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Protein Synthesis and Breakdown during Heat Shock of Cultured Pear (*Pyrus communis* L.) Cells

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Cultured pear (*Pyrus communis* L. cv Passe Crassane) cells were subjected to temperatures of 39, 42, and 45°C. Heat-shock protein (hsp) synthesis was greater at 39°C than at temperatures above 40°C and continued for up to 8 h. Both cellular uptake of radiolabeled methionine and total protein synthesis were progressively lower as the temperature was increased. Polysome levels decreased immediately when cells were placed at 39 or 42°C, although at 39°C the levels began to recover after 1 h. In cells from both temperatures, reassembly occurred after transfer of cells to 25°C. Four heat-shock-related mRNAs—hsp17, hsp70, and those of two ubiquitin genes—all showed greatest abundance at 39°C and decreased at higher temperatures. Protein degradation increased with time at 42 and 45°C, but at 39°C it increased for the first 2 h and then decreased. In the presence of cycloheximide, which prevented hsp synthesis, protein degradation at 39°C was as great as that at 45°C in the absence of cycloheximide. The data suggest that hsps may have a role in protecting proteins from degradation at the permissive temperature of 39°C. At temperatures high enough to inhibit hsp synthesis, protein degradation was enhanced. Although ubiquitin may play a role in specific protein degradation, it does not appear to be involved in increased protein degradation occurring above 40°C.

Protein turnover in organisms is regulated by both synthesis and degradation, and the rates of these processes will change in response to environmental stresses such as heat. It is commonly found that, with exposure to high temperatures, there is a decline in normal protein synthesis, together with increased, selective translation of mRNAs for characteristic sets of hsps (Gurley and Key, 1991). In plants the hsps include an abundant group of low mol wt polypeptides in addition to the higher mol wt families (Vierling, 1991). Some of the high mol wt hsps have been found to function as chaperones and can minimize high-temperature stress damage by associating with partially denatured proteins, preventing breakdown or aggregation.

Plants respond to heat conventionally, with reduction in protein synthesis concomitantly with selective synthesis of new or constitutive hsps. Total protein synthesis has been shown to decline at temperatures of 40°C and above in soybeans (Key et al., 1981), although both uptake and incorporation of radiolabel were maintained for more than 9 h at this temperature (Kimpel et al., 1990). Disassociation of polyribosomes also occurred rapidly in soybean hypocotyls at 40°C (Key et al., 1981) and in germinating maize seeds at 41°C (Riley, 1981). Heat pretreatment, however, may result in enhanced synthetic capability. Pretreatment of wheat seedlings for 24 h at 36°C resulted in a subsequent increase in polysome formation (Fehling and Weidner, 1986); ribosomal inactivation occurred at 45°C.

Heat-induced protein degradation has been less characterized. In mammalian cells, heat shock induces an initially rapid increase in protein degradation (Parag et al., 1987; Gropper et al., 1991). However, the association of hsps with protein breakdown is not clear, since in one study using CH, which inhibits hsp synthesis, there was no effect on such protein breakdown (Parag et al., 1987). Heat-induced increases in proteases have also been characterized and are implicated in breakdown of abnormal proteins (Gottesman, 1989). However, the control of heat-induced protein breakdown and its relationship to synthesis and hsp induction in mammalian cells are still unclear. Apart from a study showing more than a doubling of total breakdown in wheat roots at high temperatures (Ferguson et al., 1990), heat-induced protein degradation is largely uninvestigated in plants.

An important function of stress-induced protein breakdown in animal cells is the elimination of abnormal proteins. In both plant and animal cells, there are ATP-dependent cytosolic proteases that specifically degrade proteins targeted by the small polypeptide ubiquitin (Hershko and Ciechanover, 1992). Ubiquitin is enzymically conjugated to the target protein in ATP-dependent reactions. The consequent ubiquitin-protein complex becomes subject to proteolysis, and the ubiquitin is released. This ubiquitin-dependent pathway is an important means of controlling protein turnover in animals (Hershko and Ciechanover, 1992) and is likely to be so in plants (Vierstra, 1993).

Ubiquitin has also been shown to be associated with high-temperature-stress responses in animals (Bond and Schlesinger, 1986; Parag et al., 1987; Bond et al., 1988) and yeast (Finley et al., 1987). This has been demonstrated in several ways, including differential expression of ubiquitin genes during heat shock (Bond and Schlesinger, 1986; Fornace et al., 1989), sensitivity to high-temperature stress of yeast mutants with altered ubiquitin metabolism (Finley et al., 1987), reduced breakdown under heat stress of short-lived proteins in mammalian cell lines deficient in E1 (Gropper et al., 1991), and changes in ubiquitin pools (free versus con-

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Abbreviations: CH, cycloheximide; E1, ubiquitin-activating enzyme; E2 ubiquitin carrier protein; hsp, heat-shock protein.

jugated forms) during heat shock (Parag et al., 1987; Bond et al., 1988). However, a specific role for ubiquitin in heat response has not been well characterized, other than a suggestion that ubiquitin might assist in removing abnormal proteins resulting from high temperatures (Carlson et al., 1987).

