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KONSTANTINOS E VLACHONASIOS

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Major professor

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PRESTORAGE HEAT TREATMENT TO INHIBIT CHILLING INJURY AND SYNCHRONIZE RIPENING IN TOMATO (LYCOPERSICON ESCULENTUM MILL.) FRUIT

By

Konstantinos E. Vlachonasios

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ABSTRACT

PRESTORAGE HEAT TREATMENT TO INHIBIT CHILLING INJURY AND SYNCHRONIZE RIPENING IN TOMATO (LYCOPERSICON ESCULENTUM MILL.) FRUIT

By

Konstantinos E. Vlachonasios

The effect of heat treatment on attenuating development of chilling injury of tomato fruit was investigated. Mature green tomatoes were held at different high temperature regimes prior to storage at 2°C for 2 or 3 weeks. Chilling injury (CI) was circumvented by heat treatment at 42°C for 36 or 48 h while non-heated fruits stored at 2°C developed typical CI symptoms and failed to ripen when returned to permissive ripening temperatures. Heat treatment-induced protection from CI was abolished if the tomatoes were transferred from high temperatures to 20°C for 1 or 3 days before the low temperature storage. The heat-treated fruits ripened normally although more slowly that non-treated tomatoes. We hypothesized that heat shock proteins (HSPs) may be responsible for increased tolerance to CI. Using mRNA differential display, we cloned and characterized a full-length cDNA that is related to heat-induced chilling tolerance (HCT); it encodes a 17.6-kD cytosolic class II smHSP (named *Le*HSP17.6). In a screen of a cDNA library from heat-treated tomato fruit, a 17.4-kD cytosolic class II smHSP (*LeHSP17.4*) was isolated. In addition, three cDNAs encoding a cytosolic class I smHSP and partial cDNA clones for a chloroplastic smHSP, a mitochondrial smHSP and a heat-inducible HSP90 member were isolated. Northern analyses indicated that the expression

of the smHSPs genes induced by heat continued at low temperature only if the fruits had been exposed to heat shock. Fruit that received only low temperatures were not stimulated to express smHSPs (cytosolic class I and II as well as chloroplastic smHSP). The re-induction of the transcripts in the cold was more favorable after continued exposure to high temperatures. *Le*HSP90 and its constitutively expressed homologue *Le*HSC80 were induced by low temperatures. However, the heat-treated fruits had a higher *LeHSP90* transcript level than the non-heated fruits at low temperatures. In addition to mRNA expression pattern of the smHSPs and HSP90s, the protein level followed a similar pattern. More importantly, these proteins were still detectable when the fruits were transferred to 20°C for 3 days. The cold-inducible expression of HSP genes in the heat-treated fruits raised the possibility that both high- and low-molecular-weight HSPs may play critical roles in resistance to chilling stress. The heat-shock-induced protection against CI was lost when the fruits were transferred to 20°C after heat shock and before cold storage. Surprisingly, the re-induction of the HSPs at low temperatures was not prevented. These data suggest that heat shock proteins are not the only factor(s) that contribute to the chilling-tolerant phenotype of tomatoes.

To my parents, Epaminondas and Anna, and my brother Ioannis

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INTRODUCTION

Tomatoes as well as other subtropical fruits develop chilling injury if held below 12°C during low temperature storage and/or after being returned to permissive ripening temperatures. The degree of injury is related to the magnitude and duration at chilling temperatures. Typical symptoms appear after transfer from storage and include failure to ripen, irregular ripening, surface pitting, and increased susceptibility to decay. Chilling injury can be prevented by storage at temperatures slightly above the critical chilling range (Hatton, 1990), or holding the tissue above 35°C prior to chilling (Lurie and Klein, 1991).

Postharvest heat treatment of temperate, subtropical and tropical fruit has been examined by numerous research groups with the objective of developing non-chemical, alternative strategies for insect and disease control of fruits exported to or imported from various countries (Couey, 1989; Lay-Yee and Rose, 1994; Shellie and Mangan, 1994; Ben-Yehoshua et al., 1995). These tests and others (Dentener et al., 1996) have employed hot water or heated air at various temperatures ranging from 38 to 50°C for brief periods (minutes) at the higher temperatures or extended periods (days) at the lower temperatures. Interestingly in these studies, heat treatment of several fruits diminished the incidence of some important physiological disorders related to low temperature storage such as chilling injury and superficial scald (Klein and Lurie, 1992). When apples were heat-treated at 38°C for 3 days and subsequently stored for 6 months at low temperatures (0-3°C), they ripened and softened slower than non-heated fruits.

Moreover, the incidence of superficial scald was dramatically reduced (Lurie et al., 1990).

Mature green tomato fruits normally must be stored at temperatures above 10°C to avoid chilling injury and, therefore, have a temperature-limited postharvest life of only a few days to a week before ripening is initiated. Heat treatment of tomato fruits has very significant potential for commercial application for several reasons: (1) Wholesomeness of the fruit may be improved, avoiding the use of applied chemicals to control some important physiological and pathological disorders; 2) ripening can be delayed; and 3) the storage duration can be doubled without evoking chilling injury disorders and the accompanying decay.

More importantly, heat treatment endows the mature green tomatoes with the ability to be stored in air at 2°C for several weeks and subsequently ripen normally without symptoms of chilling injury (Lurie and Klein, 1991). Heat treatment was recently found to induce resistance to chilling injury in avocado (Sanxter et al., 1994; Woolf et al., 1995; Florissen et al., 1996), citrus fruits (Rodov et al., 1995) cucumber (McCollum et al., 1995), mango (McCollum et al., 1993), mung bean hypocotyls (Collins et al., 1993; 1995), sweet pepper (Mencarelli et al., 1993), persimmons (Burmeister et al., 1997; Lay-Yee et al., 1997), and zucchini squash (Wang, 1994). It has been hypothesized that the induction of heat-shock proteins during heat treatment is responsible for acquisition of chilling injury tolerance.

The purpose of this study was i) to develop, optimize and characterize a commercially applicable method of postharvest heat treatment for storage to chilling

injury and ii) to identify and investigate the involvement of heat-shock proteins in the resistance to chilling injury.

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LITERATURE REVIEW

1. Prestorage Heat treatments

Heat treatments have been used to control diseases and insect infestation of fruits for many years (Baker, 1952). However, with the development of effective fungicides and insecticides, which could be applied easier and cheaper, interest in heat treatments waned. Recently, EPA (Environmental Protection Agency) has withdrawn the registration of many agricultural chemicals due to safety issues (Couey, 1989). The cost of developing new fumigants increased due to regulatory restrictions, and therefore interest in heat treatments or alternative methods has been revived (Klein and Lurie, 1992). Heat may be applied in several ways: by exposure to hot water, to vapor heat (water-saturated hot air), to hot dry air, to infrared and microwave radiation (Couey, 1989). Commercially, only vapor heat or hot water treatments have been used. Heat treatments have the advantage of effective insecticidal and fungicidal action, easy of application, and absence of chemical residues. In contrast, the potential of fruit damage and the relatively high cost of application are the major disadvantages.

1.1 Heat treatment for Control of Postharvest Diseases and Insect Pest of Fruits

Prestorage heating of fruits is promising as a nonchemical method for managing postharvest pathological disorders (Paull, 1990; Klein and Lurie, 1991). In the early 1920's, citrus packers in California started using hot water to improve the effectiveness of a detergent wash. Evidently by accident, this treatment reduced decay of oranges (Fawcett,

1922). Fungal germination and growth are inhibited by heat shock (Barkai-Golan and Phillips, 1991). Hot water has been used more widely to control postharvest diseases than for insect control (Couey 1989). Water dips at 38°C to 60°C for 2 to 60 min has been reported to control *in vivo* and *in vitro* spore germination and decay development of postharvest fungi in apples (Edney and Burchill, 1967), mangoes (Coates and Johnson, 1993; Johnson et al., 1997), melons (Teitel, et al., 1991), papayas (Akamine and Arisumi, 1953), strawberries (Couey and Follstad, 1966) and tomatoes (Barkai-Golan, 1973).

In contrast to the beneficial effects of short hot-water dips used to control fungal pathogens, these treatments were insufficient to disinfest fruit and vegetables. Therefore, vapor heat treatment regimes between 42 and 48°C have been used (Couey, 1989). Heat-treatments for disinfesting fruit were first developed by Baker and co-workers before 1929 (Baker, 1952) in order to protect the citrus region from the Mediterranean fruit fly (*Ceratitis capitata*). Citrus were treated using an 8h approach time and treatment at 43°C for another 8h. Vapor heat has been used as disinfestation treatment providing an assurance to the authorities of an importing country that the commodity is free of pests (Johnson and Heather, 1995). For instance, treatment of 46°C from 30 min to 6h is used to disinfect mangoes from fruit flies (Johnson et al., 1997). Moreover, the USDA-APHIS-PPQ (The United States Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine) approved high-temperature forced-air as a quarantine treatment for mangoes imported into USA from Taiwan. In 1993, APHIS approved a similar heat-treatment to disinfest grapefruit of the Mexican fruit fly (U.S. Department of Agriculture, 1993). Vapor heat or hot water disinfestation treatments has been used on mango (Johnson et al., 1997), papaya (Akamine and Arisumi, 1953), persimmons (Dentener et al., 1996).

1.2 The Effect of High Temperature on Fruit Ripening

Ripening, the phase of fruit development preceding senescence, includes developmental changes to enhance the probability of seed dispersal (Giovanonni, 1993). The ripening process involves biochemical and physiological changes occurring by various cellular and subcellular compartments and organelles, which contribute to the desired organolyptic attributes of texture, flavor and aroma observed during ripening (Brady, 1987). These changes include; flesh softening, increase in sugar/acid ratio, enhanced color development, and increase in aromatic compounds (volatiles related to flavor development). These physio-chemical changes are preceded by a dramatic increase in ethylene production which sparks an increase in metabolic activities as seen by ascent in respiratory activity, increase in activity of alternative oxidase and elevated rates of mRNA and protein synthesis (Frenkel et al., 1968). Molecular and genetic studies showed that ripening in tomato is regulated at the level of gene expression (Gray et al., 1992; Fray and Grierson, 1993; Giovanonni, 1993). Regulation by posttranscriptional events is also implicated (Blume and Grierson, 1997). Ethylene biosynthesis and action plays an important role in initiating and sustaining the metabolism in ripening fruit (Zarembinski and Theologis, 1994; Lelievre et al., 1997).

More than 50 years ago Hansen (1942) reported that ripening of pear fruit was reversibly inhibited by holding them at 40°C for extended periods. Heat treatment evokes a reversible inhibition of ripening in several other fruits including apple (Porritt and Lidster, 1978; Klein, 1989; Lurie and Klein, 1990), avocado (Eaks, 1978), papaya (Chan, 1986), pears (Maxie et al., 1974), and tomato (Biggs et al., 1988; Lurie and Klein, 1991; Lurie et al., 1996).

The effect of heat treatment on ethylene biosynthesis and action.

A common observation derived from all of the heat treatment studies with climacteric fruits is reversible inhibition of ethylene synthesis and action (Burg and Thimann, 1959; Maxie et al., 1974; Eaks, 1978; Biggs et al., 1988; Lurie et al., 1996). The effect of high temperature on ethylene production in excised tissues has been investigated (Saltviet and Dilley, 1978; Field, 1981). They demonstrated that basal and wound-induced ethylene increased up to 37°C, thereafter declined rapidly and there was no detectable ethylene production above 42°C.

Tomato fruits incubated at 37°C showed reduction of ripening- associated ethylene, regardless of the ripening-stage (Biggs et al., 1988). Both ACC synthase and ACC oxidase activity were reduced by high temperatures (Atta-Aly, 1992; Biggs et al., 1988). Moreover, a rapid loss of ACC oxidase activity occurs in papaya and other fruits exposed for short periods to temperatures greater than 40°C (Chan, 1986; Klein and Lurie, 1990). Upon transferring the fruits to 25°C, there is a rapid increase in ACC synthase activity and a slower recovery for ACC oxidase activity. Full recovery of ACC oxidase occurs within 3 days for apples (Klein and Lurie, 1990). The recovery of ethylene production required *de novo* synthesis of protein since cycloheximide inhibits that process (Biggs et al., 1988). Picton and Grierson (1988) reported that high temperatures not only inhibit ethylene biosynthesis but also its action, since ethylene application during heat shock, did not overcome the high temperature inhibition of fruit ripening.

The effect of heat treatment on respiration.

Carbon dioxide production and O_2 uptake (respiratory activity) rise as the fruits ripen. In the case of heated tomatoes and avocados, an increase in CO_2 production occurs during exposure to high temperatures (Kerbel et al., 1987; Lurie and Klein, 1991). When the fruits are subsequently transferred to 20°C, the respiration rate plunges to a lower level than the control fruit (Lurie and Klein, 1991). In persimmons, however, CO_2 production increases after heat treatment and then decreases (within 24 h), but remains at a higher level than the non-heated control (Burmeister et al., 1997).

The effect of heat treatment on color development.

In tomato fruit, heat stress delays color development during postharvest ripening (Buescher, 1979; Cheng et al., 1988). The retardation in color development is attributed to an induced attenuation of lycopene formation (Vogele, 1937; Sayre et al., 1953; Ogura et al., 1975; Yang et al., 1990) and chlorophyll degradation (Tomes, 1963; Lurie and Klein, 1991). The color development of heated fruit resumes after the temperature stress is removed (Cheng et al., 1988; Lurie and Klein, 1992). Furthermore, hot water treatment can delay yellowing of green tissues (Kazami et al., 1991; Tian et al., 1996, 1997; Wang, 1998). For example, immersion of broccoli in 50°C water for 2 minutes is the most effective treatment for reducing yellowing and decay while not inducing off-odors or accelerating weight loss.

The effect of heat treatment on firmness

Tissue softening is another parameter affected by high temperatures. Prestorage heating of tomatoes leads to enhanced retention of fruit firmness during ripening (Hall, 1964; Ogura et al., 1975; Yoshida, et al. 1984; Cheng, et al. 1988; Sozzi et al., 1996). The rate of softening increased when the heat-treated fruit were returned to 20°C, but it was still less than the control fruit. Mitcham and McDonald (1992) reported that the rate of cell wall degradation is reduced while the synthesis of soluble polyuronides continues after heat treatment, resulting in firmer fruits. BenShalom et al. (1996) showed that heat treatment partially inhibited the degradation of uronic acids in the apple fruit cell wall. In heat-treated apple fruit, the content of galactose and arabinose in the cell wall decreased during storage, suggesting that the inhibition of solubilization of the carbonate soluble pectin fraction is one of the main factors contributing to the firmness retention caused by heat- treatment. In contrast, softening-related phenomena, such as loss of neutral sugars, pectin methylesterase activity and pectin deesterification, were not changed by heating (BenShalom et al., 1993; Klein et al., 1995). The retardation of softening by heat treatment is accounted for by the absence of polygalactorunase (PG) activity (Yoshida et al., 1984). For many year PG was thought to be the primary enzyme for tomato fruit softening. However, recent work with transgenic plants proved that the breakdown of cell wall polyuronides by PG was not sufficient to induce softening (Smith et al., 1988; Giovanonni et al., 1989; DellaPenna et al., 1990). Other enzymes, including α- and β-galactosidases and an endo-β-mannanase, that appear earlier than PG in fruit ripening could play alternative role in fruit softening (Fischer and Bennett, 1991). Heat treatment at 40°C for 2 days had a profound incidence on the levels of α- and β-galactosidase

activity in tomatoes (Sozzi et al., 1996). The recovery in β-galactosidase activity upon transfer of fruits to 21°C was complete after 10 days. On the contrary, the recovery of the α-galactosidase activity was very slow. Moreover, endo-β-mannanase activity, an enzyme involved in the breakdown of mannose-containing polymer, was undetectable after heat treatment at 40°C for 2 days, and the recovery of the activity at the permissive temperature was incomplete (Sozzi et al., 1996). The level of cellulase activity, which is associated with softening in avocados, was lower in heat-treated fruit than in nonheated controls (Klein and Lurie, 1991).

The effect of heat treatment on sugar/acid ratio.

The taste of tomato fruit is mainly determined by sweetness induced by the reducing sugars and the sourness or acidity caused by the organic acid content. Heat-treatment increases the sugar/acid ratio by 10 to 30%, which make the fruit more attractive to the consumers (Lurie and Klein, 1992). Higher ratio of sugar versus acid has been reported also for heat-treated apples (Klein and Lurie, 1990; 1992). Similar results have been reported in broccoli florets (Tian et al., 1997). Sucrose content in broccoli stems and florets increased dramatically within a short period of hot water treatment (47°C for 7.5 min) and then declined.

The effect of high temperature on gene expression during fruit ripening.

Fruit ripening, involves a co-ordinated and complex set of biochemical changes and requires the expression of specific genes (Giovanonni, 1993). As fruit ripens protein synthesis (Frenkel et al., 1968) occurs. Molecular and genetic studies showed that ripening

in tomato is regulated at the level of gene expression (Gray et al., 1992; Fray and Grierson, 1993; Giovanonni, 1993).

Picton and Grierson (1988) demonstrated that inhibition of ripening by high temperature treatments occurred at the level of gene expression. Incubation of tomato fruit at 35°C dramatically altered the level of ripening-related mRNAs. For example, there is a reduction in the expression of proteinase inhibitor, phytoene synthase, ACC oxidase, N-hydroxycinnamoyl/benzoyl transferase, and membrane channel protein following heat shock stress (Picton and Grierson, 1988). At elevated temperatures (>35°C) a decline in the activities of polygalacturonase (PG) (Chan et al., 1981; Yoshida, et al., 1984; Picton and Grierson, 1988), pectinmethylesterase (PME) (Kagan-Zur, et al., 1995), ACC synthase (Biggs, et al., 1988), ACC oxidase (Biggs, et al., 1988; Atta-Aly, 1992; Chan, et al., 1996; Lurie, et al., 1996) and phytoene synthase (Lurie, et al., 1996) was observed. However, full recovery of the ACC synthase, ACC oxidase, phytoene synthase (Biggs, et al. 1988; Lurie, et al., 1996) and PME (Kagan-Zur, et al., 1995) activities were reported when the heat-treated fruit were returned to room temperatures. The level of the PG mRNA was partially restored at room temperature, but the rate of PG mRNA accumulation declined exponentially with the duration of heat stress suggesting that PG gene expression was gradually and irreversible shut-off during heat stress (Picton and Grierson, 1988; Kagan-Zur, et al., 1995). The recovery of the PME gene expression was unaffected by the duration of heat stress (Kagan-Zur et al., 1996). Although the accumulation of PMEs mRNA was reduced by heat shock, PME protein and activity were unaffected, suggesting a greater stability of the PME to elevated temperatures (Kagan-Zur et al., 1996).

Heat treatment appears to evoke a fundamental mechanism in gene regulation that operates to temper metabolism while the tissue is under heat stress and restore normal metabolism when the temperature is subsequently lowered. This might be similar to the heat shock response common to prokaryotes and eukaryotes (Vierling, 1991). The heat shock response involves up-regulation of synthesis and action of heat shock proteins, while at the same time causes down-regulation of many proteins and metabolic enzymes at high temperatures and this process is reversed when the temperature is subsequently lowered. Kato et al., (1993) reported that heat treatment (37°C for 24 hours) induces substantial amounts of nuclear and cytosolic small HSPs in tomato fruit. Further, cytosolic class I smHSPs (Lurie et al., 1996; Sabehat et al., 1998a; This study), cytosolic class II smHSP (Kadyrzhanova et al., 1998; This study) and chloroplastic smHSP (Sabehat et al., 1998a; This study) were induced after exposure of tomato fruit at 38°C or 42°C. Moreover, cytosolic class I smHSP has been isolated during tomato fruit ripening (Fray et al., 1990).

1.3 Heat treatment Induces Resistance to Chilling Injury

While thermotolerance can be induced in plants and plant organs by heat treatment, heat stress is also known to induce tolerance to some other environmental stresses (Orzeck and Burke, 1988; Kuznetsov et al., 1997). Prestorage heat treatment of apples was found to inhibit superficial scald (a chilling injury phenomenon) during storage at 0°C (Lurie et al., 1990) while heat treatment inhibited chilling injury of avocados (Woolf et al., 1995; Florrisen et al., 1996). Mature green tomatoes are very susceptible to chilling injury by storage for only a few days at temperatures below 10°C;

symptoms normally become evident subsequent to returning them to 20°C. However, if tomatoes are heated at 38 to 40°C for 3 days prior to storage at 2°C for 3 weeks they were found to ripen normally and without development of chilling injury symptoms (Lurie and Klein 1991; 1992; This study). This protection disappears if the tomatoes are transferred from 38 to 20° C for 4 days before 2° C storage (Lurie and Sabehat, 1997). Nonheated tomatoes remained green after removal from 2°C storage and developed typical surface pitting and extensive microbial spoilage. Protection of tomatoes from chilling injury afforded by prestorage heat treatment has been correlated with the induction of transcription of smHSPs mRNA during the heat treatment and translation of the smHSPs which persisted during subsequent storage of the fruit at 2°C (Sabehat et al., 1996; 1998; Kadyrzhanova et al., 1998). An interim of holding the tomatoes at 20°C after heat and before storage at low temperatures, however without decreasing the expression in HSPs led to loss of resistance to low temperatures (Sabehat et al., 1998; This study). This suggests that the synthesis and action of HSPs afforded by heat treatment may be involved in protecting the fruit during heating and during subsequent exposure to chilling temperature. However, it appears that the HSPs are not the only factor contributing to chilling tolerance phenotype (This study).

Neven et al. (1992) identified that HSP70 is involved with a metabolic adjustment of spinach seedlings to low non-freezing temperature during cold acclimation. Low temperature stimulated the expression of most spinach HSP70 members, with cytosolic *HSC70-12* to be the most inducible and the *HSC70-2* (an ER luminal member) the least inducible (Guy and Li, 1998). This findings is consistent with the identification of the cold shock *DnaK* homologue in *E.coli* (Levilent and Kawula, 1995) and the cold sensitive

phenotypes derived by mutations in yeast stress 70 members (Schilke et al., 1996). Moreover, Krishna et al. (1995) reported a role for HSP90 in adaptation to cold temperature in *B. napus* tissues. Interestingly, an endoplasmic reticulum smHSP from potato has been isolated during cold storage (Van Berkel et al., 1994). The cold-induced expression of HSP genes raises the possibility that heat shock proteins may play a critical role in the cold acclimation or the chilling resistance process in plants.

