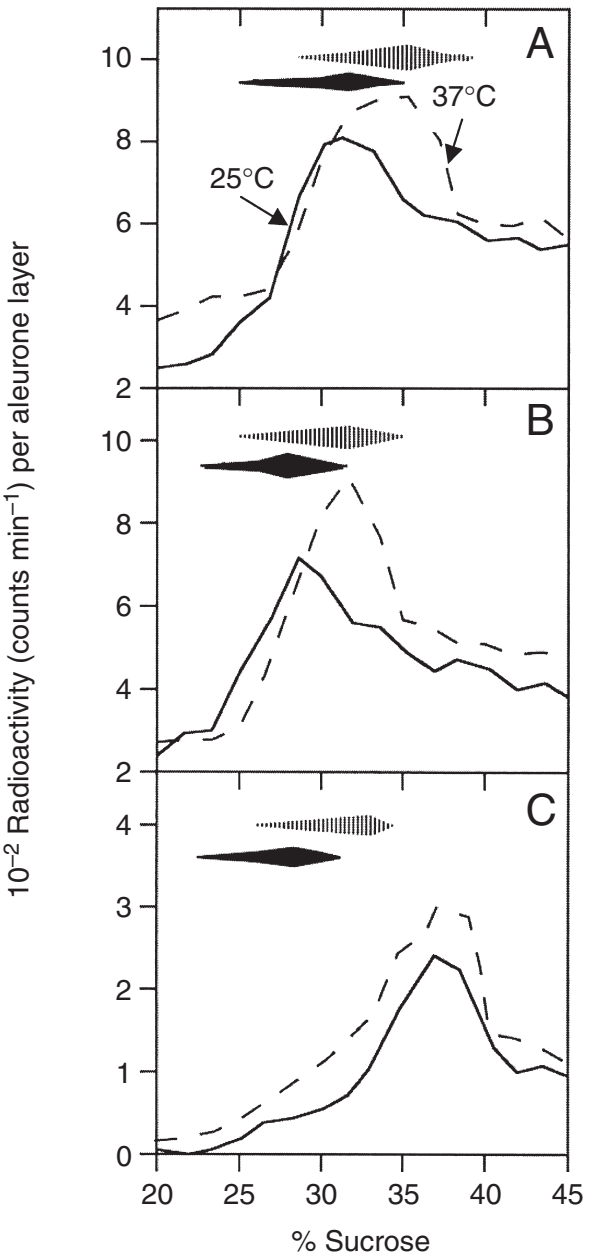


Figure 6. Distribution of [^{35}S]methionine-labelled proteins in heat-shocked developing barley aleurone layers. Samples were incubated at either 25 or 37°C for 3 h and radiolabelled with [^{35}S]methionine during the final 20 min of incubation (A and B). Some of the samples were subsequently chased with non-radioactive methionine and allowed to incubate at 25°C for 6 h longer (C). Following incubation, the samples were lysed by chopping in buffers containing Mg^{2+} (A) or EDTA (B and C), and the resulting lysates were fractionated on 20–50% continuous sucrose gradients, containing either Mg^{2+} or EDTA. The distribution of trichloroacetic acid (TCA)-precipitable radioactivity in each fraction is indicated by the black line for samples incubated at 25°C, and by the dashed line for samples incubated at 37°C. The relative distribution of NADH cytochrome *c* reductase activity is indicated by the lens-shaped bars at the top of each graph; the black and striped shadings correspond to 25 and 37°C samples, respectively.

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important. Cereal aleurone development is governed by what is often described as an 'antagonistic' balance of the phytohormones GA and abscisic acid (ABA) (Taiz and Zeiger, 2002). Whereas GA plays a fundamental role in germination, early development of the aleurone layer is chiefly governed by ABA (Naumann and Dorffling, 1982). In addition to its role in development, ABA is also thought to act as a common mediator for plant stress responses (Zeevaart and Creelmann, 1988). It may be that ABA-induced proteins are important in protecting components of the secretory machinery that are otherwise heat labile, playing a role analogous to HSPs (Vierling, 1991). However, addition of ABA to mature, GA-treated aleurone layers did not provide protection to secretory protein synthesis and ER lamellae against heat shock (M. Brodl, unpublished). Timing may be another general factor. Dry-down and dormancy are major events separating developing and mature, GA-stimulated barley aleurone layers, and these events bring about dramatic changes in metabolism and membrane biochemistry (Bewley and Black, 1982) that may also be important in regulating responses to heat shock. Understanding the reason why developing and mature, GA-treated barley aleurone layers respond quite differently to heat shock will likely yield helpful insights into the regulation of the heat-shock response.