In plants, responses to heat have included increases in binding of ubiquitin to high mol wt protein conglomerates (Shimogawara and Muto, 1989; Ferguson et al., 1990; Wettern et al., 1990) and both increases and decreases in expression of ubiquitin transcripts (Burke et al., 1988; Christensen and Quail, 1989; Hoffman et al., 1991; Christensen et al., 1992; Garbarino et al., 1992). Apart from the recent study of Ferguson et al. (1990), in which increases in protein breakdown, hsp synthesis, and ubiquitin conjugation were found in response to high temperatures in wheat roots, there has been no other work directly relating ubiquitin to protein breakdown or turnover in response to stress in plant tissues.

In the present study we have attempted to assess the extent of protein breakdown in cultured pear (*Pyrus communis* L.) fruit cells in response to high temperatures. We have associated this with induction of hsps during moderate and severe heat stress and attempted to examine whether ubiquitin is possibly involved in protein degradation during heat stress.

MATERIALS AND METHODS

Cells

Pear (*Pyrus communis* L. cv Passe Crassane) cells, initially derived from fruit, were cultured in liquid medium with 4.5 μM 2,4-D and no cytokinin, as described by Codron et al. (1979).

Uptake and Incorporation of [^{35}S]Met

Pear cells (20 mL) in the early log phase of growth and density (30–50 mg fresh weight/mL) were labeled with approximately 300 μCi of [^{35}S]Met (1220 Ci/mmol; New England Nuclear). For uptake and incorporation into protein, labeled cells were incubated at 25, 39, 42, and 45°C. At various times, including immediately after the radiolabel was added (for determining the time 0 value), 0.5-mL samples were added to 0.5 mL of 30% cold TCA. After standing for 20 min on ice, the TCA-precipitable protein and cell debris was pelleted at 12,000*g* for 2 min. Replicate samples (usually 50 μL) of the supernatant were taken for counting and represented TCA-soluble activity. TCA-insoluble activity was obtained by resuspending the pellet in the remaining supernatant and collecting the precipitate on GF/A glass fiber filters (Whatman). The filters were washed with 2 mL of cold 5% TCA and 2 mL of cold ethanol. After drying, the filters were assayed for radioactivity. All radioactivity was measured by liquid scintillation counting using a Beckman LS2800 counter and ACSII (Amersham) scintillation fluid. Where incorporation alone was assessed, cells were labeled as above for 5 min and then washed three times in fresh culture medium containing 0.2 mg/mL Met. The cells were then returned to their original density in the same Met-supplemented medium, incubated at the appropriate temperatures, and sampled as described before. In this case, the first sample taken for time 0 was after the cells were returned to their original density.

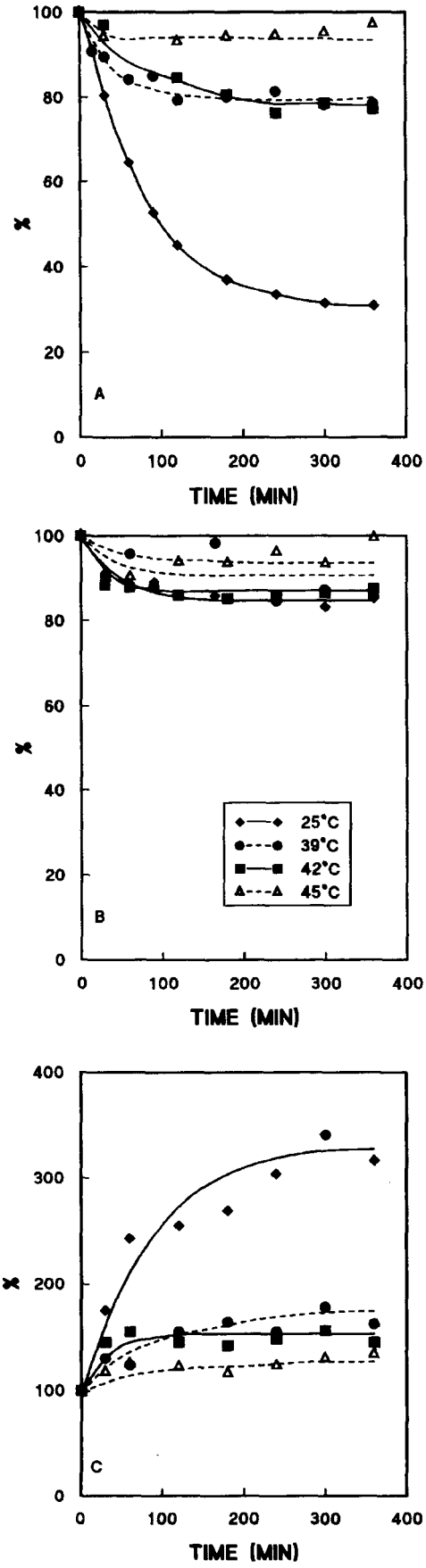
For labeling and separation of hsps, cells (approximately 700 mg/5 mL) were incubated at 25, 39, 42, and 45°C for 1 h. Cells were then labeled (20 μCi/mL for cells at 25°C, 40 μCi/mL at higher temperatures) and incubated for an additional 1 h. The cells were then washed on large GF/A filters with 2 × 15 mL of 3% Suc containing 1 mM Met, collected, frozen immediately in liquid nitrogen, and stored at −80°C. For protein extraction, the frozen cells were homogenized in a glass Kontes grinder in 0.5 mL of 10 mM Tris/Mes (pH 7.5), 1 mM PMSF, and 0.1 mM DTT. The cell debris was spun down at 12,000*g*, and TCA-precipitable radioactivity in the supernatant was assessed by spotting 5 μL onto 3MM (Whatman) paper prewashed with 10% TCA. The papers were then washed twice with 10% cold TCA (10 min), twice with 5% cold TCA (5 min), and twice with cold ethanol (5 min), dried, and counted by liquid scintillation spectrophotometry as described above. The same TCA-precipitable cpm were loaded onto lanes of 13% polyacrylamide gels, and proteins were separated by SDS-PAGE, stained, and autoradiographed as described by Veierskov and Ferguson (1991).