Chilling injury is thought to be a membrane-related disorder (Lyons, 1973). Murata (1983) found that a higher degree of fatty acid unsaturation in phosphatidylglycerol was related to chilling tolerance. Although many attempts to find a causal relationship between unsaturation of fatty acids and chilling have been made in higher plants, the results are controversial (Raison and Orr, 1990). Recent experiments, (Murata et al., 1992; Wolter et al., 1992) indicate that an increase in saturation of acyl chains attached to chloroplastic phosphatidylglycerol can increase cold-induced photoinhibition. In contrast, Wu and Browse (1995) reported that the *fab*1 *Arabidopsis* mutant, which contain 43% of high melting point phosphatidylglycerol, was unaffected by chilling temperatures. Moreover, recent evidence indicates that at low temperature, *chs* 1 *Arabidopsis* mutants are deficient in the accumulation of chloroplast proteins, probably due to the lack in a chaperonin that is required to fold chloroplast proteins at low temperature (Schneider et al., 1995). On the other hand, the function of the unsaturated fatty acids in chilling tolerance has been demonstrated using the *fad* mutants of *Arabidopsis thaliana*, which are defective in desaturation of membrane lipids (Somerville and Browse, 1991). The *fad* mutants (*fad*2, *fad*5 and *fad*6) have reduced amounts of polyunsaturated fatty acids and are more sensitive to chilling (Hugly and Somerville, 1992; Miquel et al., 1993). The *fad*7 mutants is deficient

in chloroplast ω-3 fatty acid desaturase activity, which is responsible for the formation of trienoic fatty acids (16:3 and 18:3) in leaf tissue (Iba, et al., 1993). Kodama et al. (1994) overexpressed the *FAD7* cDNA in tobacco plants and chilling injury was reduced, suggesting the importance of the polyunsaturated fatty acids in chilling tolerance. Recent data indicated that the increased fatty acid desaturation during chilling acclimation at 15°C is one of the factors involved in normal leaf development at low, nonfreezing temperatures (Kodama et al., 1995). Similarly, genes for mitochondrial catalase 3 isozyme (Prasad et al., 1994) and HSP70 related proteins (Cabane et al., 1993) are induced during chilling acclimation at 10-20°C and may act in concert to increase chilling tolerance.

Organisms adapt to low temperature by increasing the proportion of cis-acting unsaturated fatty-acyl groups in their membrane lipids and thus increase membrane fluidity (Hazel, 1995). Fatty acid desaturase activity is induced during low temperature (Nishida and Murata, 1996). Recently, Murata and Wada (1995) produced cyanobacteria that display chilling tolerance by transforming them with Δ12-desaturase. A decrease in membrane fluidity was reported to induce desaturase genes (Vigh et al., 1993).

It has been suggested that the heat treatment institutes a response to high temperature stress in the tissue that leads to strengthened membranes (Lurie et al., 1997). These treatments led to an increase in phospholipid content, a lower sterol to phospholipid ratio and more unsaturated fatty acids, relative to the unheated tissues (Lurie et al., 1997).

Interestingly, Carratu et al. (1996), using yeast as a model, proposed that the ratio of saturated fatty acid versus unsaturated fatty acid and disturbance of membrane lipoprotein complexes are involved in the perception of rapid temperatures changes, and that under heat shock conditions perturbation of the preexisting physical state of the membranes causes

transduction of a signal that induces transcription of heat shock genes. The induced HSPs may bind to membrane lipoproteins (Moczko et al., 1995) leading to stabilization of membrane topology. Indeed interaction between heat shock proteins and membranes has been reported in many organisms (Vigh et al., 1998). In *Synechocystis* cells, following heat shock, both GroEL and Hsp17 are associated with thylakoids (Kovacs et al., 1994; Horvath et al., 1988). Membrane bound HSPs might act by preventing denaturation of membrane-localized enzymes (Torok et al., 1997). Therefore, the membrane acts as a sensor and the altered saturation remotely activates a mechanism, which enhances HSP transcription (Carratu et al., 1996). Similar speculation has been proposed for the desaturase genes (Vigh et al., 1993). In contrast, no correlation was found between changes in fatty acid composition and the thermotolerance in plants (Rikin et al., 1993). Moreover, changes in heat tolerance are caused by protein factors (Nishiyama et al., 1993).

Interestingly, induction of gene expression by low temperature has been demonstrated in different plant tissues (Guy et al., 1985; Mohapatra et al., 1989; Thomashow et al., 1993). Schaffer and Fischer (1988) have shown in tomato fruit that the expression of three mRNAs including one that homologues to a cDNA clone encoding a thiol protease, increased at 4°C suggesting that proteolysis might occur during chilling. The level of these transcripts was different in cold sensitive and cold tolerant varieties, suggesting that genetically determined cold tolerance influences cold inducible gene expression. Lately, Yu et al. (1996) compared the changes in gene expression between two different chilling tolerant tomato fruits, the hybrid "NY" (*L. esculentum* x *L. pimpinellifolium*) known to be chilling tolerant, and the chilling sensitive "EC". Two low molecular weight basic translation products (18kDa, pI 8.0 and 14kDa, pI 8.2,

respectively) were noticed. The first polypeptide increased during chilling while the latter was sharply reduced during chilling and reaccumulated after transfer to 20°C. The down-regulation of many translation products might be related to the delay in ripening at low temperature. Conversely, chilling-induced translation products could play an important role in metabolic adjustments at low temperature and in the protection of existing cellular structures. The chilling sensitivity of the "EC" might involve inhibition of phosphatidylcholine transfer from the endoplasmic reticulum to the chloroplast at low temperature.

Heat shock proteins have been reported to influence protein folding (Weish et al., 1992) and probably plant HSPs assist in refolding of proteins denatured by cold temperature stress. Neven et al., (1992), proposed that accumulation of HSPs at low temperature may not only be indicative of a stress condition but also may have adaptive values. During heating of tomato fruit at 38°C five proteins of molecular weights between 15-22 kD, a HSP at 70 kD, and two above 80 kD were newly and abundantly synthesized (Lurie and Klein, 1991). Similarly sized HSPs have been described in tomato shoot and root apices (Koning et al., 1992), in tomato cell cultures (Nover et al., 1989), and tomato leaves (Fender and O'Conell, 1990). Additionally, normal proteins synthesized at 20°C are not detectable by SDS-PAGE during the heat treatment. Furthermore, mRNA for the two heat shock proteins HSP17 and HSP 70, remained abundant in heat-treated tomatoes stored at 2°C for three weeks while it disappeared rapidly from tomatoes placed at 20°C after heat treatment (Lurie et al., 1996). Recently, two other members of heat shock proteins with molecular weights 23 and 18 kD were found in heat-treated tomato fruits stored at 2°C for 21 days (Sabehat et

al., 1996). Therefore, the heat shock proteins in tomato fruits may act as chaperones and their continued presence at low temperature suggests their importance in chilling tolerance.

2. Heat Shock Response, Heat Shock Proteins and Cellular Metabolism

Thermal stress evokes rapid induction of specific proteins termed heat shock proteins (HSPs) (Key et al., 1981; Lindquist and Craig, 1988; Vierling, 1991; Parsell and Lindquist, 1994). In plants, Key et al. (1981) have demonstrated the rapid induction of specific proteins under thermal stress. The induction of genes encoding heat shock proteins is triggered by an abrupt increase in temperature of 5-10°C above the normal growing temperature (Kimpel and Key, 1985). Proteins induced by heat stress can be assigned to 11 families conserved among all organisms. They vary in size from 15 to 110 kD and within size classes share considerable homology at the amino acid level (Vierling, 1991; Nover and Scharf 1997). Isoforms within a HSP family have similar or identical biochemical function but differ in their intracellular localization and their regulation. Particularly, the low molecular weight HSPs in higher plants are encoded by six nuclear gene families (Waters et al., 1996). Classes I and II encode cytosolic proteins while classes III and IV encode chloroplast and endoplasmic reticulum localized proteins (Vierling, 1991; Helm et. al, 1993). A fifth class encodes the mitochondrial proteins (Lenne and Douce, 1994; Dong and Dunstan, 1996). For class VI, which is represented by a single 22.3 kD HSP from soybean, the intracellular location is proposed to be the endoplasmic reticulum (Lafayette et. al., 1996). In many cells it has been observed that

HSPs or homologues are expressed constitutively, under developmental or cell cycle control (Lindquist and Craig, 1988).

According to Vierling (1991), the induction of HSPs is an autonomous cellular phenomenon. Heat stress induces rapid transcriptional regulation of the HSPs by binding of the heat shock transcription factor (HSF) to a common promoter sequence called the heat shock element (HSE) (Nover et al., 1990; Gurley and Key, 1991; Sorger, 1991; Morimoto et al., 1996). HSFs in plants are encoded by at least 3 members and some of them are heat-inducible (Nover et al., 1996).

Heat shock disrupts splicing of numerous mRNA precursors (Yost and Lindquist, 1988), thus determining the nature of mature mRNAs to be transcribed. Heat stress also affects gene expression at the translational level by blocking translation of pre-existing mRNAs while pre-existing polysomes decline (Brostrom and Brostrom, 1998). HSP mRNAs appear rapidly in response to heat, become associated with polysomes and translation of HSPs accounts for them becoming the major products of protein synthesis (Lindquist, 1980). Heat shock in plants was recently found to increase mRNA stability of a reporter mRNA (Gallie et al., 1995). Mature mRNAs existing prior to heat stress remain eligible for translation in cell-free systems but *in vivo* HSP mRNAs are preferentially translated (Lindquist, 1981). This major alteration in protein synthesis while the cells are under thermal stress is fully reversible upon lowering the temperature. A normal pattern of protein synthesis is restored, depending upon the severity and duration of the heat stress and synthesis of HSPs diminishes (DiDomenico et al., 1982a). In addition, cells transferred gradually to high temperatures recover much more rapidly than cells raised suddenly to the same temperature (Lindquist and DiDomenico, 1985). The

repression of general translation likely serves to restrict the production of missense, incomplete, or misfolded proteins that might subsequently have a deleterious effect on cell growth (Duncan, 1996). The magnitude of preferential translation of some and repression of other normal proteins differs between cell types, species and heat shock conditions. For example, in *Drosophila* cells, inhibition of general translation becomes detectable at around 32°C and reaches maximum inhibition at 37°C (10 degrees above the normal temperature), while in mammalian cells, protein synthesis is inhibited at 41°C (Duncan, 1996). Following the heat stress, cells recover full translational activity. Contrary to the repression, the recovery process requires several hours (DiDomenico et al., 1982b). When more severe heat shocks are administrated, the recovery is delayed (DiDomenico et al., 1982b; Lindquist, 1993), although the non-heat shock mRNAs appear to recover synchronously.

3. Small Heat Shock Proteins

Small heat shock proteins dominate the protein synthesis profile of many plants during heat stress and particularly the cytosolic classes can accumulate to over 1.0% of the total leaf or root cell protein under certain heat shock conditions (Hsieh et al., 1992; DeRocher et al., 1991). Vierling (1991) has grouped the smHSPs into four nuclear gene families based on amino acid sequence, immunological cross-reactivity and intracellular localization. Two classes are cytosolic proteins and the other two are organelle-localized, targeting to endoplasmic reticulum (ER) (Helm et al., 1993) and chloroplasts, respectively. Recently, LaFayette et al. (1996) proposed to expand the classes to six

including a mitochondrial class (Lenne, 1995) and an additional endomembrane class of smHSPs. This diversification of the small heat shock proteins is unique to plants.

The small heat shock proteins showed less sequence similarity than the highly conserved HSP70 proteins, including both comparisons between divergent species and between different classes of plant smHSPs (Waters et al., 1996). One of the major characteristics of the low molecular weight HSPs is the conserved carboxyl-terminal domain (heat-shock domain) of about 100 amino acids, which is also found in the α-crystallin proteins of the vertebrate eye lens (DeJong et al., 1993). This domain is further subdivided into two subdomains, consensus I and II, separated by a variable hydrophilic region. The consensus I subdomain includes the motif P-$X_{(14)}$-GVL which is characteristic for all the smHSPs. Interestingly, a similar motif appears in the consensus II subdomain region P-$X_{(14)}$-X-V/L/I-V/L/I (Waters et al., 1996). Moreover, these regions have the same hydropathy profiles and secondary structure prediction (Caspers et al., 1995).

In contrast to the carboxyl terminus, the amino-terminal region is more variable with little homology among the different classes of low molecular weight HSPs (Vierling, 1991). It has been proposed that the amino-terminal region interacts with different substrates or relates to distinct functions (Vierling, 1991). Furthermore, the N-terminal region also includes sequences that determine localization of organelle-associated HSPs (Waters et al., 1996). However, within the amino-terminal regions of the mature proteins, consensus domains unique to each class of smHSP have been identified (Waters, 1995). The presence of these highly conserved domains suggests that they serve important roles in protein function (Waters et al., 1996).

With a few exceptions, the smHSPs are not expressed by vegetative tissues in the absence of heat stress. The small heat shock proteins accumulate rapidly during high temperature stress and in proportion to the temperature and duration of the stress (Vierling, 1991). Maximum synthesis and accumulation of smHSPs is observed at temperatures just below lethal temperatures (Howarth, 1991). Quantitative analysis indicates that class I proteins accumulate to over 1% of total leaf protein during heat stress (DeRocher et al., 1991; Hsieh et al., 1992) and they are considered as the most abundant class of smHSPs. The chloroplast heat shock proteins have been estimated to be 0.02% of the total protein content (Chen et al., 1990). The organelle-localized HSPs are less abundant than the cytoplasmic class I and II HSPs, which may reflect a lower protein content of the endomembrane system relative to the cytosol. The half-life of the small HSPs following the heat stress is 30-50 h (Chen et al., 1990; DeRocher et al., 1991), indicating their function may be critical for the recovery period.

The plant smHSPs, like the other HSPs, are regulated at the transcriptional level in response to heat stress (Gurley and Key, 1991). The presence of multiple genes encoding heat shock factors that bind to the heat shock element are differentially regulated by heat shock, suggesting that these transcription factors may regulate genes in response to signals other than heat stress (Waters et al., 1996).

The small heat shock proteins are also regulated by a variety of other environmental and developmental factors (Arrigo and Landry, 1994). Contrary to the high molecular weight heat shock proteins, the smHSPs are not constitutively expressed in the normal cells, indicating that the smHSP function is restricted to specialized cellular conditions shared by developmental and stressed stages (Waters et al., 1996). The best

characterized cases of developmental regulation are expression during pollen development (Bouchard, 1990; Dietrich et al., 1991; Atkinson et al., 1993; Kobayashi et al., 1994) and during seed maturation (Vierling 1991; DeRocher and Vierling, 1994). Small HSPs have been identified also during fruit ripening (Fray et al., 1990; Lawrence 1997). In addition, heat shock proteins are induced by other stress such as cold, drought, salinity, ethanol, heavy metals, ozone and oxidative stress (Anderson et al., 1994; Colmenero-Flores et al., 1997; Ruis and Schuller, 1995; Eckey-Kaltenbach et al., 1997; Banzet et al., 1998). These stress conditions could damage or denature cell proteins. In yeast, inhibition of protein breakdown with the proteasome inhibitors caused a coordinate induction of many heat shock proteins (Bush et al., 1997; Lee and Goldberg, 1998). Further, treatments with amino acids analogues as well as introduction of unfolded proteins triggers the induction of HSPs (Ananthan et al., 1986; Lee et al., 1996). The induction of the heat shock response can protect cells against a variety of other toxic insults, such as ethanol and hydrogen peroxide (Ruis and Schuller, 1995; Storz and Polla, 1996).

4. Function of the Heat Shock Proteins

Specific functions of HSPs such as HSP60, 70 and 90 have been ascribed to protein/protein interactions in protein folding and aggregation (Boston et al., 1996; Buchner, 1996; Hartl, 1996; Bukau and Horwich, 1998). The HSPs could refold stress-damaged proteins by functioning as molecular chaperones. These proteins prevent denaturation and aggregation as well as promote the refolding of denatured proteins.

Specifically, the HSP90, 70, 60 classes are thought to aid in the proper folding of the newly synthesized nascent peptides, transport across membranes, assembly of oligomeric complexes and maintenance of steroid receptor conformation. The small HSP proteins which are unique to plant kingdom, have been shown recently to maintain proper folding of reporter proteins and exhibit *in vitro* chaperone activity (Ehrnsperger et al., 1997; Lee et al., 1997; Lee et al, 1995a). The recombinant HSP 18.1 and HSP 17.7, representing class I and class II cytosolic smHSPs from pea, were able to enhance the refolding of chemically denatured model substrates citrate synthase and lactate dehydrogenase and prevented their aggregation and irreversible inactivation (Lee et al, 1997). In soybean, HSPs-enriched fractions were able to thermostabilize mainly membrane-associated proteins (Jinn et al., 1993). Based on these data, it is suggested that smHSPs act *in vivo* as a type of molecular chaperone to bind partially denatured proteins preventing irreversible protein inactivation and aggregation, and that smHSP chaperone activity contributes to the development of thermotolerance (Waters et al., 1996). In contrast to high molecular weight HSPs, the activities of the small heat shock proteins are not stimulated by nucleotides (Boston et al., 1996).

Under non-denaturing conditions in different organisms, the small heat shock proteins have been found to form high molecular weight complexes, between 200-800 kD. The class I smHSPs complexes are approximately 200-300 kD in size (Helm et al., 1993; Jinn et al., 1995). Similar sizes have been observed in class II (Helm et al, 1997), chloroplast (Clarke and Critchley, 1994; Chen et al., 1994; Osteryoung and Vierling, 1994) and mitochondrial (Lenne and Douce, 1994) smHSPs. In the presence of high salt concentration and nonionic detergent, the complexes keep their integrity (Jinn et al.,

1995) suggesting that strong ionic interactions and hydrophobic forces stabilize the high molecular weight complexes (Chen et al., 1994). Jinn and colleagues (1995) observed that the isolated 280 kD smHSP complex from soybean was able to protect up to 75% of the total soluble proteins of the cell from heat denaturation *in vitro*. These smHSP complexes can associate into insoluble larger cytoplasmic aggregates termed as "heat shock granules" (Nover et al., 1990). It has been suggested that these HS granules are transient sites for non-heat shock mRNA, preventing its degradation during heat stress (Nover 1991). These large structures may be common to all smHSPs (Osteryoung and Vierling, 1994) and their formation may be reversible and occur mainly at highly stressing temperatures (Waters et al., 1996).

The HSP18.1 (cytosolic class I from pea) has been shown to facilitate the refolding of chemically or heat-denatured model substrates (Lee et al., 1997). Furthermore, HSP 18.1 prevents aggregation of substrate proteins heated at 45°C. Using size exclusion chromatography, Lee et al., 1997 found that the binding of the mitochondrial malate dehydrogenase to smHSPs is hydrophobic in nature and that high temperatures increase the available binding surface. Moreover, based on fluorescent probe bis-ANS [1,1'-bi(4-anilino) naphthalene-5,5'-disulfonic acid] incorporation and substrate protection experiments, the consensus II region is critical for substrate binding (Lee et al., 1997). Similarly, Jinn et al. (1995) reported that immunodetectable smHSPs from heated plant extracts shifts to a higher molecular weight on native gels, while the purified smHSPs when heated alone did not show size shifting. Based on these data, Boston et al. (1996) suggested a model in which the smHSPs capture unfolding polypeptides by hydrophobic interactions and keep them in state capable to refold.

However, separation of the smHSP/substrate complex was insufficient without disassociation of the smHSPs oligomers themselves. This observation suggests that substrate release *in vivo* is triggered by synergistic interactions with other chaperones (Boston et al., 1996). In a different way, the substrate/smHSP interactions may facilitate proteolytic degradation of the substrate (Boston et al., 1996). Interestingly, Forreiter et al. (1997) using a stable transformed *Arabidopsis* cell suspension culture overexpressing luciferase as a reporter gene, demonstrated that the cytosolic class I smHSP showed chaperone activity *in vivo*, while the HSP90 protects the *Arabidopsis* cells only during recovery from the heat stress. More importantly, although the HSP70 was unable to protect the luciferase during stress, the HSP70 and the smHSPs could act synergistically during the refolding process. This is in agreement with the notion that the substrate/smHSPs complexes association is stimulated by interaction with the high molecular weight HSP.

Another important function of certain HSPs is to promote the rapid degradation of abnormal proteins (Hershko, 1988; Vierstra, 1993; Sherman and Goldberg, 1996). In eukaryotes, ubiquitin and certain ubiquitin-conjugating enzymes are involved in the rapid breakdown of denatured proteins (Parsell and Lindquist, 1994). In addition, certain molecular chaperones have been shown to serve as cofactors in the selective degradation of abnormal polypeptides (Kandror et al., 1994; Lee et al., 1996). Some of these proteins are proteases whereas others are additional components involved in substrate recognition.

