Transient Expression of HSC70 in Cherry Fruit Subjected to Heat Shock

Donna O. Perdue,[†] Elizabeth J. Mitcham,[‡] and Lisa G. Neven*,[†]

U.S. Department of Agriculture, Agricultural Research Service, 5230 Konnowac Pass Road, Wapato, Washington 98951, and Department of Pomology, University of California, Davis, California 95616

Temperature extremes are being investigated as an alternative method for postharvest control of quarantined insects. This study examined the heat shock response of cherry fruit under conditions that effectively destroy pests. Five different antibodies against members of the 70-kDa heat shock protein (HSP70) family were used to investigate the heat shock responses of treated cherry fruits. Western blots of total protein extracts revealed constitutive expression of heat shock cognate (HSC70) proteins. Each antibody recognized a pair of constitutively expressed HSC70 proteins that varied in size and relative abundance. Cherries subjected to heat treatment for 1 h at 45 °C followed by transfer to cold storage (0 °C) contained a 70-kDa polypeptide recognized by all of the antibodies used. In heat-treated fruits this induced polypeptide appeared at 12 h after heat treatment, disappeared by 24 h following treatment, and reappeared after 2 weeks in cold storage (0 °C). Cold storage alone did not alter constitutive expression or induce novel polypeptides recognized by anti-HSP70 antibodies. Thirty minutes or more of treatment at 45 °C was required to elicit the appearance of the 70-kDa polypetide recognized by all of the anti-HSP70 antibodies used.

Keywords: *Heat shock proteins (HSP); heat shock cognate proteins (HSC); postharvest heat treatment*

INTRODUCTION

Temperature extremes have been studied as potential quarantine treatments for insect pests in temperate fruits (Armstrong, 1994; Neven, 1994; Neven and Rehfield, 1995; Neven and Mitcham, 1996). These treatments are being developed as nonchemical alternatives to the use of methyl bromide fumigation to meet insect quarantine restrictions. High-quality, freshly harvested cv. Bing sweet cherries (*Prunus avium* L.) can withstand exposure to high temperatures (45 °C for 60 min or 47 °C for 25 min) without significant loss of quality (Neven and Mitcham, 1996). One combination of temperature treatments being explored is the application of a brief heat shock followed by cold storage during the transport of fresh fruit. Neven (1994) reported that a combination of 30 min at 45 °C followed by cold storage for 2 weeks resulted in 100% mortality for codling moth (*Cydia pomonella* L.), a major quarantine pest on North-American-produced pome and stone fruits. Research by others (Paull and McDonald, 1994) indicates that a heat stress prior to cold storage reduces damage incurred during cold storage. Continuing research by our laboratory indicates that cherries subjected to disinfestation heat treatments can tolerate cold storage for up to 2 weeks without loss of quality. However, fruit quality rapidly deteriorates following the 2 weeks of cold storage. We wanted to investigate the possible relationship between the production of heat shock proteins and/or heat shock cognates in cherry fruits following combination heat and cold quarantine treatments and their potential links to fruit quality. The work presented here examines the heat shock response of cherry fruits subjected to treatment at 45 °C followed by cold storage for periods lasting up to 2 weeks.

When temperatures are elevated above optimal growing temperatures, many organisms repress normal mRNA and protein synthesis and begin to transcribe and translate a set of heat shock proteins (HSPs). Crops adapted to temperate environments, including soybean, pea, maize, and wheat, can initiate HSP synthesis when their tissue temperatures exceed 32−33 °C (Vierling, 1991). HSP synthesis often occurs before leaf temperatures reach injurious levels (Hendershot et al., 1992). HSP synthesis increases with increasing temperature (Vierling, 1991). Several classes of HSPs have been described in plants: HSP110, HSP90, HSP70, HSP60, low molecular weight HSPs, and ubiquitin (Vierling, 1991). Some plant HSPs can also be induced by treatments such as cold (Neven et al., 1992), arsenite and heavy metals (Edelman et al., 1988), and wounding, water stress, or abscisic acid (Heikkila et al., 1984).