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References

Altschuler, M. and Mascarenhas, J.P. (1985) Transcription and translation of heat shock and normal proteins in seedlings and developing seeds of soybean exposed to a gradual temperature increase. *Plant Molecular Biology* **5**, 291–297.

Belanger, F.C., Brodl, M.R. and Ho, T.-h.D. (1986) Heat shock causes destabilization of specific mRNAs and destruction of endoplasmic reticulum in barley aleurone cells. *Proceedings of the National Academy of Sciences, USA* **83**, 1354–1358.

Bewley, J.D. and Black, M. (1982) *Physiology and biochemistry of seeds*, Vol. II. New York, Springer Verlag.

Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Analytical Biochemistry* **72**, 248–254.

Brodl, M.R. and Ho, T.-h.D. (1991) Heat shock causes selective destabilization of secretory protein mRNAs in barley aleurone cells. *Plant Physiology* **96**, 1048–1052.

Brodl, M.R. and Ho, T.-h.D. (1992) Heat shock in mechanically wounded carrot root disks causes destabilization of stable secretory protein mRNA and dissociation of endoplasmic reticulum lamellae. *Physiologia Plantarum* **86**, 253–262.

Campbell, J.D., Fielding, L.A. and Brodl, M.R. (1997) Heat shock temperature acclimation of normal secretory protein synthesis in barley aleurone cells. *Plant, Cell and Environment* **20**, 1349–1360.

Chirgwin, J.M., Pryzbyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.

Chrispeels, M.J. and Greenwood, J.S. (1987) Heat stress enhances phytohemagglutinin synthesis but inhibits its transport out of the endoplasmic reticulum. *Plant Physiology* **83**, 778–784.

Chu, B., Brodl, M.R. and Belanger, F.C. (1997) Heat shock inhibits release of the signal recognition particle from the endoplasmic reticulum in barley aleurone layers. *Journal of Biological Chemistry* **272**, 7306–7313.

Cooper, P. and Ho, T.-h. D. (1983) Heat shock proteins in maize. *Plant Physiology* **71**, 215–222.

Cooper, P. and Ho, T.-h.D. (1987) Intracellular localization of heat shock proteins in maize. *Plant Physiology* **84**, 1197–1203.

Fernandez, D.E. and Staehelin, L.A. (1987) Does gibberellic acid induce the transfer of lipase from protein bodies to lipid bodies in barley aleurone cells? *Plant Physiology* **85**, 487–496.

Glover, C.V.C. (1982) Heat shock induces rapid dephosphorylation of a ribosomal protein in *Drosophila*. *Proceedings of the National Academy of Sciences, USA* **79**, 1781–1785.

Gram, N.H. (1982) The ultrastructure of germinating barley seeds. I. Changes in the scutellum and the aleurone layer in nordal barley. *Carlsberg Research Communications* **47**, 143–162.

Grindstaff, K.K., Fielding, L.A. and Brodl, M.R. (1996) Effect of gibberellins and heat shock on the lipid composition of endoplasmic reticulum in barley aleurone layers. *Plant Physiology* **110**, 571–581.

Hayat, M.A. (1989) *Principles and techniques of electron microscopy: Biological applications* (3rd edition). Boca Raton, FL, CRC Press.

Jakobsen, K., Klemsdal, S.S., Aalen, R.B., Bosnes, M., Alexander, D. and Olsen, O.-A. (1989) Barley aleurone cell development: Molecular cloning of aleurone-specific cDNAs from immature grains. *Plant Molecular Biology* **12**, 285–293.

Johnston, M.K., Benson, P.A.S., Rogers, T.M. and Brodl,

https://doi.org/10.1079/SSR2003140 Published online by Cambridge University Press

M.R. (2002) Slow heating of barley aleurone layers to heat shock temperature preserves heat shock-sensitive cellular properties. *American Journal of Botany* **89**, 401–409.

Jones, R.L. (1969a) Gibberellic acid and the fine structure of barley aleurone cells: I. Changes during the lag phase of α-amylase synthesis. *Planta* **87**, 119–133.

Jones, R.L. (1969b) Gibberellic acid and the fine structure of barley aleurone cells: II. Changes during the synthesis and secretion of α-amylase. *Planta* **88**, 73–86.

Jones, R.L. (1980) The isolation of endoplasmic reticulum from barley aleurone layers. *Planta* **150**, 58–69.