Although *in vitro* experiments have demonstrated that the smHSPs potentially act as molecular chaperones, evidence for the role of smHSP in stress conditions varies dramatically among organisms. Until recently there has only been correlative evidence

that HSPs protect cells from deleterious effects of heat stress or other environmental stresses (Nover, 1991; Vierling, 1991). Lee et al. (1995b) have shown by genetically engineering *Arabidopsis* to derepress the activity of heat shock factor (*AtHSF1*) that constitutive synthesis of HSPs is enhanced and this increased thermotolerance. Schoffl and his colleagues (Prandl et al., 1998) had isolated another two HSFs from Arabidopsis (*HSF3* and *HSF4*). Transgenic *Arabidopsis* that overexpress constitutively the *HSF3* derepressed the heat shock response and conferred thermotolerance. Conversely, *Arabidopsis 35S::HSF4* transgenic plants were unable to synthesize HSPs constitutively and as a results were incapable of bestowing thermotolerance. The *At*HSF1 and *At*HSF3 are expressed in the wild type plants (Hubel and Schoffl, 1994; Prandl et al., 1998) but they require heat for activation of DNA binding and this involves aggregation from monomer to trimer forms (Hubel et al., 1995). Moreover, Lee and Schoffl (1996) indicated that the plant HSP70 is involved in early stages of HSF inactivation, possible by dissociation of the HSF trimers. It is highly plausible that the mechanism of heat activation of HSPs in *Arabidopsis* may serve as a general model for other plants. Alternatively, *in vivo* studies of the HSFs from soybean, *Arabidopsis* and tomato, conducted in a transient expression system using *GUS* as a reporter gene driven by HSE, demonstrated that plants have two classes of HSFs; the HSF class A which activate transcription (activators), and the HSF class B which are relatively abundant, *albeit* repressors of the heat shock response (Czarnecka-Verner et al., 1997). Thus, the active HSFs may be replaced by competition from repressor. The mechanism by which heat treatment evokes thermotolerance of tomato fruit to heat may fit this model.

Despite the extensive research concerning the mechanisms that regulate activation of HS gene expression, the nature of the "primary sensor" or how the signal is transferred to the nucleus is not known (Morimoto, 1993).

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CHAPTER I

PRESTORAGE HEAT TREATMENT REDUCES CHILLING INJURY IN TOMATO FRUIT

ABSTRACT

The ripening of the tomato (*Lycopersicum esculentum* Mill. cv. Mountain Springs) fruit and the development of chilling injury in relation to heat treatments was studied for three different years. Mature green tomatoes were held at 38°C, 42°C or 45°C for 8 to 48 hours prior to storage at 2°C for 2 or 3 weeks. After cold storage they were transferred to 20°C for 3 to 9 days. Fruits were also stored at 2°C without prior heat treatment and others were ripened at 20°C as controls. Chilling injury (CI) was prevented by heat treatment at 42°C for 36 or 48 hours prior to cold storage, whereas fruit stored at 2°C without preheating developed typical CI symptoms and failed to ripen at 20°C. Some protection was lost if the tomatoes were transferred from 42°C to 20°C for 1 to 3 days before low temperature storage; however, the CI was less than that of control fruits. The heat-treated fruits ripened normally although more slowly than non-treated tomatoes. In contrast, the non-heated tomatoes stored at 2°C remained green and developed typical CI symptoms upon return to a permissive ripening temperature. The heat-treated and

non-heated fruits were firmer after storage at low temperatures than the freshly harvested tomatoes. In summary, prestorage heat treatment permits storage for longer periods at low temperatures without CI development than is the case for non-treated fruits and with no loss of their ability to ripen normally.

INTRODUCTION

Mature green tomatoes, and other tropical and subtropical fruits like bananas, develop chilling injury (CI) if held below 10°C. Chilling injury is progressively more severe as the storage temperature is lowered and storage duration at low temperatures is extended (Wang, 1994a). Symptoms of CI (failure to ripen, non-uniform ripening, surface pitting and increased incidence of decay) may not be evident while the fruits are at low temperature but appear after the fruits are returned to permissive ripening temperatures of 15 to 25°C (Cheng and Shewfelt, 1988). Ripening of tomatoes is only minimally retarded at temperatures that do not cause CI; therefore, the postharvest storage life is limited to only a few days or a few weeks at best.

In recent years, there has been growing interest in heat treatments as a method to reduce chilling injury in horticultural crops, thus permitting extended storage times (Hatton, 1990; Klein and Lurie, 1991). Elevated temperatures alter tomato fruit ripening characteristics such as lycopene formation (Ogura et al., 1975; Lurie and Klein, 1991), chlorophyll degradation (Lurie and Klein, 1992), tissue softening (Yoshida, et al. 1984; Cheng, et al. 1988; Mitcham and McDonald, 1992), lipid composition (Whitaker, 1994), volatile production (McDonald et al., 1996), as well as both respiration rate (Lurie and Klein, 1991; 1992) and ethylene production (Biggs et al., 1988; Lurie and Klein, 1991). Prestorage high temperature treatments that reduce CI can be divided into two categories: long term (12 h to 4 days in air) at 38 to 46°C and short term (up to 60 min in water) at 45 to 60°C (Klein and Lurie, 1991).

The objective of this study was to develop, optimize, and characterize a commercially applicable postharvest heat treatment for cold storage of tomato fruit. Herein,

we present data from three years of several long-term heat treatments on chilling tolerance of tomatoes. Parameters evaluated included chilling injury symptoms, color development and flesh firmness. We conclude that heat treatment at 42°C for 36 or 48 hours was appropriate not only to provide chilling tolerance but also to permit normal ripening after storage.

MATERIAL AND METHODS

Experiments Conducted in 1994

Mature green tomato fruits (cv. Mountain Springs) were purchased from a grower/shipper in Southwest Michigan. Two replicates of ten fruits each were used per treatment at the various temperature regimens. The maturity/ripening indices monitored were: i) firmness with a modified Chatillon NY gauge taking 4 penetrations into the internal pericarp of the flesh of the fruit; ii) color development using a Michigan State University tomato chart scale, namely 1 (MG) to 5 (Dark red); and iii) chilling injury (CI) development expressed as chilling incidence index (1; 0% CI, 2; 10% CI, 3; 30% CI, 4; >50% CI and 5; >80% CI). The temperatures used for the heat treatment were 38, 42, and 45°C with 3 different durations for each treatment: 24, 48, and 72 h for the 38°C, 12, 24, and 48 h for the 42°C treatment, and 8, 16, and 24 h for the 45°C heat treatment. Immediately after the heat treatment, the fruit were stored at 2°C for up to 2 weeks. At weekly intervals, two replicates of each treatment were taken to 20°C, and maturity/ripening indices were determined after 1- or 7-day shelf life at 20°C. Fruits

maintained continuously at 20°C and at 2°C for 2 weeks before ripening at 20°C, respectively, served as controls.

Experiments Conducted in 1995

The 1994 experiment was repeated in 1995 with the same tomato cultivar. Three replicates of ten fruits per treatment were used. The temperatures used for the heat treatment were 38, 42, and 45°C with different durations for each treatment: 48, 72 h at 38°C, 12, 24, and 48 h at 42°C, and 12 h at 45°C. Immediately after the heat treatment, the fruits were stored at 2°C for up to 3 weeks. Color and chilling index were monitored as above.

Experiments Conducted in 1997

Mature green tomato fruits (cv. Mountain Springs) were purchased from a grower/shipper in Southwest Michigan. Three replicates of ten fruits were used per treatment at the various temperature regimens. The maturity/ripening indices monitored were: i) color development using the chromatometer (Minolta, Japan) and ii) CI development expressed as chilling index (1; 0% chilling injury (CI), 2; 10% CI, 3; 30% CI, 4; >50% CI and 5; >80% CI). Color development was measured as the Hunter "a" value. Three measurements were taken per fruit in the middle of the pericarp. Based on the previous year's data, the temperature used for the heat treatment was 42°C with 4 different durations for each treatment: 12, 24, 36 and 48 h. Immediately after the heat treatment, the fruits were stored at 2°C for up to 3 weeks. In addition, following heat treatment fruits were transferred to 20°C for 1 or 3 days and then stored at low temperatures. At weekly intervals,

three replicates of fruits from each treatment were taken to 20°C and maturity/ripening indices were determined after 3, 6 or 9 days shelf life at 20°C. Fruits maintained continuously at 20°C and at 2°C before ripening at 20°C, respectively, served as untreated and chilling controls, respectively. Standard error (SE) is indicated by bars.

RESULTS

Experiments Conducted in 1994

Flesh firmness of heated and nonheated fruits was not affected by low-temperature storage at 2°C for 7 days. However, low-temperature storage delayed tissue softening during ripening compared to the control fruits (Figure 1). After 7 days at 20°C, heat-treated and non-heated fruits were at the pink stage or color index 3 (Figure 2). There was no significant difference among the treatments. The heat-treated fruits required about twice as long at 20°C to develop full red color after chilling compared to the non-chilled control fruits. The major significant difference observed between heated and non-heated fruits was in the chilling injury incidence (Figure 3). Seven days at low temperatures was not enough to induce CI in control fruits, but 14 days was enough to cause typical symptoms. Only heat treatment at 42°C for 48 h attenuated the CI symptoms, which developed severely for fruits of the other heat treatments in relation to the control at 2°C without previous heat treatment (Figure 3). The fruits kept at 45°C ripened slowly but suffered from internal breakdown as well as heat injury. In addition, 40% of the fruits developed irregular color during 7 days at 20°C, which may be due to both heat and cold injury.

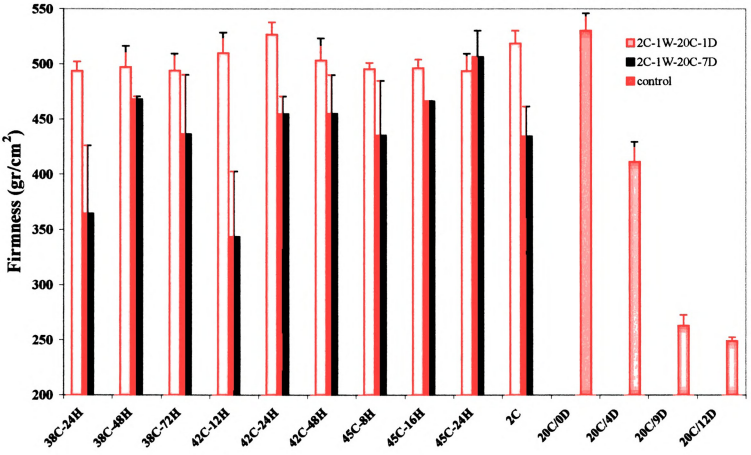

Figure 1. The effect of heat treatment on flesh firmness of tomato fruit stored at 2°C for 7 days and then ripened at 20°C (year 1994).

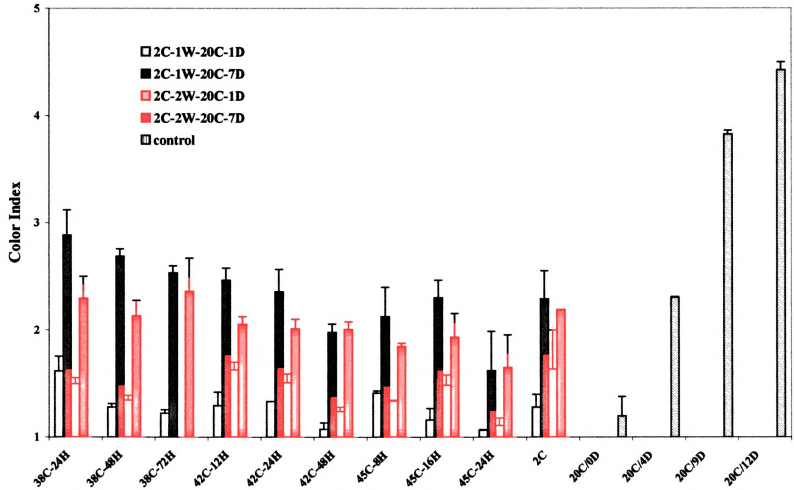

Figure 2. The effect of heat treatment on color development of tomato fruits after storage at 2°C for 7 or 14 days and during ripening at 20°C (year 1994).

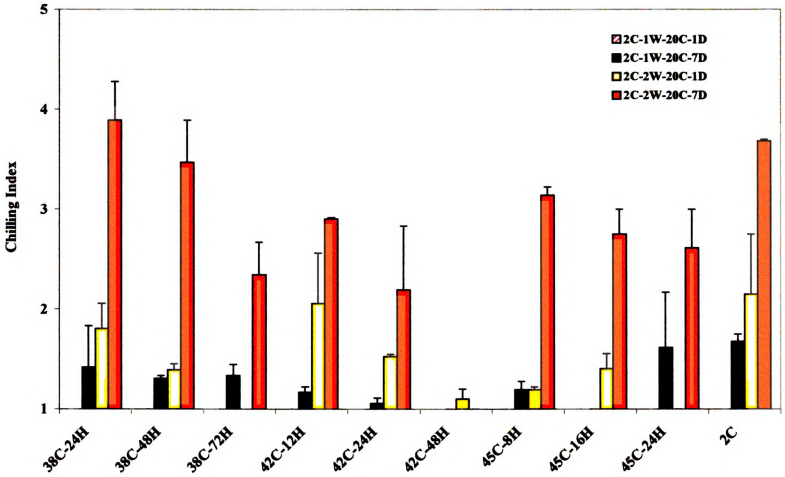

Figure 3. The effect of heat treatment on chilling injury of tomato fruit after storage at 2°C for 7 or 14 days and during ripening at 20°C (year 1994).

Experiments Conducted in 1995

Similar to the 1994 data, color development was delayed by low temperature storage in heated and nonheated fruits but the fruits ripened normally (Figure 4). However, after 3 days at 20°C there were no significant differences between the heat treatments at a given heat regimen for fruits stored for 2 or 3 weeks at 2°C. After 9 days at 20°C, the color index ranged from 2 (in the 42°C 48-h treatment) to 3.5 (in the 42°C 12-h treatment). The control fruits developed red color after 9 days at room temperature. Similar to the previous year's results, chilling injury was prevented by heat-treating tomato fruits at 42°C for 48-h (Figure 5). Control fruits developed chilling injury symptoms after storage for 3 weeks at low temperature. Surprisingly, two weeks of low temperature storage was not enough to induce chilling injury. Fruits from the other heat treatments developed chilling injury, but the incidence of the injury was lower or similar to that of the control fruits. The symptoms of the chilling injury become more evident as ripening of the stored fruits proceed.

Experiments Conducted in 1997

Color development was measured as the Hunter "a" value. Three measurements were taken per fruit in the middle of the pericarp. Negative values were obtained for mature green fruit, values near to 0 were given to turning fruit and the positive values for pink and red fruits (Figure 6). Nonheated fruits developed color normally at 20°C and reached positive values in 6 days. In contrast, fruits receiving low temperature for 2 weeks failed to develop red color and remain green even after 6 days at 20°C. The heat-treated fruits ripened slowly, and came to the pink stage after 15 days at room

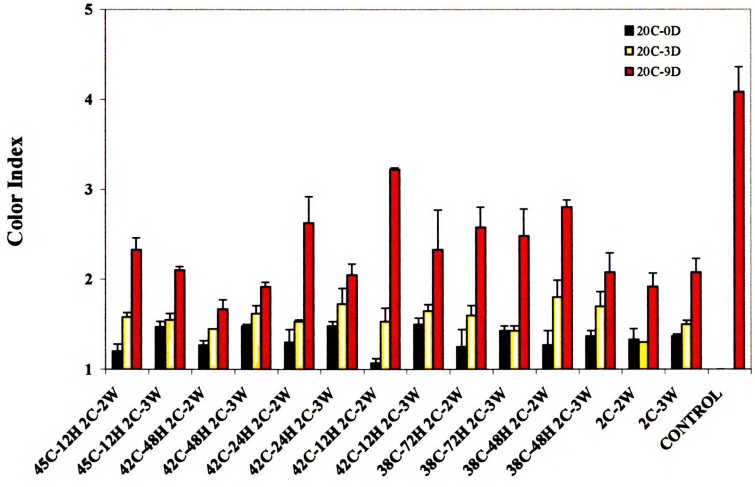

Figure 4. The effect of heat treatment on color development of tomato fruits after storage at 2°C for 14 or 21 days and during ripening at 20°C (year 1995).

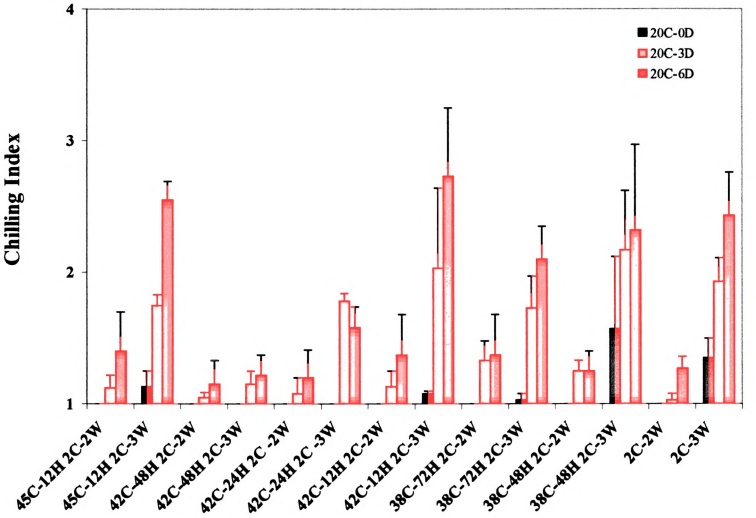

Figure 5. The effect of heat treatment on chilling injury of tomato fruit after storage at 2°C for 14 or 21 days and during ripening at 20°C (year 1995).

temperature. Overall, the shelf life of the heat-treated/cold stored tomatoes was extended to 32 days after harvest, while the control fruits attained the same ripening stage in 6 days.

Chilling injury was circumvented by heat-treating mature green tomatoes at 42°C for 36 or 48 hours prior to storing them at 2°C for two weeks, whereas fruits stored at 2°C without preheating developed typical chilling injury symptoms and failed to ripen at 20°C (Figure 7). This protection was lost if the tomatoes were transferred from 42°C to room temperature for 1 or 3 days before cold storage; nevertheless the chilling injury was less than that of the control fruits (Figure 7). Likewise, heat shock of tomatoes at 42°C for 12 or 24 hours was not sufficient to protect the fruit from low temperature. These treatments, however, were able to reduce decay significantly compared to the control fruits (data not shown). Visual difference in the incidence of chilling injury and the ability to ripen, between the heat-treated fruit at 42°C for 36 or 48 hours and the control fruits stored at 2°C for 14 days, are shown in Figures 8 and 9, respectively.

Collectively, heat treatment of 42°C for 36 and 48 h attenuates chilling injury of tomato fruit stored at 2°C up to 2 or 3 weeks; the response varied among years.

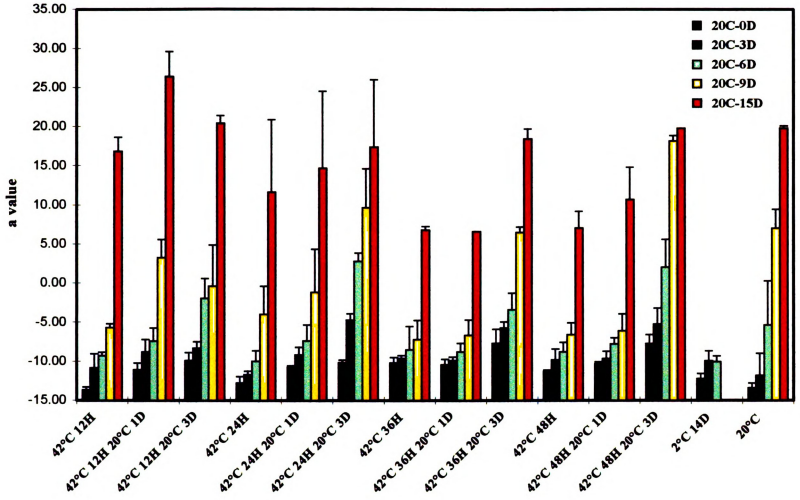

Figure 6. The effect of heat treatment and post-treatment at 20°C on color development of tomato fruits subsequently stored at 2°C for 14 days and during ripening at 20°C (year 1997).

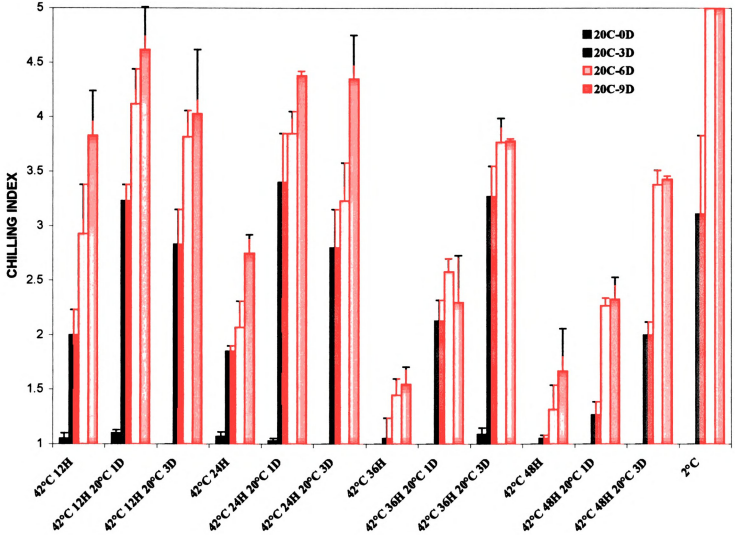

Figure 7. The effect of heat treatment and post-treatment at 20°C on chilling injury of tomato fruits subsequently stored at 2°C for 14 days and during ripening at 20°C (year 1997).

Figure 8. The effect of heat treatment (42°C for 36-h) and post-treatment at 20°C for 1 day on chilling injury of tomato fruits subsequently stored at 2°C for 14 days and ripened at 20°C.

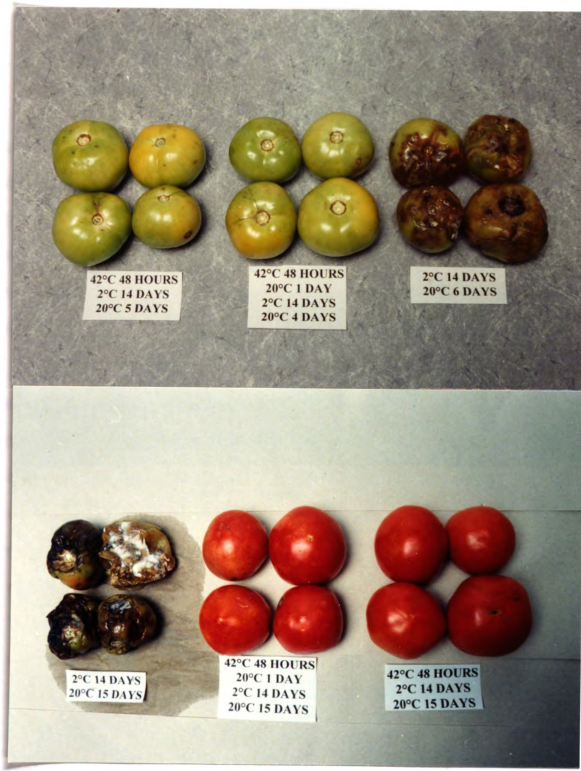

Figure 9. The effect of heat treatment (42°C for 48-h) and post-treatment at 20°C for 1 day on chilling injury of tomato fruits subsequently stored at 2°C for 14 days and ripened at 20°C.