HSP70s are a family of proteins with a high degree of evolutionary conservation. Two groups of HS70 genes have been described: (a) genes encoding heat shock cognate proteins (HSCs) that are constitutively expressed and are not strongly induced by heat shock or other stresses and (b) inducible HS70 genes (Lindquist and Craig, 1988). Although they are found throughout the cell and have a diversity of functions, all HSP70 and HSC70 proteins bind ATP, show weak ATPase activity, and can be purified by nucleotide affinity chromatography (Vierling, 1991). Constitutively expressed HSC70s in plants can be found in the cytosol, endoplasmic reticulum, mitochondrion, and chloroplast (Anderson et al., 1994). Some HSC70 proteins can be induced by exposure to cold temperatures instead of heat. In

* Address correspondence to this author at the USDA-ARS, 5230 Konnowac Pass Rd., Wapato, WA 98951 [telephone (509) 454-6556; fax (509) 454-5646; e-mail neven@yarl.gov].

[†] U.S. Department of Agriculture.

[‡] University of California.

10.1021/jf980329s CCC: $15.00
Published on Web 09/17/1998

spinach, Neven et al. (1992) purified 79-kDa polypeptides induced by chilling at 5 °C and identified them as members of the HSP70 family on the basis of microsequence and immunological cross-reactivity. Similarly, Cabane et al. (1993) demonstrated that chilling (8 °C) induces a member of the HSP70 family in soybean.

MATERIALS AND METHODS

Plant Material. Cherry fruits were obtained from field lugs at a packing facility on the day of harvest (May 24 and 31 and June 6, 1994). Fruit had not been subjected to any postharvest treatments. Cherries were transported to Davis and sorted to obtain fruit of uniform size and maturity with an absence of defects. Fruits were stored overnight at 20 °C and 90% relative humidity, and treatments were initiated the following morning.

Treatment and Storage Conditions. Fruits were carefully placed into one of three treatment bins for heat treatment in a controlled atmosphere/temperature treatment system (CATTS) (Neven and Mitcham, 1996). The heating chamber is an airtight, forced-air system with temperature, dew point, air speed, and atmosphere controls. Fruits were heated at 45 ± 0.1 °C for 1, 15, 30, 45, and 60 min. Humidity within the chamber was supplied by atomized water injected into the air stream and was monitored with a chilled-mirror dew point analyzer (EG&G, Waltham, MA). Humidity was controlled by regulating the dew point to 2 ± 0.2 °C below the fruit surface temperature to prevent condensation and water loss. Air flow was maintained at 2 ± 0.2 m/s. Surface and center (at pit) fruit temperatures were monitored throughout the heating period using thermistors. There were three surface and three center thermistors per treatment bin.

Upon removal from the heating chamber, 75 fruits per heating period were stored at 20 °C and 90% relative humidity. Three samples of five fruits each were collected at 0, 12, and 24 h after heating. An additional 75 fruits from 0 and 60 min heating times were hydrocooled to 5 °C and transferred to a 0 °C cold room for storage. Three samples of five fruits each were collected at 12, 24, 36, 48, 60, and 72 h and 1 and 2 weeks. Sampled fruits were pitted, chopped, and frozen in liquid N_2 prior to storage at −80 °C.

Protein Extraction. Total proteins were extracted as described by Schuster and Davies (1983). Frozen samples were macerated in liquid N_2 and ground to a powder. Frozen, ground tissue was extracted in 0.5 M Tris, 0.1 M KCl, 0.05 M EDTA, 0.5 M sucrose, and 0.2% (v/v) β-mercaptoethanol (pH 7.4) at a ratio of 20 mL of buffer/10 g of fresh weight of sample. Samples were homogenized for 1 min at high speed and extracted for 10 min at 4 °C. An equal volume of phenol was added, and the mixture was shaken for 30 min at 20 °C. After centrifugation at 5000*g* for 15 min, the upper (phenol) layer was removed and re-extracted with 20 mL of extraction buffer and the upper layer again removed after centrifugation. Five volumes of methanol was added to the phenol phase, and the mixture was stored overnight at −20 °C.

Precipitate protein was recovered by centrifugation at 11950*g*. The protein pellet was washed twice with 20 mL of of methanol/0.1 M ammonium acetate and once with acetone. The pellet was resuspended in 1 mL of 50 mM Tris, 0.6% SDS, and 0.2% β-mercaptoethanol (pH 7.4) and dialyzed overnight against 10 mM Tris (pH 7.4) containing 10% glycerol. Dialyzed samples were cleared by centrifugation, and the protein content was measured according to the Bradford method (Bradford, 1976).