Key, J.L., Lin, C.-Y. and Chen, Y.M. (1981) Heat shock proteins of higher plants. *Proceedings of the National Academy of Sciences, USA* **78**, 3526–3530.

Lanciloti, D.F., Cwik, C. and Brodl, M.R. (1996) Heat shock proteins do not provide thermoprotection to normal cellular protein synthesis, α-amylase mRNA and endoplasmic reticulum lamellae in barley aleurone layers. *Physiologia Plantarum* **97**, 513–523.

Lindquist, S. (1981) Regulation of protein synthesis during heat shock. *Nature* **293**, 311–314.

Lis, J. and Wu, C. (1993) Protein traffic on the heat shock promoter: Parking, stalling, and trucking along. *Cell* **74**, 1–4.

McGarry, T.J. and Lindquist, S. (1985) The preferential translation of *Drosophila* hsp70 mRNA requires sequences in the untranslated leader. *Cell* **42**, 903–911.

Morimoto, R.I., Jurivich, D.A., Kroeger, P.E., Mathur, S.K., Murphy, S.P., Nakai, A., Sarge, K., Abravaya, K. and Sistonen, L.T. (1994) Regulation of heat shock gene transcription by a family of heat shock factors. pp. 417–455 *in* Morimoto, R.I.; Tissières, A.; Georgopulos, C. (Eds) *The biology of heat shock proteins and molecular chaperones*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.

Mundy, J. and Rogers, J.C. (1986) Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: Comparison to the barley amylase/subtilisin inhibitor. *Planta* **169**, 51–63.

Nagao, R.T., Kimple, J.K., Vierling, E. and Key, J.L. (1986) The heat shock response: A comparative analysis. pp. 384–438 *in* Miflin, B.J. (Ed.) *Oxford surveys of plant molecular and cell biology*, Vol. 3. Oxford, UK, Oxford UniversityPress.

Naumann, R. and Dorffling, K. (1982) Variation of free and conjugated abscisic acid, phaseic acid, and dihydrophaseic acid levels in ripening barley grains. *Plant Science Letters* **27**, 111–117.

Nover, L. (1994) The heat shock response as part of the plant stress network: An overview with six tables. pp. 3–45 *in* Cherry, J. (Ed.) *Biochemical and cellular mechanisms of stress tolerance in plants*. Berlin, Springer-Verlag.

Nover, L., Scharf, K.-D. and Neumann, D. (1989) Cytoplasmic heat shock granules are formed from precursor particles and are associated with a specific set of mRNAs. *Molecular and Cellular Biology* **9**, 1298–1308.

Olsen, A.S., Triemer, D.F. and Sanders, M.M. (1983) Dephosphorylation of S6 and expression of the heat shock response in *Drosophila melanogaster. Molecular and Cellular Biology* **3**, 2017–2027.

Picton, S. and Grierson, D. (1988) Inhibition of expression of tomato-ripening genes at high temperature. *Plant, Cell and Environment* **11**, 265–272.

Rastogi, V. and Oaks, A. (1986) Hydrolysis of storage proteins in barley endosperms – analysis of soluble products. *Plant Physiology* **81**, 901–906.

Spiess, C., Beil, A. and Ehrmann, M. (1999) A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell* **97**, 339–347.

Staehelin, L.A. (1997) The plant ER: A dynamic organelle composed of a large number of discrete functional domains. *Plant Journal* **11**, 1151–1165.

Sticher, L., Biswas, A.K., Bush, D.S. and Jones, R.L. (1990) Heat shock inhibits α-amylase synthesis in barley aleurone without inhibiting the activity of endoplasmic reticulum marker enzymes. *Plant Physiology* **92**, 506–513.

Storti, R.V., Scott, M.P., Rich, A. and Pardue, M.L. (1980) Translational control of protein synthesis in response to heat shock in *Drosophila melanogaster* cells. *Cell* **22**, 825–834.

Taiz, L. and Zeiger, E. (2002) *Plant physiology* (3rd edition). Sunderland, MA, Sinauer Associates.

Vierling, E. (1991) The roles of heat shock proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 579–620.

Wolffe, A.P., Perlman, A.J. and Tata, J.R. (1984) Transient paralysis of hormonal regulation of gene expression. *EMBO Journal* **3**, 2763–2770.

Zeevaart, J.D.A. and Creelmann, R.A. (1988) Metabolism and physiology of abscisic acid. *Annual Review of Plant Physiology and Plant Molecular Biology* **39**, 439–473.

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