Ribosome Isolation

Pear cells (2 g) were collected on glass wool, frozen in liquid nitrogen, and stored at −80°C. To extract ribosomes, each sample was ground to a powder in liquid nitrogen with 100 mg of sand. Isolation buffer (13 mL; 0.2 M Tris-HCl [pH 8.5], 0.2 M Suc, 0.06 M KCl, 0.03 M $MgCl_2$, 0.1% PVP, 0.01 M β-mercaptoethanol) was added to the frozen powder and ground until the solution began to thaw. The homogenate was centrifuged at 15,000*g* for 20 min, and the supernatant was layered over a 2-mL Suc pad (1.2 M Suc, 0.04 M Tris-HCl [pH 8.5], 0.02 M KCl, 0.01 M $MgCl_2$) and centrifuged at 100,000*g* in a TY 40 rotor (Beckman) for 130 min. The pellet was rinsed gently with 1 mL and resuspended in 100 μL of the above solution minus the Suc. After the pellet was layered onto a 20 to 40% Suc gradient containing 0.02 M Tris-HCl (pH 8.5), 0.02 M KCl, 0.01 M $MgCl_2$, the gradient was centrifuged at 140,000*g* in an SW50 rotor (Beckman) for 60 min and then fractionated with monitoring at 254 nm with an Isco UV analyzer. The monosomal peak and polysomal fractions were collected separately, and A_{260} was measured. The percentage of polysomes were expressed as polysome *A* divided by total polysomal plus monosomal *A*.

Protein Breakdown

Cells in the log phase of growth were labeled with [^{35}S]-Met (usually 100 μCi per 50 mL of cells of approximate density 30–50 mg fresh weight/mL solution). After 20 h, the cells were washed three times in fresh medium and returned to the initial density. Aliquots of 25 mL were incubated at appropriate temperatures in shaking water baths. At the required times, replicate 0.5-mL samples of cells were taken, and TCA-soluble and -insoluble radioactivity was determined as described above. Protein breakdown was then assessed by following the increase in TCA-soluble or decrease in TCA-insoluble activity over time. For specific temperatures, break-



down was expressed as a percentage of the change in radioactivity that occurred at 25°C.

Western Analysis

Pear cells (400–500 mg/5 mL) in early log phase were incubated at various temperatures. Cells were collected by filtration and washed and frozen as described above. Frozen cells were homogenized in 0.5 mL of 10 mM Tris/Mes (pH 7.2) with 0.1 mM DTT and 1 mM PMSF. After the cells were centrifuged, proteins in the supernatant were separated by SDS-PAGE on 13% gels (30 μg/lane), using a Tricine buffer system (Schagger and von Jagow, 1987). Proteins were transferred onto 0.2-μm cellulose acetate paper and probed with antibodies to ubiquitin (bovine; Dakopat, Copenhagen, Denmark), E1 (wheat, Hatfield and Vierstra, 1989), and two proteins of the E2 family (wheat, *Arabidopsis*; gene products of *UBC1* and *UBC4*; Sullivan and Vierstra, 1991). Bound proteins were visualized with alkaline phosphatase-conjugated secondary antibodies.

Northern Analysis

Pear cells (3–5 g fresh weight) were harvested after various heat treatments, washed in 0.3 M Suc, frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted, denatured, fractionated (30 μg/lane), and transferred to a Hybond-N^+ (Amersham) membrane as described by Veierskov et al. (1992). The inserts from pKG3730 (barley polyubiquitin cDNA; Gausing and Barkodottir, 1986), pTip22 (asparagus ubiquitin-ribosomal fusion protein cDNA; Davies and King, Levin, New Zealand), and pMON9575 (petunia hsp70 cDNA; Winter et al., 1988) and a 490-bp *PstI* insert of pFS1968 (soybean hsp17 cDNA; Schoffl et al., 1984) were labeled with ^{32}P using a random primer DNA-labeling system (BRL). Hybridization was carried out according to the Amersham Hybond-N^+ protocol. Following hybridization the membrane was washed twice for 10 min in 2× SSC, 0.1% SDS at room temperature and then once in 1× SSC, 0.1% SDS at 65°C for 15 min. The membrane was exposed to Kodak XAR film with intensifying screens at −80°C.