DISCUSSION

The effect of 42°C treatment for 36 h or 48 h on reducing CI are in agreement with previous studies showing that long-term (38°C for 48 h) heat treatment conferred chilling tolerance to tomatoes (Lurie and Klein, 1991; 1992, McDonald et al., 1996; Lurie and Sabehat, 1997). Short-term heat treatment (42°C for 60 min) was also reported to beneficial for maintaining fruit quality after low temperature storage (McCollum and McDonald, 1993; McDonald et al., 1996; 1998). In our studies, however, the 38°C for 48 h did not prevent CI following storage.

Depending on the year in which the experiment was conducted, heat treatment was effective in preventing chilling injury of fruits stored at 2°C for up to 3 weeks. It appears that pre-harvest or the growing season environment factors may impact on the effectiveness of heat- treatment on CI attenuation. In addition, cultivar differences may determine whether a heat- treatment will be effective. For example, the cultivar that Lurie and Sabehat (1997) used, "Daniella", is a slow ripening variety that increases the shelf life and allows storage up to 4 weeks at 2°C after heat treatment. The variety used in the present study, "Mountain Springs" or the "Rutgers" used in other studies (Whitaker, 1994), do not appear to respond favorable to the same heat treatment (38°C for 48 h).

Heat treatment was also found to induce resistance/tolerance to chilling injury in avocado (Sanxter et al., 1994; Woolf et al., 1995; Florissen et al., 1996), citrus fruits (Rodov et al., 1995) cucumber (McCollum et al., 1995), mango (McCollum et al., 1993), mung bean hypocotyls (Collins et al., 1993; 1995), sweet pepper (Mencarelli et al.,

1993), persimmons (Burmeister et al., 1997; Lay-Yee et al., 1997), and zucchini squash (Wang, 1994b).

We wanted to test if the protection against CI conferred by heat treatment before storage would be lost if the heated fruit were held at 20°C for 1 or 3 days prior to storage at 2°C for 14 days (Figure 7). Indeed, the protection against CI was lost especially in the short-term heat treatment (42°C for 12 or 24 h). Similar results were reported by Lurie and Sabehat (1997).

The protective role of heat treatment against chilling injury in tomatoes has been correlated with the accumulation of heat shock proteins (Sabehat et al., 1996; 1998; Kadyrzhanova et al., 1998, chapter III of this study also). HSPs and especially the small HSPs are not present in fruits stored at low temperature unless they had been heat-treated (Sabehat et al., 1998; this study chapter III). HSPs accumulated in the cold-stored fruits that were held at room temperature after heat shock and prior to low temperature storage (Sabehat et al., 1998; this study chapter III). These results suggest that heat-shock proteins are not the only factor responsible for the protection of the tomato fruits from CI. Sabehat et al. (1998) suggested that the expression of the small HSP is correlated with the protection against some, but not all, symptoms of CI. Small HSPs are important components of the heat shock response and possess molecular chaperone activity *in vitro* and *in vivo* (Forreiter et al., 1997; Lee et al., 1997). At this moment, however, there is no report suggesting chaperone activity of the smHSP at low temperatures.

Another possible reason for the protection against chilling injury is related to the production of ethylene. A number of studies (Kosiyachinda and Young, 1976; Chaplin et al., 1983; Lee and Young, 1984; Florissen et al., 1996), some of them contradictory (Lipton

and Aharoni, 1979) have suggested that increased levels of ethylene are associated with increased levels of chilling injury. Avocados were more chilling sensitive during the climacteric rise and at the climacteric peak (Kosiyachinda and Young, 1976). Ethylene application increased the severity and the threshold temperature of chilling injury (Chaplin et al., 1983; Lee and Young, 1984; Florissen et al., 1996). In contrast, Lipton and Aharoni (1979) reported that ethylene reduces CI in melons. The longer the period of the heat treatment, the lower were the peak levels of ethylene production (Biggs et al., 1988; Lurie and Klein, 1991). Tomato fruits that received prolonged heat shock had gained chilling insensitivity (Lurie and Klein, 1991). Antisense ACC oxidase expression in transgenic melon has been reported to confer chilling tolerance, while ethylene application restored chilling sensitivity (JC Pech, personal communication).

In conclusion, the protection against CI afforded by heat treatment may be due to the production of HSPs as well as to the reduction in ethylene production or sensitivity. Therefore, heat treatment of tomato fruits has very significant potential for commercial application for several reasons: (1) wholesomeness of the fruit may be improved, avoiding the use of postharvest-applied chemicals to control some important physiological and pathological disorders; 2) ripening can be delayed; and 3) the storage duration can be more than doubled without evoking chilling injury disorders and the accompanying decay.

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CHAPTER II

MOLECULAR CLONING OF A NOVEL HEAT-INDUCED CHILLING-TOLERANCE RELATED cDNA IN TOMATO FRUIT BY USE OF mRNA DIFFERENTIAL DISPLAY

ABSTRACT

Chilling injury was circumvented by heat-treating mature green tomatoes (*Lycopersicon esculentum*, Mill. cv. Mountain Springs) at 42°C for two days prior to storing them at 2°C for one or two weeks, whereas fruits stored at 2°C without preheating developed typical chilling injury symptoms and failed to ripen at 20°C. Using differential display of mRNA and screening of the cDNA libraries, we have cloned from tomato fruit a full-length *HCT1* cDNA (heat induced/chilling tolerance related). The protein (17.6 kD) predicted from the coding region of *HCT1* cDNA has high identity with cytosolic class II small HSPs. The gene corresponding to *HCT1* cDNA was termed *LeHSP17.6*. Southern-blot hybridization indicates that *LeHSP17.6* belongs to a two-member gene family. Northern blot analysis indicates the heat-induced transcript of the *LeHSP17.6* remains up-regulated during subsequent exposure of the fruit to chilling temperatures for at least one week and upon transfer to ripening temperatures for one

day. Fruits that were only chilled show a low level of expression of the *LeHSP17.6* transcript. We hypothesize that *LeHSP17.6* may be involved in protecting the cell from metabolic dysfunctions leading to ripening failure caused by chilling injury. This is the first report of a cytosolic class II smHSP-encoding gene in tomato.

INTRODUCTION

The useful postharvest life of many fruits of tropical or subtropical origin is limited because they must be stored above 10-12°C to avoid chilling injury (Lyons, 1973). For example, if mature green tomatoes are stored for a few days at temperatures below 10°C and subsequently returned to a normally permissive ripening temperature, they fail to ripen and become susceptible to microbial spoilage (Hobson, 1987). Prestorage heat treatment has been found to increase chilling tolerance of tomato and other subtropical fruits (Hirose, 1985; Lurie and Klein, 1991; McCollum et al., 1993; Sanxter et al., 1994). Increased tolerance to chilling injury by high temperature treatment has been related to the accumulation of heat-shock proteins (HSPs) (Lafuente et al., 1991; Lurie and Klein, 1991). Plant HSPs consist of a few high molecular weight classes 60 kD, 70 kD, 90 kD, 100 kD and a complex group of low molecular weight proteins with molecular sizes ranging from 17 to 30 kD (Key et al., 1981; Nover et al., 1990; Vierling, 1991). The small HSPs are structurally related and are encoded by six discrete gene families (Waters et al., 1996). Classes I and II encode cytosolic proteins while classes III and IV encode chloroplast and endoplasmic reticulum localized proteins (Vierling, 1991; Helm et. al, 1993). A fifth class encodes the mitochondrial proteins (Lenne and Douce, 1994; Dong and Dunstan, 1996). For class VI, which is represented by a single 22.3 kD HSP from soybean, the intracellular location is proposed to be the endoplasmic reticulum (Lafayette et. al., 1996). Cytosolic small HSPs are particularly abundant in plants subjected to stress conditions but their specific functions are not known. Recently, *in vitro* studies demonstrated that small HSPs as well as high molecular weight HSPs

display elements suggesting molecular chaperone activities (Lee et al., 1995a). While they ostensibly function in the development of thermotolerance (Lee et al., 1994; Lee et al., 1995b; Yeh et al., 1995), some plant HSPs are expressed when the plant is subjected to other stresses such as water stress (Almoguera et al., 1993), heavy-metal toxicity (Neumann et al., 1994) and cold stress (Neven et al., 1992; Cabane et al., 1993; VanBerkel et al., 1994; Krishna et al., 1995).

To gain insight about the molecular mechanisms by which heat treatment affords subsequent protection against chilling injury in tomato fruits, we employed the differential display of mRNA technique. The differential display of mRNA method (Liang and Pardee, 1992) has been proving to be a good tool for detecting specifically expressed genes in plant tissues (Goormachtig et al., 1995; Johnson et al., 1995; Oh et al., 1995; Sharma and Davis, 1995; Wilkinson et al., 1995; Van der Knaap and Kende, 1995; Tieman and Handa, 1996). We herein report the identification and cloning of a full-length *HCT1* cDNA (heat-induced/chilling tolerance related). *HCT1* cDNA encodes a putative 17.6 kD protein which has high identity with cytosolic class II small HSPs of other plants. The gene corresponding to *HCT*1 cDNA was termed as *LeHSP17.6* (Kadyrzhanova et al., 1998). The transcript of *LeHSP17.6* is induced by heat treatment at 42°C for 2 days and remains up-regulated during a subsequent one week exposure to chilling temperatures of 2°C. The heat-treated fruits ripened normally following the low temperature storage while non-heated fruits showed typical chilling injury symptoms. We hypothesize that the translation product of *LeHSP17.6* may be involved in protecting the cell from the metabolic dysfunctions leading to ripening failure caused by chilling injury.

MATERIALS AND METHODS

Plant Material and Temperature Treatments

Tomato (*Lycopersicon esculentum* Mill. cv. Mountain Springs) fruits were harvested at the mature green stage and subjected to heat treatment at 42°C for 2 days (designated as H) followed by cold storage at 2°C for 7 days and 1 day at 20°C (designated as HC). Fruits that received only cold storage at 2°C for 7 days plus 1 day at 20°C (designated as C); fruits at the mature green stage (MG) served as controls. Fruit pericarp tissue from each treatment was frozen in liquid nitrogen and stored at -80°C.

Nucleic Acid Isolation.

Total RNA was extracted and purified according to (Grierson and Covey, 1976) and Fray and Grierson (1993) with some modifications. Frozen fruit pericarp was ground in liquid nitrogen with a mortar and pestle and extracted with buffer containing: 1% triisopropylnaphthalene sulfonate, 6% p-aminosalicylate, 5% (v/v) Tris-saturated phenol and 50 mM Tris-HCl, pH 8.0). The homogenate was extracted 4 times with an equal volume of phenol:chloroform (1:1) then once with chloroform. RNA was precipitated at -20°C by addition of 0.1 vol 3M sodium acetate, pH 5.6 and 2.5 vol of ethanol in two steps: first, 1 vol of ethanol was added and if a carbohydrate and DNA precipitate formed, this was removed and the remaining 1.5 vol of ethanol was added. RNA was precipitated with 4M lithium chloride and the pellet was dissolved in 250mM potassium acetate pH 7.0. The RNA was precipitated with 3 vols of ethanol, the pellet washed with

80% (v/v) ethanol, dried and dissolved in diethylpyrocarbonate (DEPC)-treated water. RNA was quantified and qualified by spectrophotometrically. Integrity of RNA was evaluated by fractionation of an aliquot on 1.2% agarose/formaldehyde gel.

Poly $(A)^+$ mRNA was isolated from total RNA using the PolyAT-tract^R mRNA isolation kit (Promega, Madison, WI). Concentration of poly$(A)^+$ mRNA was measured using the DNA DipStick^{TM} kit (Invitrogen, San Diego, CA).

Genomic DNA was isolated from tomato leaves as described by Doyle and Doyle (1987) except that SDS was added to the extraction buffer instead of CTAB. DNA concentration, purity and integrity were determined spectrophotometrically and by running an aliquot on 0.8% agarose gel.

Differential Display.

Differential mRNA display (Liang and Pardee, 1992) was performed using the RNA map^{TM} kit (GenHunter, Brookline, MA) according to the manufacturer's recommendation. Total RNA samples were DNase I treated as described by Liang et al., (1993) using RNase-free DNase I and human placental ribonuclease inhibitor (Boehringer Mannheim, Indianapolis, IN). DNase-free total RNA samples (0.2 μg) were used for the cDNA synthesis. The reverse transcription (RT) reaction consists of T_{12}MN anchor primers (where M is degeneration of A, C and G, while N is one of the four nucleotides). PCR amplification of one-tenth of the RT products was done in the presence of [^{33}P] dATP (Bauer et al., 1993). AmpliTaq polymerase (Perkin-Elmer Cetus Norwalk, CT) was used. Five decamers (AP_1 to AP_5) were used in combination with the respective T_{12}MN. Control reactions were performed in the absence of reverse

transcriptase. The amplified cDNAs were separated by electrophoresis on 6% denaturing polyacrylamide gel, containing 7 M urea at constant 60-W for 3 to 4 h, followed by vacuum-drying at 80°C on Whatman 3MM without fixation. X-ray film was exposed to the dried film overnight. The ^{32}P-end labeled φX174/Hae III (GIBCO-BRL, Life Technologies, Gaithersburg, MD) was used as the molecular weight markers.

Isolation of cDNA Bands.

The appropriate differential display cDNA bands were excised and eluted according to the manufacturer's instructions. Each gel was reexposed to X-ray film to verify that the band of interest had been correctly excised (Zimmerman and Schultz, 1994). cDNAs were then reamplified using the same PCR conditions and primers as above. The size of the PCR products was determined on a 1.2% agarose gel. cDNAs were purified from agarose slices using the QIAEXII™ kit (Qiagen, Chatsworth, CA).

RNA Gel Blot Analysis.

Total RNA (15 μg) was separated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to Hybond-N nylon membranes (Amersham, Aylesbury, UK) as described by Sambrook et al. (1989). The membranes were fixed by using a microwave oven for 2 min at full setting (700 W) (Angeletti et al., 1995). Prehybridization and hybridization was performed according to standard procedures (Sambrook et al., 1989). The blots were prehybridized for 3 h in hybridization buffer (50% formamide, 5X SSC, 25mM potassium phosphate, pH 7.4, 5X Denhardt's solution, 50 μg mL^{-1} denatured salmon sperm DNA) at 42°C. The eluted cDNAs were labeled

with [α-^{32}P]dCTP (DuPont/NEN Products, Boston, MA) using the random primer labeling kit (GIBCO-BRL, Life Technologies, Gaithersburg, MD) in the presence of the T_{12}MN oligomer. After alkaline denaturation, ^{32}P-labeled probe was added to hybridization buffer containing dextran sulfate in 5% (w/v) final concentration and hybridized for 16 h at 42°C. Membranes were washed twice with 1X SSC and 0.1% SDS at room temperature for 15 min and once with 0.2X SSC and 0.1% SDS at 60°C for 30 min before exposing X-ray film to the membranes overnight or periods up to 7 days. Transcript size was estimated by comparing the position of the hybridizing band to RNA molecular-weight markers (GIBCO-BRL).

The Cloning of cDNA Bands.

Following RNA blot hybridization, cDNA bands were recovered using the Northern-blot affinity capturing method (Li et al., 1994). A piece of the nylon membrane containing the captured probe was cut out, using the autoradiogram for band localization. The membrane pieces were stripped by boiling in water for 5 min, and the DNA was precipitated with 0.3 M sodium acetate and ethanol using glycogen as carrier. The reconstituted probe was reamplified by PCR using the same conditions as described above. An aliquot of the remaining reaction was ligated into pCRII vector of the TA-Cloning kit (Invitrogen, San Diego, CA). Multiple plasmid preparations were performed for each clone using the standard alkaline lysis method (Sambrook et al., 1989) and analyzed by restriction digestion with *Eco*RI. cDNA band inserts were purified from agarose slices, random primed radiolabeled and used in RNA blot analysis.

Library Construction and Screening.

Amplified cDNA libraries were constructed from heat-treated (H) and heat- and cold-treated (HC) tomato fruit poly $(A)^+$ mRNA in λgt 11 vector using the CapFinder™ PCR cDNA Library Construction kit (Clontech, Palo Alto, CA) according to manufacturer's instructions. Screening of the (H) and (HC) cDNA libraries was done by the Long-Distance (LD)-PCR based method (Barnes, 1994; Cheng et al., 1994; Ali-Osman and Akande, 1995). In a 50 μL reaction mixture, 10^6 phages from (H) or (HC) cDNA libraries were screened by amplification with 50 ng of the 3' gene-specific primer (30-mer) derived from a sequence near the poly (A) tail of *HCT1* cDNA band and 50 ng of the 5'-PCR primer (30-mer) supplied in the CapFinder™ PCR cDNA Library Construction kit (Clontech, Palo Alo, CA). The LD-PCR band was eluted from the agarose gel and cloned into pCRII vector by using TA-cloning.

DNA Sequencing and Analysis.

Double-stranded DNA sequencing was performed on an Applied Biosystems 373A (Foster City, CA) at the Michigan State University DNA Sequencing Facility, East Lansing, MI. Sequences were compared to the National Center of Biotechnology Information nonredundant sequence database (www.ncbi.nlm.nih.gov) using the default settings of BLASTN or BLASTX (Altschul et al., 1990). DNA sequence data were assembled and analyzed using DNA STAR (DNA STAR, Madison, WI).

Southern Blot Analysis.

Genomic DNA (20 μg) was digested with appropriate restriction enzymes and separated by electrophoresis on a 0.8% agarose gel. Blotting and hybridization conditions were performed as described by Sambrook et al. (1989). DNA blots were washed three times with 2X SSC and 0.1% SDS at room temperature for 15 min and twice with 0.1X SSC and 0.5% SDS at 42°C for 15 min before X-ray film was exposed to the membrane overnight with double intensifying screens at -80°C.

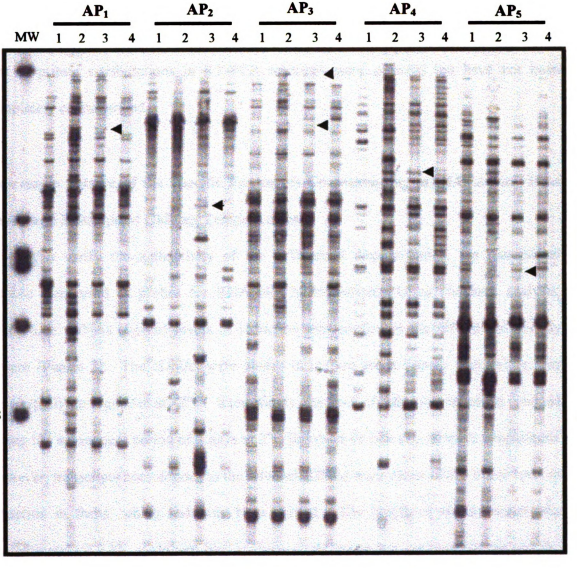
RESULTS

Differential Display.

The effect of heat and/or cold treatment on tomato fruit gene expression at the transcriptional level was studied using the differential display of mRNA (Liang and Pardee, 1992). Twenty RT-PCR reactions for each total RNA samples were conducted by combining five 5' arbitrary decamers (AP_1-AP_5) and four 3' anchor primers ($T_{12}MG$, $T_{12}MC$, $T_{12}MA$, $T_{12}MT$). An example of the differential display pattern is presented in Figure 1. About 40-80 amplified bands ranging from 100 to 600 bp in size were visible for each primer set. The anchor primers $T_{12}MA$ and $T_{12}MT$ showed lower specificity and selectivity than the $T_{12}MG$ and $T_{12}MC$ primers. From a total of about 1200 cDNA bands, 142 showed an increase as a result of heat shock (H), 41 showed high intensity

Figure 1. Differential display of total RNA from four different treatments of tomato fruit. (1) RNA was isolated from control fruits at the mature green (MG) stage, (2) heat-treated at 42°C for 2 days (H), (3) heat-treated at 42°C for 2 days followed by storage at 2°C for one week and then transferred to 20°C for 1 day (HC), and (4) fruits that were stored at 2°C for one week followed by one day at 20°C (C). Total RNA was reverse transcribed with the anchored primer $T_{12}MC$. The anchor primer and the arbitrary primer (AP_1, AP_2, AP_3, AP_4, and AP_5) were used for the amplification step of differential display. ^{32}P-end labeled φX174/Hae III (Gibco BRL) was used as molecular weight marker. The arrows indicate putative candidate, bands that were induced by heat shock (2) and maintained during low temperature storage (3).

$T_{12}MC$


AP1
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271
234
194
118

upon heat shock and subsequently cold temperatures (HC), while 18 were up-regulated during cold treatment (C) (Table I). Twenty-nine cDNAs were specifically expressed in both H and HC treatments and selected for further evaluation. After elution from the gel, these bands were reamplified with the same set of primers as used for the differential display under the same PCR conditions. The size of the fragments was verified on 1.2% agarose gels by electrophoresis. Other differential display bands derived from the same sets of primer combinations in RT-PCR reactions were detected but have not been completely characterized.

Expression Pattern of the Specific Fragments Corresponding mRNAs during Heat Shock and Subsequent Chilling Temperatures.