Gel Electrophoresis and Western Blotting. Protein samples were separated by 8% SDS−PAGE (Sambrook et al., 1989) and transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA) using a Trans-BlotSD semidry apparatus (Bio-Rad, Richmond, CA) in alkaline transfer buffer containing SDS (48 mM Tris, 39 mM glycine, 20% methanol, 1.3 mM SDS, pH 9.2; Bjerrum and Schafer-Nielsen, 1986). After transfer, blots were briefly stained with Coomassie blue (0.05% in 10% acetic acid, 25% 2-propanol) to check efficiency of transfer, then detained in methanol (25%), and rinsed in TBS (50 mM Tris, 500 mM NaCl, pH 7.4). Blots were blocked for 2 h in 1% bovine serum albumin and incubated overnight in primary antibody solution in TBS. Antibody binding was detected using alkaline phosphatase (AP) conjugated secondary antibodies (Sigma), and blots were developed in the chromogenic alkaline phosphatase substrates NBT/BCIP (Sambrook et al., 1989). Control blots were probed using only AP-conjugated secondary antibodies.

For routine analyses, proteins were separated on 5% acrylamide gels in a Bio-Rad Protean II minigel electrophoresis system. For more accurate determination of molecular mass, proteins extracted at 12 h following 1 h of heat treatment were separated on 15 × 16 cm 8% acrylamide gels in a Bio-Rad Protean electrophoresis system, and Western blotting was as described previously. Molecular masses of constitutive and induced bands were calculated by comparison with calibrated standard markers (broad range molecular weight markers, Bio-Rad).

HSC Antibodies. Five antibodies against members of the HSC70 family of proteins were used to examine cherry fruit responses to heat shock and cold storage. Descriptions of the antibody and choice of nomenclature (HSP or HSC) are as found in the literature supplied with the product. The antibodies used in this study were selected on the basis of potential cross-reactivity with members of the heat shock family of 70 kDa proteins (commercial preparations) or specific reactivity to cold-responsive heat shock cognates of the 70 kDa family (those from C. Guy).

(1) Affinity BioReagents (Golden, CO) MA3-001, monoclonal (rat) anti-heat shock protein 70 gene family antibody, was supplied as ascites fluid. This is the antibody 7.10 raised against *Drospophila* proteins as described in Kurtz et al. (1986). Monoclonal antibody MA3-001 recognizes both cognate and heat-shock-induced HSC70 proteins in a wide variety of species, including yeasts, trypanosomes, mice, humans, soybeans, and *Arabidopsis* (Wu et al., 1988). Antibody was used at a dilution of 1:250 in TBS.

(2) Affinity BioReagents MA3-006, monoclonal (mouse) anti-heat protein 70 antibody, was supplied as ascites fluid. This monoclonal antibody was raised against recombinant human HSP70 overexpressed in *Escherichia coli* (clone 3a3; Nadler et al., 1992). This antibody recognizes both constitutive (HSC70) members of the HSP70 family over a broad taxonomic range, including mouse, chicken, frog, yeast, and wheat. The antibody was used at a dilution of 1:1000 in TBS.

(3) Sigma H-5147, monoclonal (mouse) anti-heat shock protein 70, s supplied as ascites fluid. This antibody was raised against purified bovine brain HSP70 and recognizes both constitutive and inducible forms of HSP70 in a wide variety of organisms. Antibody was used at a dilution of 1:250 in TBS.

(4) Monoclonal (mouse) anti-heat shock cognate protein antibody 5B7 was a gift of C. Guy, University of Florida. This antibody was raised against spinach cytosolic HSC70 (Anderson et al., 1994) and supplied as lyophilized protein. Reconstituted antibody was used at a dilution of 1:1000 in TBS.

(5) Monoclonal (mouse) anti-heat shock cognate protein 70 antibody 1D9 was a gift of C. Guy, University of Florida. This antibody was raised against a BiP HSC70 protein from the endoplasmic reticulum lumen of spinach (Anderson et al., 1994) and supplied as lyophilized protein. Reconstituted antibody was used at a dilution of 1:1000 in TBS.

RESULTS

Expression of HSC70 following Heat Stress. The anti-HSP/HSC70 antibodies used in this study recognized one to three polypeptides in total proteins extracted from cherry fruits. Figure 1 shows representative Western blots of total cherry proteins probed with antibody 5B7, raised originally against spinach cytosolic HSC70 (Anderson et al., 1994). Table 1 summarizes the constitutive and induced HSC70s recognized by each antibody tested. The size and relative abundance of