RESULTS

Protein Synthesis at High Temperatures

Protein synthesis was reduced at high temperatures (Fig. 1A). During 6 h of incubation there was a marked reduction in TCA-soluble radioactivity at 25°C, and this reduction was

Figure 1. Cellular uptake and incorporation of radiolabeled Met into proteins of pear cells incubated at different temperatures. Uptake and incorporation were measured by TCA-soluble radioactivity with continuous labeling (A). Incorporation divorced from uptake was measured by TCA-soluble (B) or TCA-insoluble (C) radioactivity after 5 min of labeling and subsequent washes and incubation with unlabeled Met. Values in the box are incubation temperatures. Data are presented as the percentage of decrease (A and B) or increase (C) compared with that at the beginning of the experiment and are the mean values for triplicate samples averaged from at least two separate experiments.

much less at higher temperatures. This measurement of TCA-soluble activity is that from the total 0.5-mL sample, i.e. cells plus solution. Therefore, a reduction in TCA-soluble cpm is the result of both uptake of [^{35}S]Met into the cell and incorporation into protein. For instance, uptake may be reduced at high temperatures, and incorporation would be correspondingly less. These two processes needed to be separated, and this was done by washing the cells with unlabeled Met after only 5 min of uptake and then monitoring loss of TCA-soluble activity. (In this case, time 0 was immediately after the 5-min pulse.) When this was done, there was little difference between treatments (Fig. 1B), indicating that much of the large difference in TCA-soluble radioactivity associated with the heat treatments was the result of reduced cellular uptake at the higher temperatures. A more relevant and sensitive measure of incorporation was found when TCA-insoluble radioactivity was measured in the presence of unlabeled Met. Under these conditions, we found reduced incorporation of label into protein at the higher temperatures (Fig. 1C). However, although incorporation was reduced, TCA-insoluble activity increased over time, even at 45°C, amounting here to an approximately 30% increase, as against approximately 200% at 25°C.

Ribosomal association into polysomes, an indication of the potential for active protein synthesis, decreased rapidly when cells were transferred to either 39 or 42°C (Fig. 2). The decrease in percentage of polysomes was greatest at 42°C, and this low level remained constant until the cells were returned to 25°C. In contrast, cells held at 39°C showed a gradual recovery in polysome concentration at times longer than 1 h, and when they were returned to 25°C the percentage of polysomes was higher than in cells held continuously at 25°C.

When the products of protein synthesis at high temperatures were examined by one-dimensional SDS-PAGE, we found that hsp synthesis was strongly induced in the cells

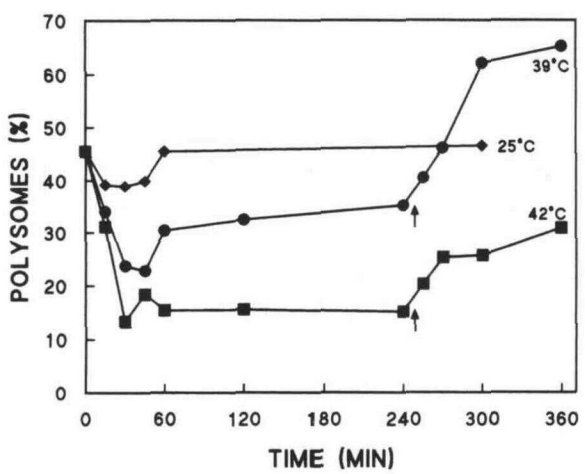


Figure 2. Aggregation of ribosomes extracted from pear cells after different heat treatments. Cells were transferred to the indicated temperatures at time 0 and returned to 25°C at the times indicated by the arrows. Data are representative values from triplicate experiments.

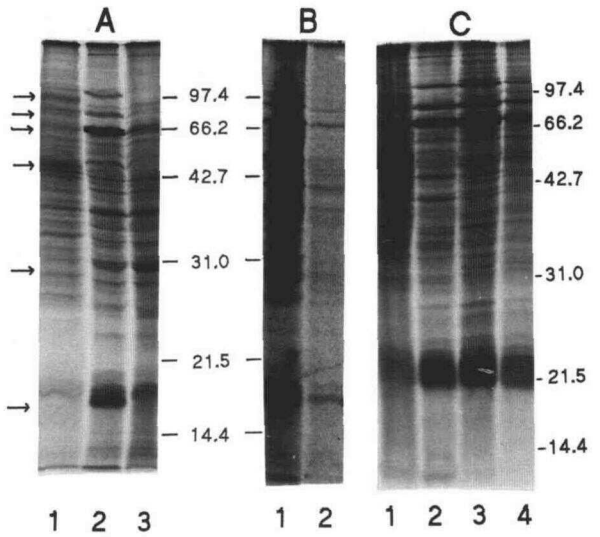


Figure 3. [^{35}S]Met-labeled proteins extracted after heat treatment of pear cells. The same amounts of labeled protein were loaded on each lane in each of A, B, and C. A, Cells labeled in the 2nd h of a 2-h exposure to 25, 39, and 42°C (lanes 1, 2, and 3, respectively). B, Cells labeled as in A but at 42 and 45°C (lanes 1 and 2, respectively). C, Cells labeled for 1 h after 1 h at 25°C (lane 1) and after 1 h (lane 2), 4 h (lane 3), and 7 h (lane 4) at 39°C. Values denote molecular mass markers (Bio-Rad) in kD and arrows indicate likely hsps.

after 1 h at 39°C (Fig. 3). Among a number of differences in polypeptide bands, high molecular mass hsps were found, denoted by arrows in Figure 3A, with an additional strong band at approximately 30 kD. The low molecular mass hsps were evident at about 17 to 20 kD. Above 39°C, synthesis of these hsps declined; at 42°C they were still at levels above control but were not present in cells after 2 h at 45°C (Fig. 3, A and B; since only very low levels of radiolabeled proteins could be extracted at 45°C, two lanes of proteins from cells at 42 and 45°C were run separately with only 5000 cpm/lane, and these are shown in Fig. 3B). Synthesis of the major hsps continued at 39°C for up to 8 h, although some minor bands declined and some increased during this time at the high temperature (Fig. 3C).