To verify the authenticity of the differential display bands, the reamplified cDNAs were used as probes for RNA blot hybridization. Upon Northern analysis, twenty-one cDNAs showed levels of induction that confirmed the differential display pattern (Figure 2). The cDNAs were absent in mature green tomato fruit, while they were rapidly up-regulated at 42°C. Interestingly, the level of induction remained elevated during the subsequent period of 7 days at 2°C followed by one day at room temperature. However, transcripts homologous to the selected cDNAs were detected at a lower level of induction in fruits, which had only been chilled. The length of the transcript was approximately 0.8 kb. Northern blot analysis could detect but not confirm the specific pattern for some cDNA fragments, while other fragments did not reveal any detectable hybridization signals.

Table I. Analysis of differential display cDNA bands corresponding to H, HC, C and MG tomato fruit RNA.

Primer combinations in RT-PCR reactions		Number of cDNA bands			
		H	HC	C	Total (including MG)
$T_{12}MG$	AP_1	8	2	4	58
	AP_2	9	1	1	53
	AP_3	15	5	0	54
	AP_4	13	2	1	57
	AP_5	5	1	0	41
$T_{12}MC$	AP_1	16	5	10	69
	AP_2	7	5	0	64
	AP_3	11	1	1	73
	AP_4	10	3	1	61
	AP_5	8	2	0	65
$T_{12}MA$	AP_1	4	2	0	77
	AP_2	1	4	0	69
	AP_3	1	1	0	67
	AP_4	5	1	0	57
	AP_5	3	1	0	41
$T_{12}MT$	AP_1	7	1	0	54
	AP_2	4	1	0	59
	AP_3	7	1	0	64
	AP_4	2	1	0	39
	AP_5	6	1	0	56
	Total	142	41	18	1196

Cloning and Sequence Analysis of the Specific cDNA Bands.

The cDNA bands confirmed by RNA blot analysis were recovered using the Northern blot affinity capturing method (Li et al., 1994), and cloned into the pCRII plasmid vector by using TA Cloning. The cloned cDNA fragments were released from the pCRII vector by digestion with *Eco*RI and random prime labeled for use as a probe in RNA blot hybridization. Northern blot analysis resulted in an autoradiogram (Figure 2)very similar to that obtained previously.

The partial cDNA clones were termed *HCT* (heat-induced/chilling tolerance related). Herein, we present one representative named as *HCT*1. The *HCT*1 nucleotide sequence contained the decamer and the oligo (dT) primer used for the differential display as well as part of the open reading frame (ORF) (underlined in Figure 3). Database searches revealed the following similarities: At the nucleotide level, the 273-bp *HCT*1 fragment showed 79% similarity with the cytosolic class II small HSP from soybean (Raschke et al., 1988), and pea (Lauzon et al., 1990). The carboxyl end (40 amino acids) of the ORF present in the *HCT1* encoding protein had 92 % identity to the soybean cytosolic class II small HSP and contained the conserved GVL motif that is common to all the small heat-shock proteins (Waters et al., 1996). The 3' non-coding region of the *HCT*1 contained a putative polyadenylation signal AATAAA 104 bp upstream of the poly (A) tail (Joshi, 1987).

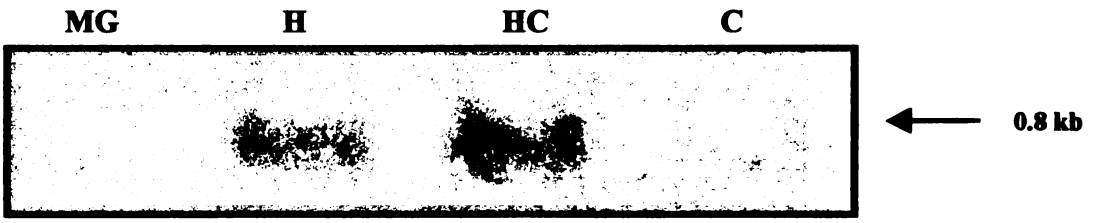

Figure 2. RNA blot hybridization analysis of total RNA from tomato fruit. Approximately 15 μg of total RNA from control mature green tomato fruit (MG), heat-treated fruit at 42°C for 2 days (H), heat-treated fruit as (H) followed by storage at 2°C for one week and then placed at 20°C for one day (HC) and non heat-treated fruit stored at 2°C for one week and transferred at 20°C for one day (C) was hybridized with ^{32}P random primed labeled reamplified *HCT1* cDNA. The mRNA molecular weight is indicated.

Construction, Screening of the cDNA Library and Sequence Analysis of a *HCT1* cDNA.

To isolate a full-length *HCT1* cDNA, amplified cDNA libraries were constructed from heat-treated and from heat- and cold-treated tomato fruit poly $(A)^+$ mRNA, respectively, in λgt11 (Clontech) according to the manufacturer's instructions. Screening of the cDNA libraries was performed by LD-PCR based screening (Ali-Osman and Akande, 1995). The screening of 10^6 independent phages using a specific 3'UTR primer (5'-GGCATCAAAACAAACAATACCCATGGCTAT-3') from *HCT1* and the specific 5' cap primer (30-mer) supplied by the CapFinder™ PCR cDNA Library Construction kit (Clontech) confirmed the presence of about 0.8 kb PCR products. DNA sequencing of the cloned LD-PCR product revealed a full-length *HCT1* cDNA 738 bp in size. The entire sequence of one of them (designated as *LeHSP 17.6*) is presented in Figure 3. Translation of the *LeHSP17.6* cDNA sequence revealed an uninterrupted reading frame of 477 nucleotides starting at the first ATG (108) and ending with a TGA 154 bases upstream of the poly (A) tail.

The deduced amino acid sequence of the *Le*HSP17.6 (Figure 3) corresponds to a polypeptide of 158 amino acids with an expected molecular mass of 17.6 kD and a pI of 6.63. A BLAST search of the nonredundant protein database showed that *Le*HSP17.6 protein is highly similar (>70%) over its entire length to cytosolic class II small heat-shock proteins (Vierling, 1991). The highest identity (75%) was found to the 17.7 kD HSP from pea (Lauzon et al., 1990), 74% to the 17.9 kD HSP from soybean (Raschke et al., 1988), 73% to the 17.9 kD HSP from parsley (Eckey-Kaltenbach, et al., 1997)

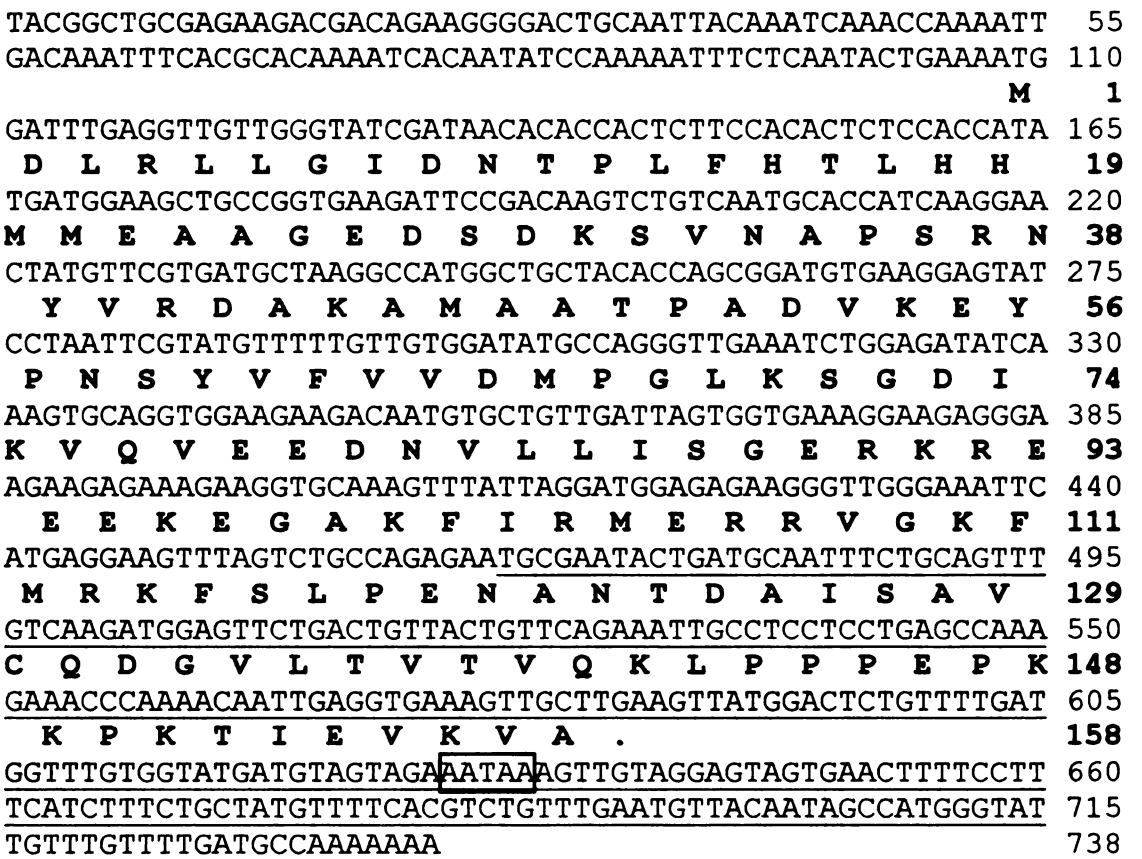

Figure 3. Nucleotide and deduced amino acid sequences of the *LeHSP17.6* cDNA. The putative polyadenylation signal is boxed. The differential display *HCT1* nucleotide sequence is underlined. The GenBank Accession Number of the *LeHSP17.6* sequence is U72396.

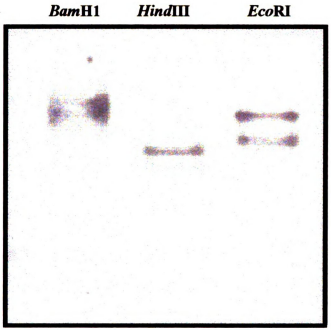

Figure 4. Genomic Southern blot analysis of the tomato *LeHSP17.6*. Tomato genomic DNA (20 μg) was digested with *Bam*HI, *Hind*III or *Eco*RI. The restriction fragments were separated on an agarose gel, blotted on nylon membrane and probed with *LeHSP17.6* cDNA without its poly(A) tail under low stringency hybridization and washing conditions.

and 70% with small HSPs from alfalfa (Keleman et al., unpublished) as well as with the 17.2 kD HSP from *Pharbitis nil* (Krishna et al., 1992).

Southern Blot Analysis.

To estimate the *LeHSP17.6* gene copy number, Southern blot analysis of genomic tomato DNA digested with three different restriction enzymes (*Eco*RI, *Bam*HI, *Hind*III) was conducted, using the entire *LeHSP17.6* cDNA without its poly(A) tail as the probe under low-stringency hybridization and washing conditions. As shown in Figure 4, two bands were detected in each restriction digestion, indicating that the *LeHSP17.6* transcript is encoded by a two-member gene family in the tomato genome.

DISCUSSION

Employing the differential display of mRNA techniques and screening the cDNA libraries, we have identified and cloned a full-length *LeHSP17.6* cDNA (heat-induced/chilling tolerance related) whose expression pattern changes during heat treatment and subsequent cold storage of tomato fruits. Northern blots confirmed the specific expression of the *LeHSP17.6* cDNA. Only transcripts from heated- and heated-and chilled-tomato tissues were preferentially expressed. These data show that *LeHSP17.6* cDNA originates from a gene(s) whose transcription is activated by heat treatment and maintained up-regulated during subsequent cold storage. The protein (17.6 kD) predicted from the coding region of *LeHSP17.6* cDNA has high identity with

cytosolic class II small HSPs from other plants. This is the first report of a cytosolic class II smHSP encoding gene in tomato.

The optimal multiple sequence alignment identified several conserved amino acids among the *Le*HSP17.6 and the other cytosolic class II smHSPs (Figure 5). The carboxyl end of *Le*HSP17.6 contains the conserved "heat shock" domain. These conserved domains consist of two subdomains I and II separated by a variable length hydrophilic region (Waters et al., 1996). The subdomain I contains the motif P-X_{14}-GVL (where X is any amino acid) and the subdomain II consists of a similar motif P-X_{14}-N-V/L/I-V/L/I (Waters et al., 1996). The significance of these conserved carboxyl terminus domains for the structure and function of smHSP has not been determined (Waters et al., 1996). Contrary to the carboxyl end, the amino terminus of the *Le*HSP17.6 differs from that of the other smHSPs. This may confer some functional specificity to the protein.

Interestingly, *Le*HSP17.6 includes a putative protein kinase C phosphorylation site S^{28}DK in the N-terminal region where the serine residue is preferentially phosphorylated by protein kinase C (Woodget et al., 1986) and a putative cAMP dependent protein kinase phosphorylation site RKFS^{85} (Zetterqvist, 1990) at the C-terminus, suggesting that phosphorylation of the *Le*HSP17.6 might occur. However, in early studies, Nover and Scharf (1984) failed to detect phosphorylation of the smHSP in tomato culture cells. Similarly, Suzuki et al. (1998) reported that the HSP21 and the cytoplasmic class I and class II smHSP do not appear to be phosphorylated during heat stress. Waters et al. (1996) suggested that the lack of the consensus RXXS phosphorylation motif is a possible explanation of the insufficient smHSP

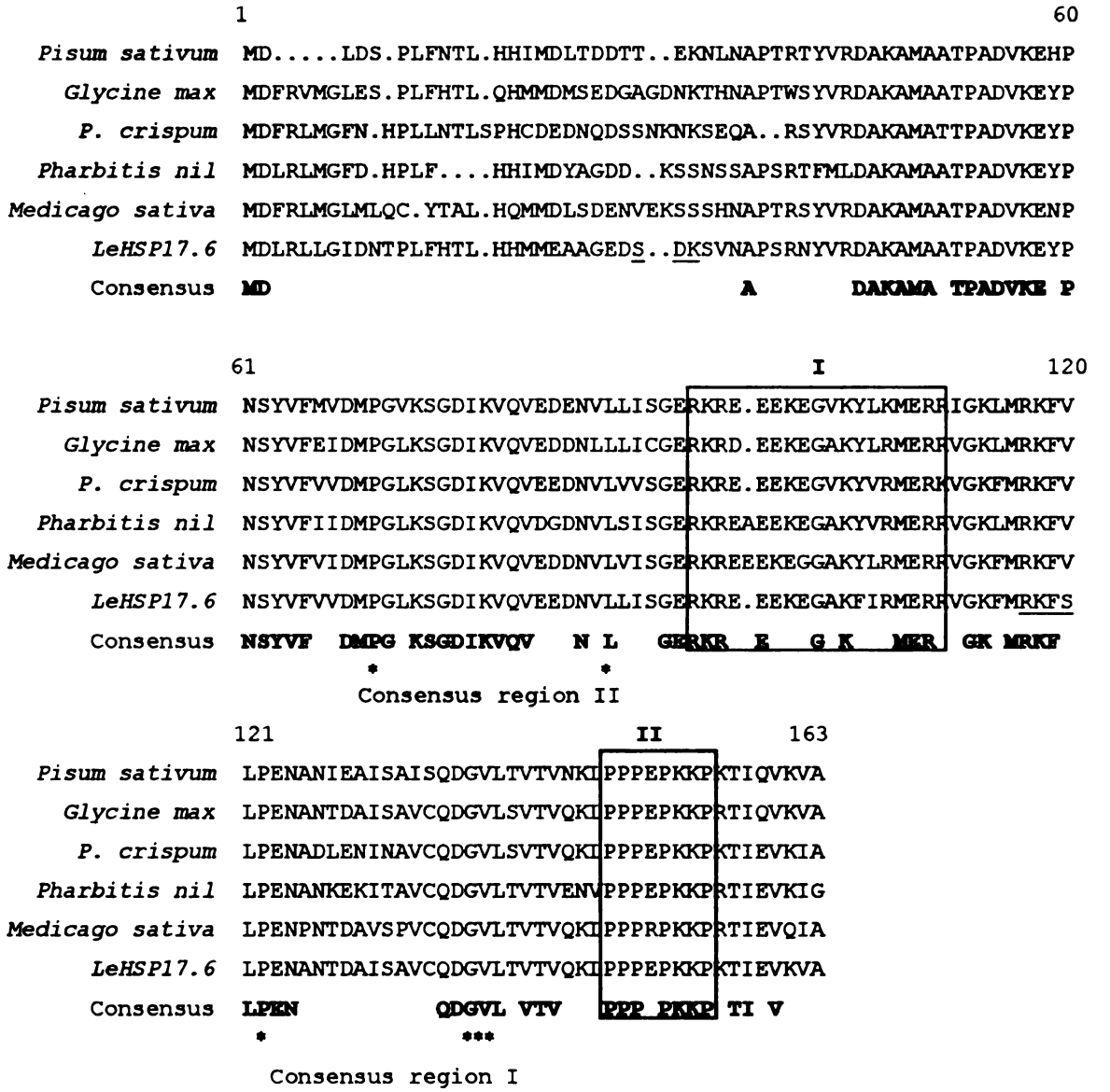

Figure 5. *Le*HSP17.6 is a cytosolic class II small HSP. The amino acid sequence alignment of the *Le*HSP17.6 tomato protein with cytosolic class II smHSPs from pea (M33901), soybean (X07159), parsley (X95716), *Pharbitis nil* (M99429) and alfalfa (X98617). Consensus sequence appears below the alignment typed in bold. A putative nuclear localization signal is indicated in box I. Box II indicates a potential polyproline motif. The underlined sequences suggest a putative protein kinase C phosphorylation sites. Asterisks define the important residues within the heat shock domain. Gaps within the alignment were introduced to optimize the alignment.

phosphorylation.

In the first box of the alignment (Figure 5) a conserved basic amino acid sequence is present (RKR) and corresponds to a putative *Xenopus* type nuclear localization signal (NLS) (Robbins et al., 1991). According to that motif, 2 basic amino acids are followed by 10 residues and the next 5 residues contain at least 3 basic residues. The two basic regions cooperate in binding, whereas the spacer may facilitate their cooperative interaction (Raikhel, 1992). Alternatively, the *Le*HSP17.6 at the C-terminus (K^{148}KKPK) contains a putative SV40 large T-antigen nuclear targeting signal (Garcia-Bustos et al., 1991). Recent studies by Wollgiehn et al. (1994) have suggested that the smHSPs move between the nucleus and the cytoplasm in a stress-dependent fashion. Small proteins, like the 13.8 kD heat-shock protein from yeast (Moreland et al., 1987), have been shown to have an NLS capable of redirecting a reporter protein to the nucleus. By histochemical analysis, it has been shown that the SV40 sequence can function as an NLS in transgenic tobacco (Van der Krol and Chua, 1991). However, not all the sequences similar to SV40 NLS are recognized by the plant nuclear import machinery (Silver, 1991). Perhaps, these putative nuclear localization signals are responsible for the shuttling of the smHSPs from the cytoplasm to the nucleus during stress.

Finally, the carboxyl end of the cytosolic class II smHSPs contains a polyproline motif PPPEPKKP (Figure 5). This motif, particularly the diproline sequence PXXP, is recognized by proteins with Src homology 3 (SH3) domains (Rickles et al. 1995; Ren et al., 1993). In a number of cell types, the SH3 domains function to regulate cellular events such as protein localization, enzyme activity and substrate requirement (Cohen et al., 1995). The sequences of these proteins determine specific signal transduction

pathways (Pawson, 1995). It remains to be elucidated if the smHSPs bind to proteins with SH3 domains.

Genomic Southern-blot hybridization indicates that *LeHSP17.6* belongs to a two-member gene family (Figure 5). Schoffl and Key (1983) reported that the soybean cytosolic class II smHSP is encoded by single gene. In contrast, the *Pharbitis nil* cytosolic class II smHSPs are encoded by a multigene family, with at least four representatives (Krishna et al., 1992).

The transcription of the *LeHSP17.6* gene is heat induced and is maintained up-regulated during subsequent exposure to chilling temperature and this correlated with tolerance to chilling injury. The mechanism remains to be determined and the function of the putative *Le*HSP17.6 is unknown. However, our data are in good agreement with results of S. Lurie and colleagues (Sabehat et al., 1996). They found that protection of tomatoes from chilling injury afforded by prestorage heat treatment is correlated with the induction of transcription of *HSP17* and *HSP70* mRNAs and with translation of HSP17 and HSP23 proteins which persist during subsequent storage of the fruit at chilling temperature. Other heat-induced transcripts may be involved as well. Recently, Collins et al. (1995) reported that heat shock of mung bean hypocotyls induced synthesis of several HSPs and only *de novo* synthesized HSP 79 and HSP 70 remained at significantly higher levels in tissue during a subsequent chilling period. These data, together with our results, suggest that the synthesis and action of HSPs attained by heat treatment may be involved in protecting the fruit and other parts of the plant from heat and chilling stress damage. One of the possible models for the mechanism by which heat treatment attenuate heat and chilling injury may be attributed to molecular chaperone activities of

HSPs (Vierling, 1991). Molecular chaperones are a group of intracellular proteins that control correct folding, oligomeric assembly, transport across membranes or disposal by degradation of other conformer unstable proteins by binding to them and release of them. Additionally, molecular chaperones prevent incorrect interaction within and between non-native polypeptides, which result in their irreversible aggregation (Hartl, 1996). Recently, the *in vitro* evidence for molecular chaperone activity of plant smHSPs was demonstrated by Vierling and colleagues (Lee et al., 1995a). They observed that recombinant HSP 18.1 and HSP 17.7 representing cytosolic class I and class II smHSPs from pea were able to enhance the refolding of chemically denatured model substrates citrate synthase and lactate dehydrogenase and prevented their aggregation and irreversible inactivation.