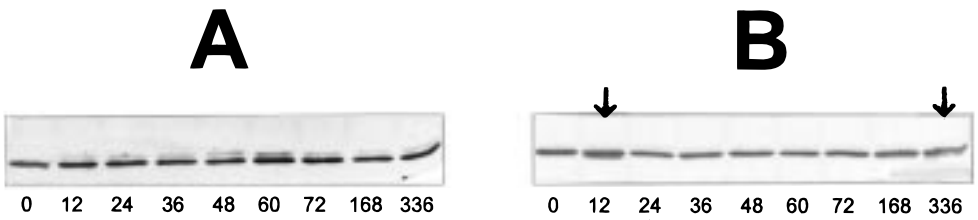

Figure 1. Western blots of total cherry proteins probed with monoclonal antibody 5B7. Proteins were extracted at the times indicated and separated on a 5% SDS−PAGE minigel; 25 μg of protein loaded per lane. (A) Cold storage only. Proteins were extracted from cherries after storage at 0 °C for the times indicated. (B) Heat shock followed by cold storage. Cherries were exposed to 45 °C for 1 h and transferred to storage at 0 °C for the times indicated. Arrows indicate HSC70 band induced at 12 h and 2 weeks of cold storage.

Table 1. Molecular Masses of Polypeptides Recognized by Anti-HSC Antibodies[a]

antibody	constitutive bands, kDa	induced band, kDa[b]
MA3-001	77 (heavy) 73 (light)	70
MA3-006	77 (light) 73 (heavy)	70
H-5147	79 (light) 73 (heavy)	70
5B7	77 (light) 73 (heavy)	70
1D9	77 (heavy) 73 (light)	70

[a] Molecular masses were determined by comparison with standard molecular mass markers. [b] Induced band seen only at 12 h and 2 weeks of cold storage after heat treatments at 45 °C (see Figure 1).

different HSC70 bands recognized varied according to the antibody used. Some of the polypeptides appeared to be constitutively expressed in cherry fruits. All of the antibodies recognized induced 70-kDa polypeptides, below the major band, at 12 h and 2 weeks of cold storage after the 45 °C heat treatment (Figure 1B). Cold storage alone did not appear to elicit up-regulation or novel expression of any polypeptides recognized by these antibodies (Figure 1A). It is unclear why only the heat-treated fruit exhibited this additional band at 12 h and 2 weeks of cold storage.

Cherry fruits exposed to 45 °C do not appear to synthesize HSP70s rapidly during the course of a heat treatment. Proteins extracted immediately after 60 min of exposure to 45 °C do not show induction of the HSP70 polypeptide(s) recognized by the antibodies used here (Figure 1B, time 0). The heat shock response was detectable after 12 h of cold storage (following 60 min of heat treatment) and had disappeared by 24 h (Figure 1; Table 1). However, proteins were extracted only at 0, 12, and 24 h after heat treatment (not cold-stored fruit); therefore, it is not possible to pinpoint the exact time course of HSP70 synthesis and decay. It is also possible that 45 °C may be too hot for cherries to produce heat shock proteins. It must be stressed, however, that this time/temperature combination was used because it has been shown to be effective in postharvest control of codling moth larvae, from which this project is derived.

Minimum Time Required for Expression of HSC70s. Cherries were exposed to heat treatment at 45 °C for 0, 15, 30, 45, or 60 min. Proteins were extracted immediately after heat treatment (Figure 2A) or after 12 h at 0° (Figure 2B). No HSC70s showed up-regulation or novel synthesis during the course of heat treatment lasting up to 1 h. Following 12 h of cold storage, increased HSC70 synthesis is seen only in fruit subjected to 30 min or more of exposure to 45 °C (Figure 2B). Blots with antibody MA3-001 are not shown since they were identical to blots with MA3-006.

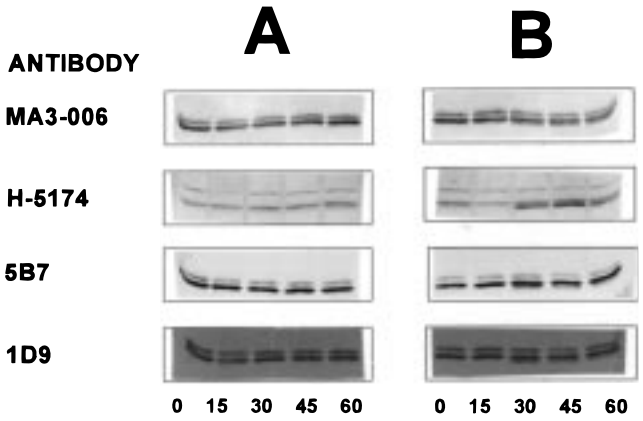

Figure 2. Duration of heat treatment required to elicit 70-kDa induced polypeptide. Cherries were treated for 0, 15, 30, or 60 min at 45 °C. Proteins were extracted immediately after heat treatment (A) or after 12 h of storage at 0 °C (B). The Western blots shown here are replicate blots probed with various anti-HSP70 antibodies as listed; each lane contains 25 μg of protein separated on 5% SDS−PAGE.