The abundance of mRNAs showing sequence homology with four heat-induced genes was examined at the different temperatures. Although the hsp17 and hsp70 cDNAs were from soybean and petunia, respectively, there was sufficient sequence homology between these and RNA from pear to provide some characterization of the pattern of expression. A single band was found when pear RNA was probed with the radiolabeled hsp17 insert. This was not expressed at 25°C and accumulated to detectable levels only at 39°C (Fig. 4A). Above this temperature its expression appeared to be inhibited. At 39°C, hsp17-related RNA levels were maximal at 2 h and were maintained at that level for up to 8 h (Fig. 4B). When cells were returned to either 25 or 4°C after 2 h at 39°C, expression of hsp17 mRNA continued to increase. Hybridization with the hsp70 cDNA also produced a single band, showing constitutive expression at 25°C (Fig. 5A). Its

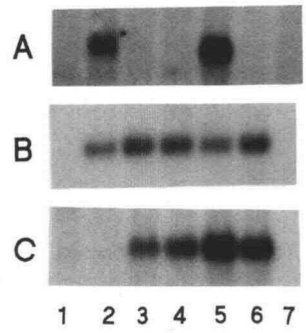


Figure 4. Northern analysis of total RNA extracted from pear cells after various heat treatments. Hybridization was with the ^{32}P-labeled insert from pSF1968 (soybean hsp17). An equal amount of RNA (30 μg) was loaded on each lane. The radioactive bands were sized from RNA markers at 2.3 kb. Lanes 1 through 7 represent, respectively, 25°C, 39°C for 1 h, 42°C for 1 h, 45°C for 1 h, 39°C for 2 h, 42°C for 2 h, and 45°C for 2 h (A); 25°C and 39°C for 1, 2, 4, 6, and 8 h (B); 25°C, 4°C for 2 h, 39°C for 1 h, 39°C for 2 h, 39°C for 2 h, 25°C for 2 h, 39°C for 2 h, 4°C for 2 h (C).

abundance increased greatly at 39°C, but expression declined at higher temperatures. The level of hsp70 mRNA was maintained after the cells were returned to 25°C from 2 h at 39°C (data not shown), as was evident for hsp17 (Fig. 4C). The two cDNAs for different ubiquitin genes gave slightly different patterns for enhanced expression at high temperatures (Fig. 5, B and C).

Hybridization with inserts from both pKG3730 and pTip22 produced bands of 1.5 and 0.7 kb. There was strong constitutive expression of both polyubiquitin and ubiquitin/fusion protein mRNAs at 25°C. Both ubiquitin mRNAs increased in strength at 39°C but not significantly so at the higher temperatures. At 45°C, there was some decline in transcript level (Fig. 5). The lower 0.7-kb band declined in strength after 2 h, particularly for the polyubiquitin transcripts (Fig. 5B).

Protein Breakdown at High Temperatures

When breakdown was followed by measuring TCA-soluble activity in prelabeled cells, we found it to be highest in cells incubated at 45°C, and it then increased after 6 h to a level 50% higher than that of control cells (Fig. 6A). Protein degradation was also higher in cells held at 39 and 42°C. Degradation at 39°C occurred in the 1st h of heating and was followed by some sign of recovery.

An increase in levels of TCA-soluble radioactivity could be interpreted as resulting from decreased protein synthesis with increasing temperatures rather than an increase in breakdown per se. However, measuring protein breakdown in the presence of the protein synthesis inhibitor CH enabled a clearer assessment of the extent of breakdown to be made. Treatment with CH resulted in an increase in the level of TCA-soluble radiolabel at 25°C to about 10% above that in the absence of CH (Fig. 6B). This was a level similar to that found at 39°C. When cells were heated to 39°C in the presence of CH, however, a large increase in TCA-soluble radioactivity was found, approaching the levels found at 45°C (Fig. 6).

Measurements of cell viability using Evans blue indicated that the increase in the number of dead cells at 39 and 42°C after 2 h was less than 10% more than that occurring during the same time at 25°C. Cell death at 45°C was only 30 to 40% after 2 h, by which time the major breakdown has taken place.

Ubiquitin Conjugation

There was little change in ubiquitin conjugation in response to heat treatments of cells. Western analysis of cell extracts after various temperature treatments showed characteristic binding to high molecular mass regions and several bands, but no specific changes associated with heat were evident (binding at 25°C is shown in Fig. 7C). Many of the bands are likely to result from nonspecific binding. When the blots were probed with antibodies for E1 and the E2 proteins from UBC1 and UBC4, the only changes associated with heat were found in bands responding to UBC4 and E1 protein antibodies. There was a marked degradation in a polypeptide recognized by the UBC4 protein antibody at 42°C after 1 h and at all elevated temperatures after 2 h (Fig. 7E). Intensity of the major E1 protein band declined at 42 and 45°C after 2 h (Fig. 7A). The major E1 band is the highest molecular mass band, the others possibly being breakdown products (P. Hatfield, personal communication). Intensity of binding of a polypeptide cross-reactive to the UBC1 protein remained constant during the 2 h at different temperatures (binding at 25°C is shown in Fig. 7B). The upper UBC1 protein band (arrow) has a mass of about 25 kD, and the lower has a mass of about 20 kD. Both polypeptides that were cross-reactive with the E2 protein antibodies appear to have greater molec-