Plant smHSPs can assemble into multimeric units and form soluble high molecular weight complexes between 200-400 kD (Lee et al., 1995a; Waters et al., 1996). Lin and colleagues (Jinn et al., 1995) observed that the isolated 280 kD smHSP complex from soybean was able to protect up to 75% of the total soluble proteins of the cell from heat denaturation *in vitro*. These smHSP complexes can associate into insoluble larger cytoplasmic aggregates termed as "heat shock granules" (Nover et al., 1989). It has been suggested that these HS granules are transient sites for non-heat shock mRNA, preventing its degradation during heat stress (Nover, 1991). These large structures may be common to all smHSPs (Osteryoung and Vierling, 1994) and their formation may be reversible and occur mainly at highly stressing temperatures (Waters et al., 1996). Another role of plant HSPs is the protection of some proteins from and the targeting of other proteins to proteolysis (Vierstra, 1993). This putative function of HSPs may be

involved in the protection of plants from different stress damages such as heat shock and chilling injury. Genetically engineering transgenic plants that constitutive overexpress *LeHSP17.6* should prove useful to elucidate the function of the *Le*HSP17.6 in attenuation of chilling injury in tomatoes.

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CHAPTER III

HEAT TREATMENT ATTENUATES CHILLING INJURY: INVESTIGATION OF THE INVOLVEMENT OF HEAT SHOCK GENES AND HEAT SHOCK PROTEINS IN THE RESISTANCE OF TOMATO FRUIT TO LOW TEMPERATURES

ABSTRACT

Chilling injury (CI) was prevented by heat treating mature green tomato (*Lycopersicum esculentum* cv. Mountain Springs) fruit at 42°C for 36 or 48 h prior to storage at 2°C for two weeks, whereas fruit stored at 2°C without preheating developed typical CI symptoms and failed to ripen at 20°C. The protection afforded by heat treatment was lost if the tomatoes were transferred from 42°C to 20°C for 1 to 3 days before low temperature storage, however the CI was less than that of control fruits. We hypothesized that heat-shock proteins (HSP) may be responsible for increased tolerance to CI. Using differential display of mRNA we recently cloned and characterized a full-length cDNA that encodes a cytosolic class II smHSP (*Le*HSP17.6) termed *HCT1* (heat induced/chilling tolerance). Screening a cDNA library from heat-treated tomato fruits, a 17.4 kD cytosolic class II smHSP (*HCT2*) was isolated. It has about 90% similarity at the amino acid level to *Le*HSP17.6. Northern analysis indicates that both transcripts were

induced during heat shock, and the level of transcription remained high during subsequent storage at chilling temperatures. Using gene-specific differential display, six heat shock genes were identified including, three members of cytosolic class I smHSP, a chloroplastic smHSP, a mitochondrial smHSP and a cytosolic HSP90. The transcripts for the cytosolic class I smHSPs and the chloroplastic smHSP were up-regulated by heat treatment and are increased slightly during cold storage; fruits receiving only chilling temperatures had a low level of expression. When the fruits were warmed to 20°C for 3 days, the mRNA for all the smHSP declined slowly. HSP90 was induced by heat, but when the fruits were exposed to 2°C, the mRNA level increased 5-fold, suggesting that the expression of HSP90 is cold regulated. In a similar manner to the smHSPs, the HSP90 mRNA decreased slowly upon return to 20°C. HSP90 has 70% similarity at the amino acid level with the developmentally-regulated *Le*HSC80 protein and is more similar to stress-induced cytosolic HSP90 from other species. These data suggest that the synthesis and action of several HSPs whose transcripts increase during heat treatment may be involved in protecting the fruit from chilling stress.

INTRODUCTION

When plants are exposed to temperatures that exceed their normal growth temperature, they respond by inducing the synthesis of several polypeptides referred to as heat-shock proteins (Vierling, 1991). Pre-exposing plants to a mild heat shock, will induce resistance to higher lethal temperatures, a phenomenon called thermotolerance (Nover, 1991; Vierling, 1991). While thermotolerance can be induced in plants by heat-treatment, heat stress is also known to develop tolerance against other environmental stresses (Bonham-Smith et al, 1987; Orzeck and Burke, 1988; Kuznetsov et al., 1997; Sabehat et al., 1998b). The cross-protection response observed in plants is similar to that observed in yeast (Mager and Varela, 1993; Piper, 1993; Schuller et al., 1994); Exposure to one stress induces tolerance to another stress unrelated to the first one. Heat shock proteins are induced by other stress such as cold, ethanol, drought, amino acid analogues, salinity, ozone and oxidative stress (Anderson et al., 1994; Ruis and Schuller, 1995; Coca et al., 1996; Lee et al., 1996; Colmenero-Flores et al., 1997; Eckey-Kaltenbach et al., 1997; Banzet et al., 1998). The induction of the heat shock response can protect cells against a variety of other toxic insults, such as ethanol and hydrogen peroxide (Ruis and Schuller, 1995; Storz and Polla, 1996).

Mature green tomatoes are very susceptible to chilling injury by storage for only a few days at temperatures below 10°C. Heat treatment has been found to protect tomatoes against chilling injury (Klein and Lurie, 1991). We hypothesized that heat-shockproteins (HSP) may be responsible for increased tolerance to chilling injury following heat

treatment. Using differential display of mRNA we cloned and characterized a full-length cDNA (*LeHSP17.6*) that encodes a cytosolic class II small heat-shock protein (Kadyrzhanova et al., 1998). We confirmed our hypothesis that heat-shockproteins are correlated with chilling tolerance. This raised question whether other members of the HSPs are induced during heat shock and are stable during cold temperatures.

MATERIALS AND METHODS

Nucleic Acid Isolation

Total RNA was extracted and purified according to (Grierson and Covey, 1976) and Fray and Grierson (1993) with some modifications. Frozen fruit pericarp was ground in liquid nitrogen with a mortal and pestle and extracted with buffer containing: 1% triisopropylnaphthalene sulfonate, 6% p-aminosalicylate, 5% (v/v) Tris-saturated phenol and 50 mM Tris-HCl, pH 8.0). The homogenate was extracted 4 times with an equal volume of phenol:chloroform (1:1) and once with chloroform. RNA was precipitated at -20°C by addition of 0.1 vol 3M sodium acetate, pH 5.6 and 2.5 vols of ethanol in two steps: first, 1 vol of ethanol was added and if a carbohydrate and DNA precipitate formed, this was removed and the remaining 1.5 vol of ethanol was added. RNA was precipitated with 4M lithium chloride and the pellet was dissolved in 250mM potassium acetate pH 7.0. The RNA was precipitated with 3 vols of ethanol, the pellet washed with 80% (v/v) ethanol, dried and dissolved in diethylpyrocarbonate (DEPC)-treated water.

RNA was quantified and qualified by spectrophotometrically. Integrity of RNA was evaluated by fractionation of an aliquot on 1.2% agarose/formaldehyde gel.

Differential Display

Differential display was performed as described by (Kadyrzhanova et al., 1998) with a some modifications. Instead of the arbitrary decamer, primers designed from a conserved amino acid region of the specific HSP (mitochondrial, chloroplastic and cytosolic class I smHSP as well as HSP90) were combined with the anchored primers (T_{12}MN). The primers used were: 5'-GATCAAAGGTCCCAGGGTGGTGAGCATTGC-3' for mitochondrial smHSP, 5'-GGGGGAACACAAAAAGGAAGAAGACGGAG-3' for the chloroplastic smHSP, 5'-GGATCAAGTTAAGGCGTCTATGGAGAATGG-3' for the cytosolic class I smHSP and 5-GAGACTGCCCTTCTCACCTCAGGTTTCAGG-3' for the HSP90. Total RNA isolated from fruits, at the mature green (MG) stage, or heat-treated at 42°C for 2 days (H), or heat-treated at 42°C for 2 days following by storage at 2°C for one week and then transferred at 20°C for 1 day (HC). RNA from fruits that were stored at 2°C for one week following by one day at 20°C (C) was also obtained. Total RNA was DNAse I treated as described by Liang et al., (1993). DNAse-free RNA was reversed transcribed with the anchored primer T_{12}MN. PCR amplification of 1/10 of the first strand synthesis cDNA product was done in the presence of [α-^{32}P] dATP. Amplified cDNAs were separated through a 6% denaturing polyacrylamide gel containing 7M urea. Electrophoresis was done at constant 60W for 3-4hrs followed by drying on Whatman paper. The PCR fragments were resolved on 1.2% agarose gel, purified and then inserted into a *EcoR*I linearized plasmid pCRII vector using TA-cloning

system (Invitrogen). The cDNA clones released from the pCRII were random primer labeled and used as probes in Northern blots.

cDNA Library Screening

Screening of the cDNA library was done by the Long-Distance (LD)-PCR based method using gene-specific primers (Barnes, 1994; Cheng, 1994; Ali-Osman and Akande, 1995). About 10^6 phages from a heat- and cold-treated tomato fruit cDNA library (Kadyrzhanova et al., 1998) were screened by amplification with 3'gene-specific primer derived from sequence close to the poly (A) tail of the differential display products and the 5'-PCR primer provided by the CapFinterTM PCR cDNA library Construction kit (Clontech). The screening of the cDNA library for the isolation of the *LeHCT2* cDNA was performed according standard procedures (Sambrook et al., 1989).

DNA sequencing

The putative gene-specific clones were sequenced by double-stranded DNA sequencing. DNA sequence determination was performed on Applied Biosystems 373A (Foster City, CA) at the MSU Instrumentation Facility. DNA sequence data were assembled and analyzed using DNA STAR (DNA STAR, Madison, WI).

RNA Gel Blot Analysis

Total RNA (25μg) was separated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to Hybond-N nylon membranes (Amersham, Aylesbury, UK) as described by Sambrook et al. (1989). The membranes were fixed by using a microwave oven for 2 min at full setting (700W). The blots were prehybridized for 3 hrs

(50% formamide, 5XSSC, 25mM potassium phosphate, pH 7.4, 5X Denhardt's solution, 50μg/ml denatured salmon sperm DNA) at 42°C. The eluted cDNAs were [α-^{32}P]dCTP labeled using Random Primer Labeling kit (Gibco BRL, Gaithersburg, MD) in the presence of the T_{12}MN oligomer. After denaturation, the radiolabeled probe was added to hybridization buffer containing 5% (w/v) dextran sulfate and hybridized for 16 hrs at 42°C. Membranes were washed twice for 15 min at room temperature with 1X SSC and 0.1% SDS, followed by 15 min at 60°C with 0.2 X SSC and 0.1% SDS before exposure to X-ray film overnight to 7 days.

Protein Isolation and Western Analysis

Total proteins were extracted by grinding tissue in SDS sample buffer containing 60mM Tris-HCl pH 8.0, 60mM DTT, 2% SDS, 15% sucrose, 1mM PMSF, using 5 mLg^{-1} fresh weight of tomato pericarp. After grinding, samples were boiled for 5 min, and the insoluble debris was removed by centrifugation (10 min at 17,000g). Proteins were precipitated with 4 volume of cold acetone overnight at -20°C. Protein concentration was determined using Bradford assay (Bio-Rad). Twenty μg of each sample were separated on a 10% or 13% (w/v) polyacrylamide gel using the method of Laemmli (1970). After electrophoresis, gels were either stained with Coomassie brilliant blue R or transferred to a nitrocellulose membrane (Bio-Rad) for Western blot analysis. Membranes were blocked with TBST (20mM Tris-HCl, pH 7.5, 500mM NaCl and 0.5% (v/v) Tween 20) supplemented with 5% (w/v) non-fat dry milk for 1 h and then incubated for 1 or 2 h with the same solution containing anti-HSP antiserum. Antibodies used were the *Arabidopsis thaliana* cytosolic class I HSP 17.6 (1:2000 dilution) and the wheat cytosolic class II HSP 18.0 (1:500 dilution), both were gifts from Dr. Vierling, (University of Arizona, Tuscon),

as well as the *Pharbitis nil* HSP83 (1:5000 dilution), kindly provided by Dr. Krishna (University of Western Ontario, Canada). Bound antibodies were detected using goat anti-rabbit horseradish peroxidase diluted 1:2000 (v/v), an enhanced chemiluminescent system (Amersham), and exposed to x-ray film (Amersham) for 1 to 5 min, depending on signal strength.

RESULTS

Cloning and Characterization of Cytosolic Class II smHSP *LeHSP 17.4.*

Genomic Southern blot hybridization indicates that the cytosolic class II smHSP *LeHSP17.6* belongs to a two-member gene family (Kadyrzhanova et al., 1998). To isolate the other member of the family we screened 10^6 phages of the amplified cDNA library from heat-and cold-treated tomato fruit with the *LeHSP17.6* cDNA. The screening resulted in isolation of a full-length cDNA *LeHCT2* (heat induced/cold tolerance). Figure 1 shows the nucleotide sequence and the deduced amino acid sequence of the *LeHCT2*. The cDNA consists of 759 bp upstream of the polyadenylation tail, which includes a 79-bp 5' leader sequence, followed by 466 bp of an open reading frame (ORF), and a 213-bp 3'-untranslated region. A polyadenylation signal 174bp before the poly(A) tail is marked. The translation product of the *LeHCT2* is 155 amino acids long with a predicted molecular mass of 17.4kD and a pI of 7.24. The *LeHCT2* was further designated as *LeHSP17.4*.

The *Le*HSP17.4 has 92% similarity at the amino acid level with *Le*HSP17.6 indicating that is another member of the tomato cytosolic class II smHSP family. Table I indicates that sequence identities ranged between 54% (*Arabidopsis thaliana* HSP17.6b) and 95% (*Lycopersicum peruvianum* HSP17.4). A comparison of amino acids sequence of the *Le*HSP17.4 and the cytosolic class II smHSPs from other higher plants is shown on Figure 2. The cytosolic class II smHSP have several motifs, including the putative bipartite nuclear localization signal $R^{87}KRX_{10}RMERR^{104}$ (marked in purple) suggesting that these proteins may be targeted to the nucleus (Garcia-Bustos et al., 1991; Robbins et al., 1991; Silver, 1991; Raikhel, 1992), the putative cAMP protein kinase phosphorylation $R^{110}XXS$ site (blue) which is unique to tomato homologues and the polyproline motif (red) which is similar to known SH3-binding sites (Ren et al., 1993; Pawson, 1995; Rickles et al., 1995).

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GNGGCCGCTNCGACACGGCTGCGAGAAGACGACAGAAGGGGGACAGAATAAAATT  55
CCATTTTCAAACACGATAGAAGAATGGATTTGAGGTTGATGGGTATTGATAACAC 110
                          M  D  L  R  L  M  G  I  D  N  T  11
ACCACTCTTCCACACTCTTCAGCATATGATGGAAGCTGCTGGTGAAGATTCCGTG 165
  P  L  F  H  T  L  Q  H  M  M  E  A  A  G  E  D  S  V  29
AATGCACCACCAAAGAAGTATGTTCGTGATGCTAAGGCAATGGCTGCGACACCAG 220
 N  A  P  P  K  K  Y  V  R  D  A  K  A  M  A  A  T  P  47
TGGACGTGAAAGAGTATCCTGATTCATATGTTTTCGTTGTGGATATGCCAGGGTT 275
V  D  V  K  E  Y  P  D  S  Y  V  F  V  V  D  M  P  G  L  66
GAAATCTGGAGATATCAAAGTGCAGGTAGAAGAAGACAATGTGCTGTTGATTAGT 330
  K  S  G  D  I  K  V  Q  V  E  E  D  N  V  L  L  I  S  84
GGTGAAAGGAAGAGGGAAGAAGAGAAAGAAGGTGTAAAGTTTATTAGAATGGAGA 385
 G  E  R  K  R  E  E  E  K  E  G  V  K  F  I  R  M  E  102
GAAGGGTTGGGAAATTCATGAGGAAGTTTAGTCTGCCGGAGAATGCGAATACTGA 440
R  R  V  G  K  F  M  R  K  F  S  L  P  E  N  A  N  T  D 121
TGCAATTTCTGCAGTTTGTCAAGATGGAGTTCTGACTGTTACTGTTCAGAAGCTG 495
  A  I  S  A  V  C  Q  D  G  V  L  T  V  T  V  Q  K  L  139
CCTCCTCCTGAGCCAAAGAAGTCCAAAACCATTCAGGTCAAAGTTGCTTGAAAAT 550
 P  P  P  E  P  K  K  S  K  T  I  Q  V  K  V  A  .       155
ATAAAGTTACTCTGTTTTCTTGCTCTGTTTTGATGTAATAAAGCAATTGCTGCTC 605
TAGATTACCATATTTTGATGCATCCAAGGATTAACAAAATACAAATTTTAATGCA 660
TGTATCTTGTTTGATAAAGAATCGAATTTTAATTACTTTTTGCCTCATCTCCTTG 715
ATTGTGTGTTATAACTGTTTCACGAAAAGCCATTTACTTTAATCAAAAAAAAAAA 770
AAAAAAAAAAAAAAAAA                                        787
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Figure 1. Nucleotide and deduced amino acid sequences of the *LeHSP17.4* (*LeHCT2*) cDNA. Nucleotides and amino acids are represented as normal and bold letters, respectively. The putative polyadenylation signal is boxed.

Table I. Amino acid sequence identity of *LeHSP17.4* to those of the cytosolic class II smHSPs from various species

Species	Protein	Sequence Identity %	Accession Number	Reference
Arabidopsis thaliana	*At*HSP17.6a	63	X63443	Bartling et al. (1992)
Arabidopsis thaliana	*At*HSP17.6b	59	Y14070	Prandl et al. (1998)
Arabidopsis thaliana	*At*HSP17.0	54	X89504	Grellet et al. (1995)
Picea glaucum	*Pg*HSP17.0	64	L47717	Dong and Dunstan (1996)
Picea glaucum	*Pg*HSP17.1	63	L47740	Dong and Dunstan (1996)
Picea abies	*Pa*HSP16.9	64	X99346	Schubert et al. (1997)
Zea mays	*Zm*HSP17.5	57	X54075	Goping et al. (1991)
Zea mays	*Zm*HSP18.1	60	S59777	Atkinson et al. (1993)
Zea mays	*Zm*HSP17.8	61	X54076	Goping et al. (1991)
Triticum aestivum	*Ta*HSP17.3	60	X58279	Weng et al. (1991)
Pisum sativum	*Ps*HSP17.7	74	M33901	Lauzon et al. (1990)
Glycine max	*Gm*HSP17.9	75	X07159	Raschke et al. (1988)
Petroselinum crispum	*Pc*HSP17.9	74	X95716	Eckey-Kaltenbach et al. (1997)
Medicago sativa	*Ms*HSP17.0	73	X98617	Kelemen et al. (1996)
Lycopersicum esculentum	*Le*HSP17.6	92	U72396	Kadyrzhanova et al. (1998)
Lycopersicum peruvianum	*Lp*HSP17.4	95	AJ225049	Forreiter and Loew (1998)
Helianthus annuus	*Ha*HSP17.9	71	Z29554	Coca et al. (1994)
Pharbitis (Ipomoea) nil	*Pn*HSP17.2	71	M99429	Krishna et al. (1992)
Pharbitis (Ipomoea) nil	*Pn*HSP18.8	62	M99430	Krishna et al. (1992)