DISCUSSION

Time Course of Protein Expression. The time course of heat-shock-stimulated expression of HSP70s was identical for all of the polypeptides recognized by a variety of anti-HSP70 antibodies. Transient heat-shock-induced expression of 70-kDa polypeptides is seen after 12 h of storage following heat treatments of 30 min or longer (Figures 1B and 2B). In some fruits, cold shock alone can induce expression of HSC70 proteins. For example, Neven et al. (1992) demonstrated that 2 days of exposure to 5 °C leads to increases in the levels of HSC70 proteins in spinach, bean, green pepper, tomato, petunia, and broccoli. In cherry fruit, cold storage alone did not increase or induce expression of HSC70 proteins recognized by the diverse anti-HSC70 antibodies employed here (Figure 1A).

Although some plants produce heat shock proteins within 30 min of the onset of high temperatures (Kanabus et al., 1984), cherry fruits require more than an hour to synthesize detectable amounts of heat-induced HSC70 proteins at 45 °C (Figure 2). This is when proteins are extracted 1 h following exposure to high temperature, not including any cold storage period. In cherries, a minimum of 30 min of exposure to 45 °C is required to induce expression of HSC70 at 12 h after treatment. Heat-shock-induced HSPs usually decay within 24 h (Paull and McDonald, 1994). The heat-shock-induced HSC70s seen in cherry fruit decayed before 24 h (Figure 1B).

Diversity of Antibodies Used. The BiP proteins (immunoglobulin heavy chain binding proteins) are HSC70 proteins that show significant sequence divergence from cytoplasmic HSC70s (Vierling, 1991). This difference is such that there is often greater similarity among eukaryotic HSC70s from differing species than among HSC70s from different cellular compartments of a single species (Vierling, 1991). For example, antibody MA3-006 prepared against recombinant human HSC70 overexpressed in *E. coli* does not recognize the human glucose-regulated BiP protein GRP78, although it cross-reacts with proteins from the animal, fungal, and plant kingdoms. Anderson et al. (1994), using an antibody monospecific against spinach lumen ER BiP, found constitutive expression of a 70-kDa protein that is up-regulated during cold acclimation but is not expressed in response to heat shock. In cherry fruits, the polypeptides recognized by anti-BiP antibodies (1D9 against spinach lumen BiP) show the same pattern of constitutive and induced expression as those recognized by antibodies against the more highly conserved HSC70 family of proteins. Only two-dimensional electrophoresis would permit us to determine exactly which proteins are recognized by each antibody and to determine whether the 70-kDa polypeptide induced by heat treatment of cherry fruits consists of one or several isoforms.

Heat Shock and Fruit Quality. Neven and Mitcham (1996) reported that freshly harvested cv. Bing sweet cherries can withstand a 60 min exposure to 45 °C with little loss of quality. Cherries exposed to these temperatures showed no significant changes in titratable acidity, soluble solids, or firmness for up to 2 weeks after heat treatment (Neven and Mitcham, 1996). The transient expression of HSC70 polypeptides at 12 h following heat treatment was not associated with a decline in fruit quality as determined by standard measures of fruit status. Following 2 weeks of cold storage of heat-treated cherries at regular atmospheres, quality repidly declines. However, subsequent research (Neven and Drake, unpublished results) has shown that if fruits are packaged directly into modified atmosphere packaging (MAP) after heat treatment and hydrocooling, quality is maintained for >2 weeks. It is possible that the HSC70 seen at 2 weeks of storage in heat-treated fruit is a BiP indicative of cellular breakdown and/or protein denaturation leading to the rapid decline in fruit quality. It would be interesting to determine whether this protein is present in MAP-packaged cherries after 2 weeks of cold storage.

ACKNOWLEDGMENT

We thank C. L. Guy, Department of Environmental Horticulture, University of Florida, for the kind gift of antibodies 5B7 and 1D9 and You mei Wu, Bill Biasi, and Dan Coleman of U. C. Davis and Mac Morford of USDA-ARS for their technical support.