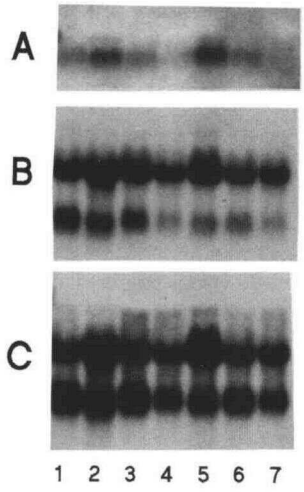


Figure 5. Northern blots of total RNA extracted from pear cells after heat treatments hybridized with ^{32}P-labeled inserts from pMON9575 (petunia hsp70; A), pKG3730 (barley polyubiquitin; B), pTip22 (asparagus ubiquitin/fusion protein; C). An equal amount of RNA (30 μg) was loaded on each lane. The radioactive bands were sized from RNA markers at 3.1 kb for A and 1.5 and 0.7 kb for the two bands in B and C. Lanes 1 through 7 represent, respectively, 25°C, 39°C for 1 h, 42°C for 1 h, 45°C for 1 h, 39°C for 2 h, 42°C for 2 h, and 45°C for 2 h.

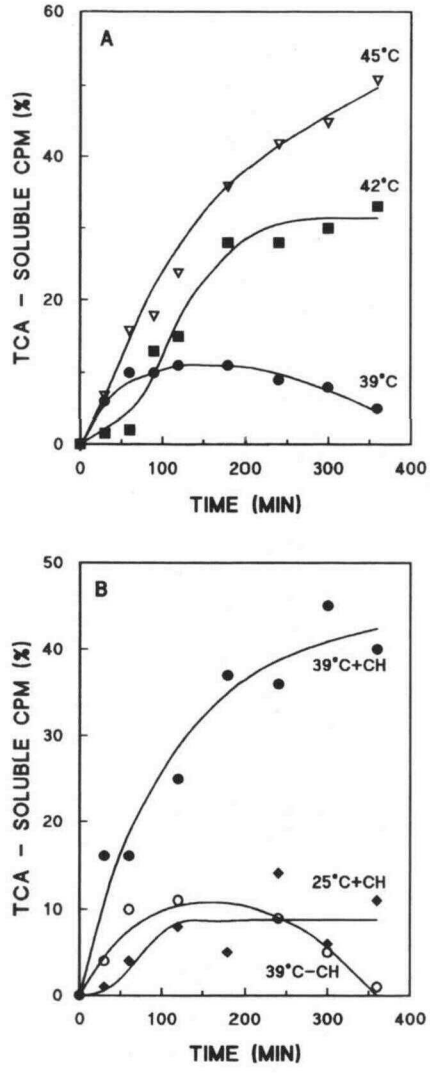


Figure 6. Increase in TCA-soluble radioactivity in pear cells during incubation at different temperatures. Cells were prelabeled, and TCA-soluble radioactivity was determined. Data are presented as percentages of increase in TCA-soluble cpm over that measured for cells at 25°C and are the means of triplicate values averaged from at least two experiments. Data in A are from cells treated at the given temperatures; data in B are from cells incubated in the presence or absence of 3.5 μM CH added at the beginning of the heat treatment.

ular masses than the 16- and 23-kD masses of the original wheat and *Arabidopsis* proteins (Sullivan and Vierstra, 1991).

DISCUSSION

A temperature of between 39 and 42°C appears to be critical for cultured pear cells. Protein breakdown increases above 39°C, but synthesis of hsps declines at temperatures above this. This manifestation of heat response is accompanied by a reduction in expression of the heat-induced transcripts of hsp17 and hsp70 (Figs. 4 and 5). A further indication that 39°C is a permissive temperature is that cells recover from both protein breakdown (Fig. 1) and ribosomal deaggregation (Fig. 2) after varying times at 39°C but not with continuous exposure to higher temperatures. A similar temperature sensitivity was found in the same cultured cells when regrowth was monitored as a viability test (Wu and Wallner, 1983). Temperatures below 40°C have also been shown to be permissive with these cells, with stimulated calcium uptake occurring at 38°C (Klein and Ferguson, 1987). Continued hsp synthesis during 8 h at 39°C was similar to that found in discs of heated apple (Lurie and Klein, 1990) and tomato (Klein and Lurie, 1990) fruit. Our results appear to differ slightly from those from soybean (Kimpel et al., 1990), in which, despite continued uptake and incorporation of radiolabel, hsp synthesis declined after about 3 h of continuous exposure to 40°C. It is worth noting that cells in solution may not be subject to the same range of stresses encountered in whole plants, but, although they are less useful in gauging the reaction of intact tissues, they may provide a clearer indication of cellular responses to heat.