Figure 2. Amino acid sequence comparison among the cytosolic class II smHSPs. Consensus amino acid sequence is showed in bold face. The secondary structure of the protein is shown as predicted by the PHDsec (Profile network prediction HeiDelberg) method (www.embl-heidelberg.de/predictprotein/predictprotein.html; Rost and Sander, 1994). Helices are indicated with red box while the strands are presented as green arrows. The putative bipartite nuclear localization signal is in purple, the putative polyproline motif is in red and the putative cAMP protein kinase phosphorylation site of tomato homologues is in unbolded blue. The consensus I and II domains are indicated in blue. Gaps within the alignment were introduced to optimize the alignment. The nucleotide and amino acid sequences of the cytosolic class II small heat-shockproteins were obtained from the GeneBank and the Accession Numbers are listed in Table II. Amino acid sequences were aligned using CLUSTAL V method in DNASTAR.

```
AtHSP17.6  MDL...GRF.....PIISILEDMLEVPEDHNNEKTRNN......PSRVYMRDAKAMAATPADVIEHPNAYAFVVDMPGIKGDEIKVQVENDNVLVVSGER.QRENKENEG.VKYVRMERRMGK
AtHSP17.6  MDLE.FGRF.....PIFSILEDMLEAPEEQT.EKTRNN......PSRAYMRDAKAMAATPADVIEHPDAYVFAVDMPGIKGDEIQVQIENENVLVVSGKR.QRDNKENEG.VKFVRMERRMGK
AtHSP17.6  MDLE.FGRF.....PIFSILEDMLEAPEEQT.EKTRNN......PSRAYMRDAKAMAATPADVIEHPDAYVFAVDMPGIKGDEIQVQIENENVLVVSGKR.QRDNKENEG.VKFVRMERRMGK
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PgHSP17.1  MAMD..........PLLNTVQQLLVVPDDLER.........ILHAPTRSYMRDTEATASTPVDVKEYPNSYVFIVDMPGLKSNDIKVQVEDENILNISGER.KRNEK.EEGEVKYIRMERRVGK
PaHSP16.9  MAMD..........PLLSTVQQLLGVPDDLER.........ILHAPTRSYMRDTEATASTPVDVKEYPNSYVFIVDMPGLKSNDIKVQVEDENVLNISGER.KRNEK.EEGEVKYIRMERRVAK
ZmHSP18.0  MDAVMFGLET....PLMAALQHLLDVPDGDAGAGGDNKTGSGGSATRTYVRDARAMAATPADVKELPGAYAFVVDMPGLGTGDIRVQVEDERVLVVSGER.RR..EEREDDAKYLRMERRMGK
ZmHSP18.1  MDARMFGLET....PRVAALHHLLDVPDGDKAGGG.........ATRTYVRDARAMAATPADVKELAGAYAFVVDMPGLSTGDIRVQVEDERVLVISGER.RR..EERED.AKYLRMERRMGK
ZmHSP18.0  MDGRMFGLET....PLMVALQHLLDVPDGDAGAGGDK..AGGGGPTRTYVADARAMAVTPADVKELPGAYAFVVDMPGLGTGDIKVQVEDERVLVISGER.RR..EERED.AKYLRMERRMGK
TaHSP17.3  MAGMVFGLDA....PMMAALQHLLDIPDGEAEPPPEKQ.....GPTRAYVRDARAMAATPADVKELPGAYAFVVDMPGLGSGDIKVQVEDERVLVISGER.RR..EEKED.AKYLRMERRMGK
PsHSP17.7  MD.....LDS....PLFNTLHHIMDLTDDTT..E....KNLNA.PTRTYVRDAKAMAATPADVKEHPNSYVFMVDMPGVKSGDIKVQVEDENVLLISGER.KRE.EEKEG.VKYLKMERRIGK
GmHSP17.9  MDFRVMGLES....PLFHTLQHMMDMSEDGAGDN....KTHNA.PTWSYVRDAKAMAATPADVKEYPNSYVFEIDMPGLKSGDIKVQVEDDNLLLICGER.KRD.EEKEG.AKYLRMERRVGK
PcHSP17.9  MDF.R..LMGFNH.PLLNTLSPHCDEDNQDSSN.....KNKSE.QARSYVRDAKAMATTPADVKEYPNSYVFVVDMPGLKSGDIKVQVEEDNVLVVSGER.KRE.EEKEG.VKYVRMERKVGK
MsHSP17.0  MDF.R..LMGLMLQC.YTALHQMMDLSDENVEKS....SSHNA.PTRSYVRDAKAMAATPADVKENPNSYVFVIDMPGLKSGDIKVQVEDDNVLVISGER.KRE.EEKEGGAKYLRMERRVGK
LeHSP17.6  MDL.R..LLGIDNTPLFHTLHHMMEAAGEDS..D....KSVNA.PSRNYVRDAKAMAATPADVKEYPNSYVFVVDMPGLKSGDIKVQVEEDNVLLISGER.KRE.EEKEG.AKFIRMERRVGK
LeHSP17.4  MDL.R..LMGIDNTPLFHTLQHMMEAAGEDS.........VNA.PPKKYVRDAKAMAATPVDVKEYPDSYVFVVDMPGLKSGDIKVQVEEDNVLLISGER.KRE.EEKEG.VKFIRMERRVGK
LpHSP20.2  MDL.R..LLGIDNTPLFHTLHHMMEAAGEDS.........VNA.PSKIYVRDAKAMAATPADVKEYPNSYVFVVDMPGLKSGDIKVQVEEDNVLLISGER.KRE.EEKEG.AKFIRMERRVGK
HaHSP17.9  MDIDS..LMGFD..PLLRNLHYILEATDDNTTGN....KSNNSGPSRAYVRDARAMAATPADVKECPNSYVFIVDMPGLKSGDIKVQVERDNVLVISGKR.NRE.EEKEG.VKYVRMERRMGK
PnHSP17.0  MDL.R..LMGFDH.PLF...HHIMDYAGDD..KS....SNSSA.PSRTFMLDAKAMAATPADVKEYPNSYVFIIDMPGLKSGDIKVQVDGDNVLSISGER.KREAEEKEG.AKYVRMERRVGK
PnHSP18.0  MDLRNFGLSNFGLEPQLLSTIQDMLDFADDHDRAG...RAPPEQPIRAYVRDAKAMAATPADVKEYPNSYVFIADMPGVKAAEIKVQVEDDNVLVVSGERTEREKDEKDG.VKYLRMERRVGK
Consensus  M               P                                    Y RD  AMA TP DV E P  Y F  DMPG      I VQVE    VL  SG R  R       E   K  RMERR GK
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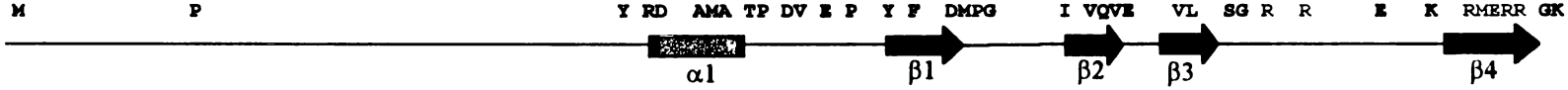


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AtHSP17.6  FMRKFQLPENADLDKISAVCHDGVLKVTVQKLPPPEPKKPKTIQVQVA
AtHSP17.6  FMRKFQLPDNADLEKISAACNDGVLKVTIPKLPPPEPKKPKTIQVQVA
AtHSP17.6  FMRKFQLPDNADLEKISPACNGGVLEVTNPKLPPPRNKKPRQNKVQFA
PgHSP17.0  FMRKFTLPADCNLEAISAACQDGVLTVTVPKLPPPEPKKPKTIEVKIG
PgHSP17.1  FMRKFSLPADCNLEAISAACQDGVLTVTVPKLPPPEPKKPKTIAVQIG
PaHSP16.9  FMRKFSLPADCNLEAISAACQDGVLTVTVPKLPPPEPKKPKTIAVKIG
ZmHSP18.0  FMRKFVLPDNADVDKVAAVCRDGVLTVTVEKLPPPEPKKPKTIEVKVA
ZmHSP18.1  FMRKFVLPDNADVDKVAAVCRDGVLTVTVEKLPPPEPKKPKTIEIKVA
ZmHSP18.0  FMRKFVLPDNADMDKISAVCRDGVLTVTVEKLPPPEPKKPKTIEVKVA
TaHSP17.3  LMRKFVLPENADMEKISP.CRDGVLTVTVDKLPPPEPKKPKTIQVQVA
PsHSP17.7  LMRKFVLPENANIEAISAISQDGVLTVTVNKLPPPEPKKPKTIQVKVA
GmHSP17.9  LMRKFVLPENANTDAISAVCQDGVLSVTVQKLPPPEPKKPRTIQVKVA
PcHSP17.9  FMRKFVLPENADLENINAVCQDGVLSVTVQKLPPPEPKKPKTIEVKIA
MsHSP17.0  FMRKFVLPENPNTDAVSPVCQDGVLTVTVQKLPPPRPKKPRTIEVQIA
LeHSP17.6  FMRKFSLPENANTDAISAVCQDGVLTVTVQKLPPPEPKKPKTIEVKVA
LeHSP17.4  FMRKFSLPENANTDAISAVCQDGVLTVTVQKLPPPEPKKSKTIQVKVA
LpHSP20.2  FMRKFSLPENANTDAISAVCQDGVLTVTVQKLPPPEPKKPKTIEVKVA
HaHSP17.9  FMKKFALPEDANTDKISAICQDGVLTVTVEKLPPPEPKKPKTIQVQVA
PnHSP17.0  LMRKFVLPENANKEKITAVCQDGVLTVTVENVPPPEPKKPRTIEVKIG
PnHSP18.0  FMRKFVLPENANVEAINAVYQDGVLQVTVEKLPPPEPKKPKTVEVKVA
Consensus  MRKF LP          C DGVL VTV KLPPPEPKKP T
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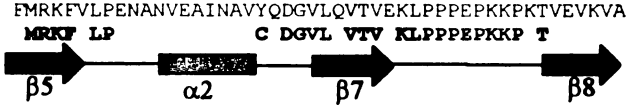

Differential Display, Cloning and Sequence of Heat-Induced Chilling Tolerance-Related HSPs.

Using differential display of mRNA, we cloned a cytosolic class II smHSP (*LeHSP17.6*) that was heat induced and related to chilling tolerance of tomato fruit (Kadyrzhanova et al. 1998). To test the hypothesis that other HSPs may also be related to cold tolerance, a modification of differential display of mRNA was used where the arbitrary primers are replaced with gene-specific primers for other HSP encoding genes. Based on sequence information we designed primers specific for smHSPs and high molecular weight HSPs. Gene-specific differential display fragments that were induced during heat shock (H) and subsequently at cold temperatures (HC) were selected, cloned into the pCRII plasmid vector and sequenced.

The characteristics of the heat-inducible chilling tolerance cDNAs isolated by gene specific differential display are summarized in the table II. Three members of cytosolic class I smHSPs (*LeHCT3*, *LeHCT4* and *LeHCT8*), a chloroplastic smHSP (*LeHCT5*), a mitochondrial smHSP (*LeHCT6*) and a cytosolic HSP90 (*LeHCT7*) were cloned. The nucleotide and the deduced amino acid sequence of the *LeHCT3* is presented in Figure 3. The differential display fragment *LeHCT3* is 324 bp long and contained the sequence of the cytosolic class I smHSP specific primer. This piece is identical to pTOM66 (Fray et al., 1990). The 3'UTR, however, is longer than the pTOM66, suggesting that the *LeHCT3* has a complete 3' end, which contains a putative poly(A) signal. LD-PCR screening of the HC cDNA library, with a *LeHCT3* 3'-end primer derived from a sequence near the polyadenylation signal and of the 5'-PCR primer

supplied from CapFinder™PCR cDNA library construction kit, resulted in isolation of two full-length cDNAs; the *LeHCT3* and *LeHCT8*, respectively. The nucleotide sequence of the *LeHCT3* is 724 bp long and contains an ORF from 62 to 526, a 5'-UTR of 61 nucleotides and a 3'-UTR of 198 nucleotides (Figure 3). A putative poly A signal (AATAA) is found at the nucleotide position 701. The deduced amino acid sequence of the *LeHCT3* corresponds to a polypeptide of 154 amino acids with a predicted molecular weight of 17.7 kD and a pI of 5.9. *LeHCT3* was further designated as *LeHSP17.7* and is identical to the tomato cytosolic class I smHSP, pTOM66 (Fray et al., 1990) with one substitution in the amino acid sequence; the P^{57} is an A^{57}.

The nucleotide sequence of the *LeHCT8* is 742-bp long and includes an 86-bp 5'-leader sequence, followed by a 467-bp sequence of an ORF and a 189-bp of an 3' UTR (Figure 4). The deduced amino acid sequence of the *LeHCT8* corresponds to a 17.8 kD protein with a pI of 5.6. The *LeHCT8* was further named as *LeHSP17.8* and has 98% similarity in the coding region with *LeHSP17.7*.

In addition to *LeHCT3*, the same combination of primers generated another differential display fragment designated as *LeHCT4*. The *LeHCT4* is 320-bp in length and has 97% similarity in the coding region with *LeHCT3*, but the 3' UTR is different, indicating that it is another member of cytosolic class I smHSP family (Figure 5). Analogous to the *LeHCT3*, the *LeHCT4* contains a putative polyadenylation signal 137-bp before the poly(A) tail. A gene-specific primer before the poly A tail of the *LeHCT4* was designed to screen the (HC) cDNA library using the LD-PCR approach using this primer in combination with the 5'-PCR primer provided from CapFinder™PCR cDNA library construction kit. This screening provided the full-length cDNA *LeHCT4*.

Table II. Characteristics of the cDNA clones corresponding to heat-inducible chilling tolerance genes

Clone Name	Clone Size (DD size)* (bp)	Predicted Protein Size (kD)	Poly(A) signal	Sequence Homology	Accession No.
*Le*HCT2	787	17.4	Yes	Cytosolic Class II smHSP	AF090115 (This study)
*Le*HCT3	725 (304)	17.7	Yes	Cytosolic Class I smHSP	X56138 (Fray et al.,1990); (This study)
*Le*HCT4	745 (320)	17.6	Yes	Cytosolic Class I smHSP	This study
*Le*HCT8	742	17.8	Yes	Cytosolic Class I smHSP	This study
*Le*HCT5	(327)	-	Yes	Chloroplastic smHSP (*Le*HSP21)	U59917 (Lawrence et al., 1997)
*Le*HCT6	(660)	-	Yes	Mitochondrial smHSP (*Le*HSP22)	This study
*Le*HCT7	1610* *(447)	-	No	HSP90	This study

*The number in parenthesis indicates the size of the differential display fragment
** The *Le*HCT7 is a partial cDNA

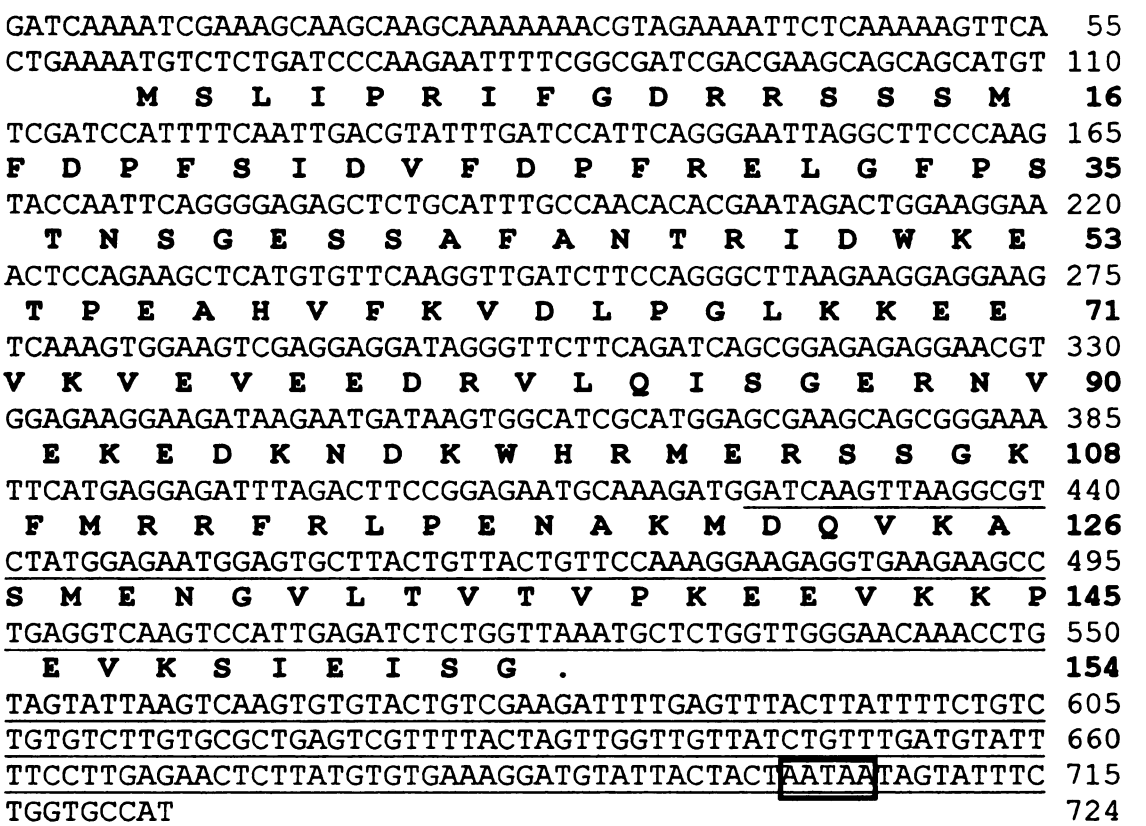

Figure 3. Nucleotide and deduced amino acid sequences of the cytosolic class I *LeHSP17.7* (*LeHCT3*) cDNA. Nucleotides and amino acids are represented as normal and bold letters, respectively. The putative polyadenylation signal is boxed. The differential display *LeHCT3* nucleotide sequence is underlined.

```
ACGGCTNCCAGAAGACGACAGAAGAGGCATCAAAATCGAAAGCAAGCAAGCAAAA  55
AAACGTAGAAAATTCTCAAAAAGTTCACTGAAAATGTCTCTGATCCCAAGAATTT 110
                                      M  S  L  I  P  R  I    7
TCGGCGATCGACGAAGCAGCAGCATGTTCGATCCATTTTCAATTGACGTATTTGA 165
F  G  D  R  R  S  S  S  M  F  D  P  F  S  I  D  V  F  D   26
TCCATTCAGGGAATTAGGCTTCCCAAGTACCAATTCAGGGGAGAGCTCTGCATTT 220
  P  F  R  E  L  G  F  P  S  T  N  S  G  E  S  S  A  F   44
GCCAACACACGAATAGACTGGAAGGAAACTCCAGAAGCTCATGTGTTCAAGGTTG 275
 A  N  T  R  I  D  W  K  E  T  P  E  A  H  V  F  K  V    62
ATCTTCCAGGGCTTAAGAAGGAGGAAGTCAAAGAGGAAGTCGAGGAGGATAGGGT 330
D  L  P  G  L  K  K  E  E  V  K  E  E  V  E  E  D  R  V   81
TCTTCAGATCAGCGGAGAGAGGAACGTGGAGAAGGAAGATAAGAATGATAAGTGG 385
  L  Q  I  S  G  E  R  N  V  E  K  E  D  K  N  D  K  W   99
CATCGCATGGAGCGAAGCAGCGGGAAATTCATGAGGAGATTTAGACTTCCGGAGA 440
 H  R  M  E  R  S  S  G  K  F  M  R  R  F  R  L  P  E   117
ATGCAAAGATGGATCAAGTTAAGGCGTCTATGGAGAATGGAGTGCTTACTGTTAC 495
N  A  K  M  D  Q  V  K  A  S  M  E  N  G  V  L  T  V  T  136
TGTTCCAAAGGAAGAGGTGAAGAAGCCTGAGGTCAAGCCCATTGAGATCTCTGGT 550
  V  P  K  E  E  V  K  K  P  E  V  K  P  I  E  I  S  G   154
TAAATGCTCTGGTTGGGAACAAACCTGTAGTATTAAGTCAAGTGTGACTGTCGAA 605
GATTTTGAGTTTACTTATTTTCTGTCATGGCTTGGGCCCTGAGTCGTTTACTAGT 660
TGGTTGGTATCTGTTTGATGTATTTTCCTTGAGAACTCTTGAGAACTCTTATGTG 700
TGAAAGGATGTATTACTACTAATAATAGTATTTCTGGTGCCAT              742
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Figure 4. Nucleotide and deduced amino acid sequences of the cytosolic class I *LeHSP17.8* (*LeHCT8*) cDNA. Nucleotides and amino acids are represented as normal and bold letters, respectively. The putative polyadenylation signal is boxed.

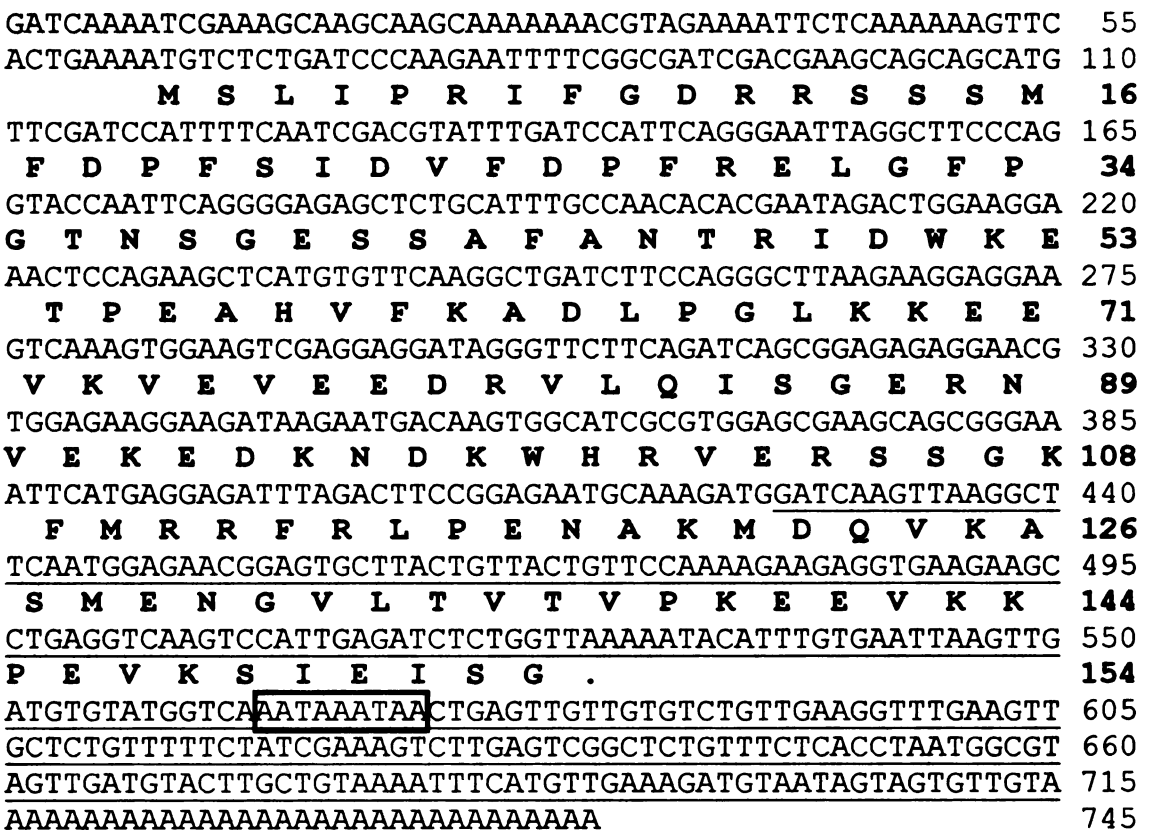

Figure 5. Nucleotide and deduced amino acid sequences of the cytosolic class I *LeHSP17.6* (*LeHCT4*) cDNA. Nucleotides and amino acids are represented as normal and bold letters, respectively. The putative polyadenylation signal is boxed. The differential display *LeHCT4* nucleotide sequence is underlined.

The length of the *LeHCT4* is 745 bp. It contains a 62-bp sequence of the 5'-untraslated region, followed by 466-bp sequence of an ORF and a 217-bp 3'-untranslated region, including the 31 A residues of the poly(A) tail. The translation product is 154 amino acids long with a predicted molecular weight of 17.6kD and pI of 5.9. The *LeHCT4* was designated as cytosolic class I smHSP *LeHSP17.6*.

The derived amino acid sequences of the three tomato cytosolic class I smHSP are compared with the other sequences present in the GeneBank databases in Figure 6. Table III shows that the sequence similarities among the tomato and the other higher plant cytosolic class I smHSPs ranged from 60% (*Hordeum vulgare HSP17.0b* and *Agrostis stolonifera HSP16.5*) to 95% (*Lycopersicum peruvianum HSP17.8*).