LITERATURE CITED

Anderson, J. V.; Li, Q.-B.; Haskell, D. W.; Guy, C. L. Structural organization of the spinach endoplasmic reticulum-luminal 70-kilodalton heat-shock cognate gene and expression of 70-kilodalton heat-shock genes during cold acclimation. *Plant Physiol.* **1994**, *104*, 1359−1370.

Armstrong, J. W. Heat and Cold Treatments. In *Insect Pests and Fresh Horticultural Products: Treatments and Responses*; Paull, R. E., Armstrong, J. W., Eds.; CAB International, Wallingford, U.K., 1994.

Bjerrum, O. J.; Schafer-Nielsen, C. *Analytical Electrophoresis*; Dunn, M. J., Ed.; Verlag Chemie: Weinheim, Germany, 1986; p 315.

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principles of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Cabane, M.; Calvet, P.; Vincens, P.; Boudet, A. M. Characterization of chilling-acclimation-related proteins in soybean and identification of one as a member of the heat shock protein (HSP 70) family. *Planta* **1993**, *190*, 346−353.

Edelman, L.; Czarnecka, E.; Key, J. L. Induction and accumulation of heat shock-specific poly (A^+) RNA's and proteins in soybean seedlings during arsenite and cadmium treatments. *Plant Physiol.* **1988**, *86*, 1048−1056.

Heikkila, J. J.; Papp, J. E. T.; Shultz, G. A.; Bewley, D. Induction of heat shock protein messenger RNA in maize mesocotyls by water stress, abscisic acid, and wounding. *Plant Physiol.* **1984**, *76*, 270−274.

Hendershot, K. L.; Weng, J.; Nguyen, H. T. Induction temperature of heat-shock protein synthesis in wheat. *Crop Sci.* **1992**, *32*, 256−261.

Kanabus, J.; Pikaad, C. S.; Cherry, J. H. Heat shock proteins in tobacco cell suspension during growth cycle. *Plant Physiol.* **1984**, *17*, 481−486.

Kurtz, S.; Rossi, J.; Petko, L.; Lindquist, S. An ancient developmental induction: heat-shock proteins induced in sporulation and oogenesis. *Science* **1986**, *231*, 1154−1157.

Lindquist, S.; Craig, E. A. The heat-shock proteins. *Annu. Rev. Genet.* **1988**, *22*, 263−77.

Nadler, S. G.; Tepper, M. A.; Schacter, B.; Mazzucco, C. E. Interaction of the immunosuppressant deoxyspergualin with a member of the HSP70 family of heat shock proteins. *Science* **1992**, *258*, 484−486.

Neven, L. G. Combined heat treatments and cold storage effects on mortality of fifth-instar codling moth (*Lepidoptera: Tortricidae*). *J. Econ. Entomol.* **1994**, *87*, 1262−1265.

Neven, L. G.; Mitcham, E. J. CATTS (Controlled Atmosphere/Temperature Treatment System): A novel tool for the development of quarantine treatments. *Am. Entomol.* **1996**, *42*, 56−59.

Neven, L. G.; Rehfield, L. M. Comparison of prestorage heat treatments on fifth-instar codling moth (*Lepidoptera: Tortricidae*) mortality. *J. Econ. Entomol.* **1995**, *88*, 1371−1375.

Neven, L. G.; Haskell, D. W.; Guy, C. L.; Denslow, N.; Klein, P. A.; Green, L. G.; Silverman, A. Association of 70-kilodalton heat-shock cognate proteins with acclimation to cold. *Plant Physiol.* **1992**, *99*, 1362−1369.

Paull, R. E.; McDonald, R. E. Heat and cold treatments. In *Insect Pests and Fresh Horticultural Products: Treatments and Responses*; Paull, R. E., Armstrong, J. W., Eds.; CAB International: Wallingford, U.K., 1994; pp 191−222.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. Detection and analysis of proteins expressed from cloned genes. In *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989; pp 18.47−18.74.

Schuster, A. M.; Davies, E. Ribonucleic acid and protein metabolism in pea epicotyls. I. The aging process. *Plant Physiol.* **1983**, *73*, 809−816.

Vierling, E. The roles of heat shock proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1991**, *42*, 579−620.

Wu, C. H.; Caspar, T.; Browse, J.; Lindquist, S.; Somerville, C. Characterization of an HSP70 cognate gene family in *Arabidopsis*. *Plant Physiol.* **1988**, *88*, 731−740.

Received for review March 29, 1998. Revised manuscript received August 13, 1998. Accepted August 14, 1998. This work was partially funded by the Washington State Tree Fruit Commission.

JF980329S