Our results concerning the reduction in protein synthesis

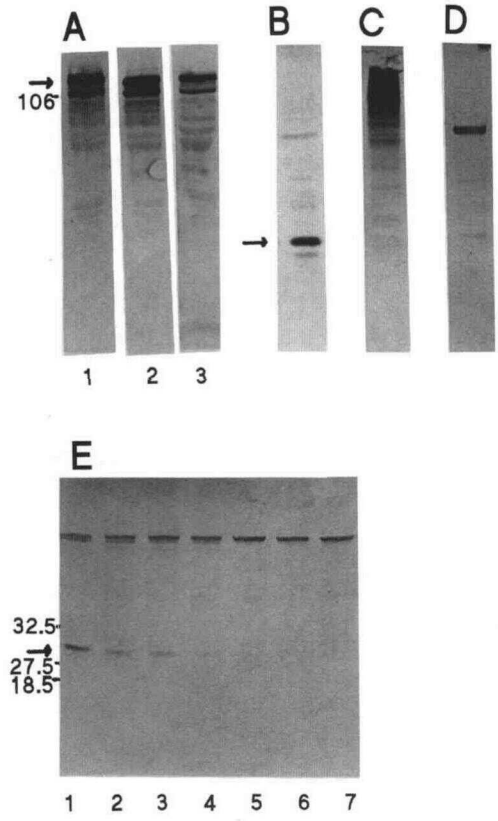


Figure 7. Western analysis of pear cell proteins extracted and separated after heat treatments. Western blots were probed with antibodies to E1 (A), UBC1 (B), ubiquitin (C), preimmune serum (D), and UBC4 (E). Lanes in A are 25°C, 45°C for 1 h, and 45°C for 2 h (lanes 1, 2, and 3, respectively). B, C, and D, Representative lanes of binding at 25°C. E, Lanes 1 through 7 are 25°C, 39°C for 1 h, 42°C for 1 h, 45°C for 1 h, 39°C for 2 h, 42°C for 2 h, 45°C for 2 h, respectively. Arrows denote: A, E1 protein; B, protein cross-reactive with UBC1 protein antibody; E, protein cross-reactive with UBC4 protein antibody. Molecular masses (in kD) were determined from prestained marker proteins (Bio-Rad).

with high temperatures are consistent with those previously reported (Key et al., 1981). Protein breakdown has been studied less, although our results agree with the increased breakdown found in wheat roots exposed to 40°C (Ferguson et al., 1990). It is unlikely that the increased breakdown that we found is due to enhanced cell death at the higher temperatures. Although there was significant mortality at 45°C, there was little difference in viability between cells in the control and the lower heat treatments in the first 2 h.

Transient increases in proteolysis in response to heat shock have been found in nonplant cells, such as mammalian cells (Parag et al., 1987; Gropper et al., 1991) and in *Escherichia coli* (Gottesman, 1989). At higher temperatures, there appears to be a reduction in proteolytic activity, and it is possible that some proteolytic activity is heat labile (Carlson et al., 1987). Plant cells may respond differently; we found what may be a transient increase in proteolysis at the permissive temperature of 39°C, and this was enhanced at higher temperatures. Whether this increase represents true temperature sensitivity of proteases or is a more indirect response of the cells at temperatures above about 40°C remains to be established.

Our results with CH run counter to those with animal cells. Parag et al. (1987) found that CH had little effect on heat-induced proteolysis and concluded that the measured transient protein breakdown was not dependent on hsp synthesis. In contrast, we found that CH markedly increased the levels of TCA-soluble radiolabel at 39°C (Fig. 6). This increase may be due to one of two processes. One is that CH, by inhibiting protein synthesis, reveals the true extent of protein breakdown. If this is correct, then there is still a considerable turnover (synthesis-breakdown-resynthesis) of protein occurring at permissive high temperatures. The other is that CH, by inhibiting hsp synthesis, prevents the protection of protein degradation afforded by hsps. Thus, degradation at 39°C, normally low because of the presence of hsps, in the presence of CH, reaches levels achieved at 45°C, a temperature at which hsps do not accumulate. Detailed analysis of turnover at the various temperatures is needed before a clear conclusion can be determined concerning the processes involved.

Given a pattern of reduced protein synthesis and increased levels of protein breakdown at high temperatures, then we need to consider possible relationships of this with hsps and ubiquitin. The induction of hsps by the temperatures used in our experiments is conventional. The moderate heat-shock temperature of 39°C induces rapid and high accumulation of hsps, an increase in mRNA of two hsp genes and polyubiquitin, and an initial increase in protein degradation that later ceases or stabilizes. This cessation of protein degradation may be due to the presence of high concentrations of hsps, which recognize and stabilize partially unfolded proteins and cause dissolution of protein aggregates due to denaturation. That these hsps can be produced in very large amounts has been shown recently by DeRocher et al. (1991). Hsp accumulation is much less and protein degradation is higher at 42 or 45°C than at the moderate heat-shock temperature. There seems, therefore, to be a relationship between the extent of protein degradation and hsp accumulation, where the general increase in hsps may protect against protein breakdown.

There appears to be sensitive control of expression for the hsp17 gene, with a rapid decrease in transcript level above 39°C. The low mol wt family is strongly expressed in plants (Vierling, 1991), and our results showing continued protein and gene expression during 8 h at 39°C and after return to ambient or low temperature are consistent with recent results from pea hsp18.1 (DeRocher et al., 1991). Temperature sensitivity may also be a feature: expression of β-glucuronidase through the hsp18.2 promoter in transgenic *Arabidopsis* also follows a temperature pattern reminiscent of our results with hsp17 transcripts, where maximal levels of β-glucuronidase activity at 35°C declined to negligible levels at 40°C (Takahashi et al., 1992). The continuation of hsp synthesis at 39°C for up to 8 h, whereas synthesis and gene expression are only transiently induced at 42 and 45°C, suggests that there are regulatory controls that limit hsp synthesis during prolonged heat treatment above 40°C. Whether this control is due to mRNA breakdown or reduced transcription and/or translation is unknown.

Although ubiquitin is involved in the heat-shock response in mammalian systems, we found only two effects of heat on ubiquitin in our study. The first was an increase in levels of ubiquitin transcripts, and the second was a decrease in the protein cross-reacting with the UBC4 antibody. In some ways the lack of further effects is not surprising given the equivocal results with ubiquitin and heat in the literature. After the initial recognition that ubiquitin could be heat induced (Bond and Schlesinger, 1986), the major evidence for involvement of ubiquitin in heat response was in work on yeast and mammalian cells; mutants lacking a polyubiquitin gene, or with a thermolabile E1, were more temperature sensitive (Finley et al., 1987; Parag et al., 1987; Gropper et al., 1991).

Despite the high cross-reactivity with ubiquitin antibodies in the high molecular mass region (Fig. 7), we were unable to find an increase associated with heat. In both *Chlamydomonas* and wheat roots, high mol wt ubiquitinated protein conglomerates increased, with a concomitant reduction in free ubiquitin (Shimogawara and Muto, 1989; Ferguson et al., 1990; Wettern et al., 1990). We did not find significant levels of free ubiquitin in western analyses. To some extent, unless particular ubiquitinated proteins are at high levels, we should not expect to see significant ubiquitination under conditions of rapid protein breakdown. This is particularly the case if we assume that most ubiquitin involvement is primarily with abnormal and short-lived proteins (although evidence for involvement in degrading long-lived proteins has been published recently by Gropper et al., 1991). Until antibodies specific to plant ubiquitin conjugates are used, we will not be sure of the appearance of specific conjugates as a heat response.

The effects of heat on the conjugating enzymes are in agreement with conjugation results, except for the decline in levels of protein cross-reacting with UBC4 protein antibody. It is obvious that, despite a decrease in intensity of the E1 antibody binding, levels of the E1 and UBC1 proteins are largely retained over the heating treatments, suggesting maintenance of conjugating facility. The increase in ubiquitin transcripts that we found at 39 and 42°C (Fig. 5) is in agreement with similar increases found in other species. In maize, levels of a 2.1-kb polyubiquitin mRNA increased substantially with heat, although smaller mRNAs were less

affected, and two larger transcripts (3.1 and 5.2 kb) appear in response to heat (Christensen and Quail, 1989; Christensen et al., 1992). Differential effects with transcripts were also found in *Arabidopsis*, in which a 1.7-kb transcript increased in abundance after heat shock and a 1.35-kb transcript decreased. Heat-induced increases in transcript levels were also found in two sunflower ubiquitin genes, with one gene showing putative heat-shock sequences (Binet et al., 1991). Transcripts of the parsley polyubiquitin gene *ubi4* showed no change with heat (Kawalleck et al., 1993). It appears that there is a general pattern emerging where polyubiquitin genes tend to be heat inducible or insensitive, whereas those for the ubiquitin/fusion proteins are heat sensitive and tend to decline in abundance with high temperatures (Hoffman et al., 1991; Garbarino et al., 1992).

In summary, although we measured enhanced protein breakdown at all elevated temperatures, ubiquitin transcripts accumulated only at the least deleterious high temperature, 39°C, at which protein breakdown was lowest. Conjugation of ubiquitin to proteins was not responsive to heat insofar as we could detect possible changes. However, there is good published evidence in the nonplant literature for ubiquitin involvement in heat response but at a much more selective level, in terms of transcription, postranscriptional sensitivity, and conjugation. We should expect to find evidence for increased ubiquitination of abnormal proteins and short-lived proteins if the turnover of these can be adequately analyzed in plants. A moderate heat shock (43°C) of mammalian cells caused a transient acceleration of protein degradation, whereas higher temperatures inhibited proteolysis. Protein degradation at 43°C appeared to be mediated by the ubiquitin system (Parag et al., 1987). The same kinetics of degradation were found even when CH was present during the heating to inhibit the synthesis of hsps. Therefore, the ubiquitin involved in heat-induced protein degradation was present before the heat treatment was given. In cultured pear cells a moderate heat shock (39°C) also caused a transient increase in protein degradation, but higher temperatures stimulated protein breakdown instead of inhibiting proteolysis, as in the study with mammalian cells. This stimulation was consistent with reduced hsp synthesis and gene expression. It is also possible that ubiquitin has a dual role. Whereas the animal research suggests that it can remove abnormal proteins as a heat response, we also would suggest that it has an hsp-like protective function. Although it is constitutively expressed more strongly than the hsp genes that we have looked at, ubiquitin transcripts are also subject to reduced expression at nonpermissive temperatures. The functions of hsps and ubiquitin in heat response will only be clarified with more detailed study of protein breakdown.

Our study of heat response over the 39–45°C temperature range, and the separation of protein synthesis and breakdown, has revealed a pattern of response that highlights the possible importance of the regulation of protein breakdown in thermotolerance. The patterns of hsp and ubiquitin mRNA, hsp synthesis, and protein degradation support our suggestion that the protection of probably a wide range of proteins from breakdown as a heat response is critical to thermotolerance.

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