At the N-terminus, the tomato cytosolic class I smHSPs contain a putative cAMP protein kinase phosphorylation site ($R^{11}XXS$), which is also observed in the *Lycopersicum peruvianum* (Forreiter and Loew, 1998) and Douglas fir (*P. menziesii*) (Dong and Dunstan, 1996) homologues shown in blue (Figure 6). These proteins also share a distinct region of conserved amino acids near the N-terminus (F^{17}DPFXXDXXDPF) followed by a large variable region (10 to 28 amino acids), and a large region of conservation to the C-terminus. The C-terminus region contains the conserved "heat shock domain", which consists of two subdomains, the consensus I and II separated by a variable length domain (Vierling, 1991). The subdomain consensus region II, which contains the putative substrate-binding region (Lee et al., 1997), is highlighted in red. Secondary structure prediction as well as the crystal structure of the *Methanoccocus jannaschii* HSP16.5 (Kim et al., 1998) suggests that this region is a loop

between β3 and β4 (Figure 6). In this region, the hydrophobic residues are involved in dimer interaction.

Another differential display fragment isolated was the *LeHCT5* (Figure 7). The partial cDNA *LeHCT5* is 327 bp long and is identical to the *pTOM111* (chloroplastic smHSP *LeHSP21*) isolated by Lawrence et al. (1997) and Sabehat et al. (1998a). In parallel with chloroplastic smHSP, a mitochondrial related smHSP differential display fragment *LeHCT6* was isolated. *LeHCT6* is 660 bp long, containing 113-bp 3' end of an ORF and a large 3'-untranslated region of 547 nucleotides, with a poly (A) signal 66-bp before the polyadenylation tail (Figure 8). The ORF encodes a predicted polypeptide of 36 amino acids and has 50% similarity with the other mitochondrial smHSP from plants. To isolate a heat-inducible chilling tolerance HSP90 homologue, a primer was designed from the conserved C-terminus domain of the HSP90 family. Using the differential display approach a fragment of 447 nucleotide designated as *LeHCT7* was isolated (Figure 9). *LeHCT7* is 447 bp long, containing a 406-bp 3' untranslational region. There was no consensus eukaryotic polyadenylation motif (AATAAA), which is not unusual for the HSP90 family. For instance, the *LeHSC80* did not contain a polyadenylation signal (Koning et al., 1992). To obtain a larger clone, we used a primer designed from 5' terminal portion of the *LeHCT7* to screen the (HC) cDNA library by LD-PCR as described for the cytosolic class I smHSPs. The longest cDNA clone found was about 1.61kb long. This fragment was designated as *LeHSP90*. Efforts to get a full-length cDNA clone by LD-PCR were unsuccessful.

Table III. Cytosolic class I smHSP similarity to *Le*HSP17.7

Species	Protein	Sequence Similarity %	Accession Number	Reference
Pennicetum glaucum	HSP16.9	68	X94192	Caven et al. (1996)
Pennicetum glaucum	HSP17.0	68	X94191	Caven et al. (1996)
Zea mays	HSP17.2	67	X65725	Jorgensen and Nguyen (1994)
Oryza sativa	HSP16.9a	72	M80938	Tzeng et al. (1992)
Oryza sativa	HSP16.9b	72	M80939	Tzeng et al. (1992)
Oryza sativa	HSP16.9c	71	U81385	Huang et al. (1996)
Triticum aestivum	HSP16.9a	65	X64618	Weng et al. (1995)
Hordeum vulgare	HSP17.0a	67	Y07844	Slocombe et al. (1996)
Hordeum vulgare	HSP17.0b	60	X64560	Marmiroli et al. (1993)
Triticum aestivum	HSP16.9b	67	P12810	McElwain and Spiker (1989)
Agrostis stolonifera var. palustris	HSP16.5	60	AF007762	Park and Luthe (1998)
Chenopodium rubrum	HSP18.3	64	Q05832	Knack et al. (1992)
Helianthus annuus	HSP17.7	72	U46545	Coca et al. (1996)
Helianthus annuus	HSP17.6	69	X59701	Almoguera and Jordano (1992)
Dancus carota	HSP17.8	72	X53851	Darwish et al. (1991)
Dancus carota	HSP17.9	70	X53852	Darwish et al. (1991)
Glycine max	HSP17.5a	80	M11318	Nagao et al. (1985)
Glycine max	HSP17.6	76	M11317	Nagao et al. (1985)
Glycine max	HSP18.5	74	X07160	Raschke et al. (1988)
Glycine max	HSP17.5b	77	P04794	Czarnecka et al. (1985)
Glycine max	HSP17.3	78	P02519	Schoffl et al. (1984)
Medicago sativa	HSP18.2	78	X58711	Gyorgyey et al. (1991)
Pisum sativum	HSP18.1	78	M33899	Lauzon et al. (1990)
Lycopersicum esculentum	HSP17.7	100	X56138	Fray et al. (1990)
Lycopersicum esculentum	HSP17.8	98	This study	
Lycopersicum esculentum	HSP17.6	98	This study	
Lycopersicum peruvianum	HSP17.8	94	AJ225047	Forreiter and Loew (1998)
Lycopersicum peruvianum	HSP17.6	95	AJ225048	Forreiter and Loew (1998)
Lycopersicum peruvianum	HSP17.7	92	AJ225046	Forreiter and Loew (1998)

Table III (cont'd)

Oryza sativa	HSP17.3b	65	M80186	Tseng et al. (1992)
Oryza sativa	HSP17.8	62	X75616	Lee et al. (1995b)
Oryza sativa	HSP18.0	65	U83670	Guan et al. (1998)
Oryza sativa	HSP17.3a	67	U83669	Guan et al. (1998)
Oryza sativa	HSP17.4	67	D12635	Nishi et al. (1992)
Oryza sativa	HSP17.7	65	U83671	Guan et al. (1998)
Pennisetum glaucum	HSP17.9	69	X94193	Caven et al. (1996)
Helianthus annuus	HSP18.6	69	U46544	Coca et al. (1996)
Nicotiana tabaccum	HSP18.0	72	X70688	Zarsky et al (1995)
Cuscuta japonica	HSP17.6	73	AB017273	Yamada (1998)
Arabidopsis thaliana	HSP17.4	68	X17293	Takahashi and Komeda (1989)
Brassica rapa	HSP17.6	69	AF022217	Kim and Jo (1997)
Arabidopsis thaliana	HSP17.6	71	X16076	Helm and Vierling (1989)
Arabidopsis thaliana	HSP18.2	72	X17295	Takahashi and Komeda (1989)
Fragaria ananassa	HSP17.4	70	U63631	Medina-Escobar et al. (1998)
Pseudotsuga menziesii	HSP18.2a	68	X92983	Kaukinen et al. (1996)
Pseudotsuga menziesii	HSP18.2b	67	X92984	Kaukinen et al. (1996)

Figure 6. Amino acid sequence comparison among the cytosolic class I smHSPs. Consensus amino acid sequence is showed in bold face. The secondary structure of the protein is indicated as predicted by the PHDsec method (www.embl-heidelberg.de/predictprotein/predictprotein.html; Rost and Sander, 1994). In addition, the secondary structure of a small heat shock protein from *Methanococcus jannaschii*, a hyperthermophilic archaeon, is showed based on its crystal structure (Kim et al., 1998). Helices are indicated with red box while the β-strands are presented as green arrows. The putative cAMP protein kinase phosphorylation site of tomato homologues is underlined in blue. In addition the consensus I and II domains are underlined. The amino acids in the consensus domain II responsible for substrate recognition are in red. Gaps within the alignment were introduced to optimize the alignment. The DNA and amino acid sequences of the cytosolic class I small heat-shock proteins were obtained from the GeneBank. Accession Numbers are listed in Table III. Amino acid sequences were aligned using CLUSTAL V method in DNASTAR.

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PgHSP16.9 MSLV........RRGN.VFDPFSMD.LW.DPFDNM.FRS.......IVPS...SSSSDTAAFANARIDW
PgHSP17.0 MSLV........SRSS.VFDPFSMD.LW.DPFDSM.FRS.......IVQSAG.SPDSDTAAFAAARIDW
ZmHSP17.2 MSLV........RRSN.VFDPFSMD.LW.DPFDTM.FRS.......IVPSAT.STNSETAAFASARIDW
OsHSP16.9 MSLV........RRSN.VFDPFSLD.LW.DPFDSV.FRS.......VVPA...TSDNDTAAFANARIDW
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OsHSP16.9 MSLV........RRSN.VFDPFA.D.FW.DPFDGV.LRS.......LVPA...TSDRDTAAFANARVDW
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HvHSP17.0 MSIV........RRSN.AFDPFA.D.LWADPFDT..FRS.......IVPAFS.GN.SETAAFANARVDW
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GmHSP18.5 MSLIP..NFFGGRRNN.VFDPFSLD.VW.DPFKDFPF..PNTLSSAS...FPEFSR.ENSAFVSTRVDW
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PsHSP18.1 MSLIP..SFFSGRRSN.VFDPFSLD.VW.DPLKDFPF.SNSSP.SAS...FPR....ENPAFVSTRVDW
LeHSP17.7 MSLIP..RIFGDRRSSSMFDPFSID.VF.DPFRELGFPSTNSG.............ESSAFANTRIDW
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LeHSP17.6 MSLIP..RIFGDRRSSSMFDPFSID.VF.DPFRELGFPGTNSG.............ESSAFANTRIDW
LpHSP19.9 MSLIP..RIFGDRRSSSMFDPFSID.VF.DPFRELGFPGTNSG.............ETSAFANTRIDW
LpHSP20.1 MSLIP..RIFGDRRSSSMFDPFSID.VF.DPFRELGFPGTNSR.............ETSAFANTRIDW
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OsHSP17.4 MSMI........RRSN.VFDPFSLD.LW.DPFDGFPFGSG......SGSLFPRANS.DAAAFAGARIDW
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FaHSP17.4 MAL.S..LFGNSRRSN.VFDPFSLD.TW.DPFQGFGPLMN...........SSSTAGDTSAFAQTRIDW
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PmHSP18.2 MSIIP..SFFG.RRSSSAFDPFSLD.VW.DPFRAFTDLAAGGPSGQFVN........EASAIANTQIDW
Consensus MS                FDPF  D    DP                                  DW
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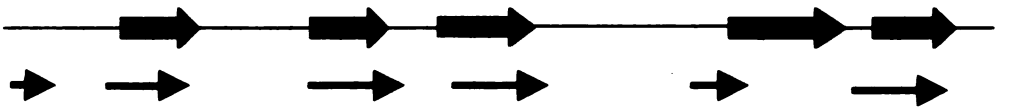

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AsHSP16.5 KETP.EAHVFKADLPGVKKEEVKVEVEGG.NVLVVSGER.KGEGGQERQVATLERSSGKFVRRFRLPE
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FaHSP17.4 KETP.EAHVFKADLPGLKKEEVKVELEEG.NVLQISGERSKEQEEKNDKWHRVERSSGKFVRRFRLPD
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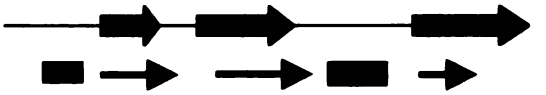

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OsHSP16.9 NAKVDQVKAGLENGVLTVTVPKAEV.KKPEVKAIEISG    151
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TaHSP16.9 DAKVEEVKAGLENGVLTVTVPKAEV.KKPEVKAIEISG    152
AsHSP16.5 NAKVEEVKAGLENGVLTVTVPKAEV.KKPEVKAIEISG    151
CrHSP18.3 NAKVDQVKAGMENGVLTVTVPKNEA.PKPQVKAINVYE    163
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HaHSP17.6 NAKMDEVKAMMENGVLTVVVPKEEEEKKPMVKAIDISG    154
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BcHSP17.6 NAKVDEVKASMENGVLSVTVPKMAE.RKPEVKSIDISG    155
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FaHSP17.4 NAKVDQVKAAMENGVLTVTVPKAPE.PKPQVKSIDISGA   154
PmHSP18.2 NAKVEEIKAAMENGVLTVTVPKQPEPQPPQPKSIEISG    159
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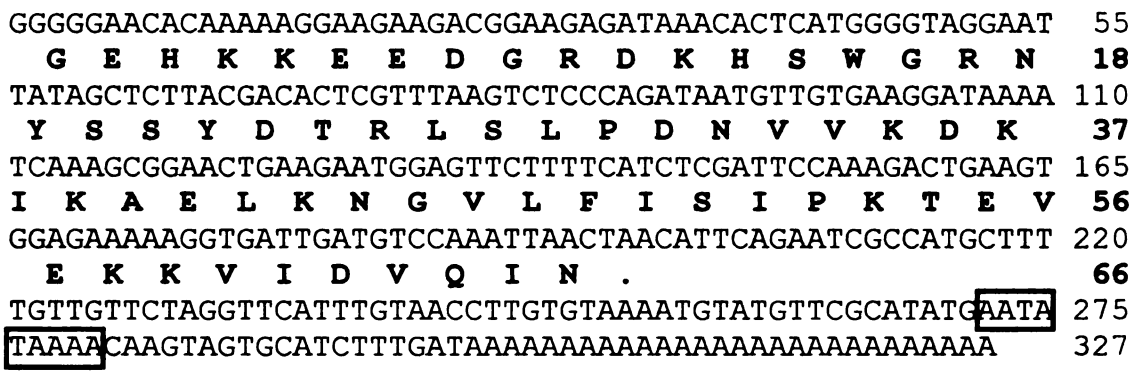

Figure 7. Nucleotide and deduced amino acid sequences of the *LeHSP21.0* cDNA (*LeHCT5*). Nucleotides and amino acids are represented as normal and bold letters, respectively. The putative polyadenylation signal is boxed.

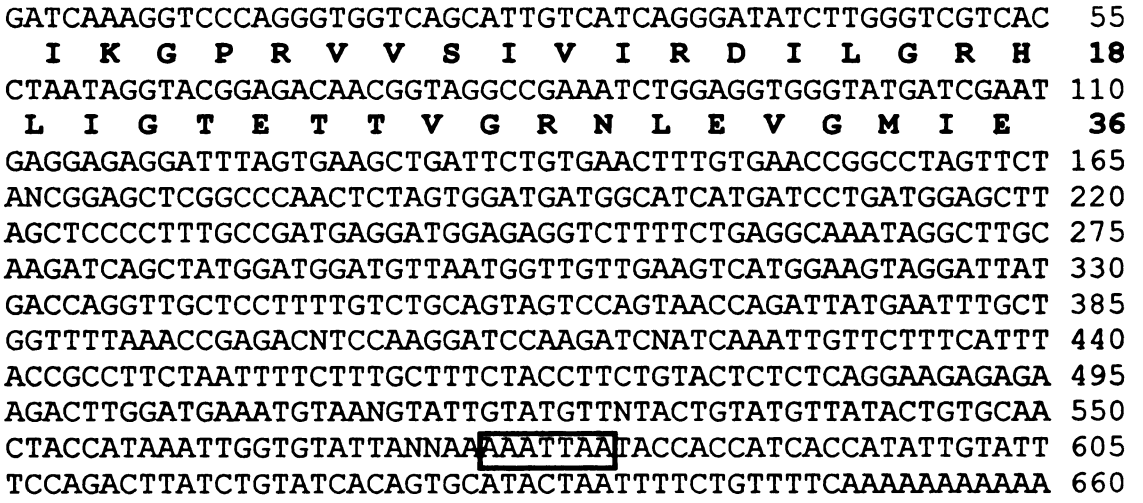

Figure 8. Nucleotide and deduced amino acid sequences of the putative *LeHSP22.0* cDNA (*LeHCT6*). Nucleotides and amino acids are represented as normal and bold letters, respectively. The putative polyadenylation signal is boxed.

The nucleotide and deduced amino acid sequence of the *LeHSP90* are shown on Figure 9. This partial cDNA of *LeHSP90* contain an ORF of 1202 bp encoding a predicted polypeptide of 406 amino acids. Comparison of the predicted amino acid sequence between *Le*HSP90 and the other higher plants HSP90s reveals sequence identities ranging from 82% (*Le*HSC80) to 92% (*Nicotiana tabacum* HSP82) (Table IV). The amino acid alignment of *Le*HSP90 with the some of the cytosolic HSP90s is presented in Figure 10.

The eukaryotic HSP90s were dissected by proteolysis into three independently folded domains, the N-terminus domain 1-236, the middle domain 272-615 and the C-terminal domain 621-730 (Nemoto et al., 1997; Stebbins et al., 1997). *Le*HSP90 is lacking the N-terminal domain and part of the middle domain. The ~25-kDa N-terminal domain (underlined in Figure 10) was identified as the binding site for the benzoquinoid ansamycin drug geldanamycin (Stebbins et al., 1997) and for ATP (Grenert et al., 1997; Prodromou et al., 1997). The putative ATP-binding sites GXXGXG are marked blue. This domain was shown to have a chaperone activity by binding to unfolded polypeptides and preventing aggregation *in vitro* (Young et al., 1997; Scheibel et al., 1998). The middle domain is located between the two highly charged domains of the protein (marked green). In this domain the *Le*HSP90 contains a putative leucine zipper motif (marked red), which may be involved in the specificity and stability of hetero- or homodimers. The 12kD C-terminal domain (double underlined in Figure 10) has been shown to bind to partially folded proteins in an ATP-independent way potentially regulated by cochaperones (Young et al., 1997; Scheibel et al., 1998). In addition, the ~200 C-terminal residues of HSP90, including the C-terminal domain and part of the middle

Figure 9. Nucleotide and deduced amino acid sequences of the *LeHSP90* cDNA. Nucleotides and amino acids are represented as normal and bold letters, respectively. The differential display *LeHCT7* nucleotide sequence is underlined.

```
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  L  F  V  P  K  R  A  P  F  D  L  F  D  T  R  K  K  M   36
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  N  N  I  K  L  Y  V  R  R  V  F  I  M  D  N  C  E  E   54
ACTTATCCCTGAGTACCTTGGATTCGTGAAGGGTGTTGTTGACTCTGATGATTT  212
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  V  I  K  D  I  L  G  D  K  V  E  K  V  V  V  S  D  R  270
GATTGTTGATTCTCCATGTTGCTTAGTGACAGGTGAGTATGGTTGGACAGCTAA  848
  I  V  D  S  P  C  C  L  V  T  G  E  Y  G  W  T  A  N  288
CATGGAAAGGATCATGAAAGCTCAAGCTTTGAAGGACAATAGCATGAGCTCTTA  901
  M  E  R  I  M  K  A  Q  A  L  K  D  N  S  M  S  S  Y  306
CATGTCTAGCGAGAAGACAATGGAAATCAACCCTGATAATGGCATTGTGGAGGA  954
  M  S  S  E  K  T  M  E  I  N  P  D  N  G  I  V  E  E  324
GTTGAGGAAGAGAGCTGAAGTTGACAAGAATGACAAGTCGGTGAAAGATCTTGT 1007
  L  R  K  R  A  E  V  D  K  N  D  K  S  V  K  D  L  V  342
GCTGCTGCTGTTTGAGACAGCTTTGCTAACATCTGGTTTTAGTCTTGATGACCC 1060
  L  L  L  F  E  T  A  L  L  T  S  G  F  S  L  D  D  P  360
GAATACATTTGCTGCAAGAATTCATAGAATGCTGAAGTTGGGTTTGAGCATTGA 1113
  N  T  F  A  A  R  I  H  R  M  L  K  L  G  L  S  I  D  378
CGAAGAAGAGGAAGCTGGTGTGGATGTTGATGATATGCCTCCTCTGGAGGATGT 1169
  E  E  E  E  A  G  V  D  V  D  D  M  P  P  L  E  D  V  396
TGGTGAGGAAAGCAAGATGGAAGAAGTGGACTAATCATTGAAATCAGTTGAGAC 1222
  G  E  E  S  K  M  E  E  V  D  .                        406
TTTTGAGATGCGTGAAAATACAATTTGAGCGTGTTGCTTTTTTTCTCATCTTGT 1275
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GTCTAGTATAGTTTTTTTTTTTAGGCAAGAAAAGCTGTTCAATCAAAATGATCA 1328
ATTAACAAAGGTTGGCATATTACATTGAAAGTAACTTTAGTTTCATGGGTGTCA 1381
ACTGGGACTTTGGACCAAAGCCTTATGCAACTAACCCTTCACAACAACCTGAAG 1434
TACTCACAATTACAATTACAACATAGTGTTGATAGACAAAAGGTAAAGAAGTGA 1487
TAAAACAATTATCCAACTGTATATTGGAGAATTGCTTAGTTGCTGCCACCGCCG 1540
AAGAGATAACCCAAAGAAGATCCACCTCCAGGATTTGCCATAGG 1610

Table IV. HSP90 Accession Numbers

HSP90 (organisms)	Protein	Sequence Similarity %	Accession Number	Reference
Arabidopsis thaliana	*At*HSP81	88	P27323	Yabe et al. (1994)
Arabidopsis thaliana	*At*HSP82	88	D00710	Takahashi and Komeda (1991)
Arabidopsis thaliana	*At*HSP83	88	M62984	Conner et al. (1990)
Arabidopsis thaliana	*At*HSP90	86	Y07613	Milioni and Hatzopoulos (1997)
Lycopersicum esculentum	*Le*HSP90	100	This study	
Nicotiana tabacum	*Nt*HSP82	92	X63195	Severin et al. (1991)
Oryza sativa	*Os*HSP82	84	Z11920	Van Breusegem et al. (1994)
Pharbitis(Ipomoea) nil	*Pn*HSP90	91	M99431	Felsheim and Das (1992)
Zea mays	*Zm*HSP82	86	S59780	Mars et al. (1993)
Lycopersicum esculentum	*Le*HSC80	82	M96549	Koning et al. (1992)

Figure 10. Amino acid sequence comparison among the HSP90s. Consensus amino acid sequence is shown in bold face. The ATP and geldanamacin-binding domain are underlined and the ATP-binding region is in blue. The leucine zipper motif is shown in red. The C-terminal domain responsible for oligomerization activity is double underlined. In green are the K/E rich regions. Gaps within the alignment were introduced to optimize the alignment. The DNA and amino acid sequences of the HSP90 were obtained from the GeneBank. Accession Numbers are listed in Table IV. Amino acid sequences were aligned using CLUSTAL V method in DNASTAR.

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AtHSP81 MA..DVQMA...DAETFAFQAEINQLLSLIINTFYSNKEIFLRELISNSSD
AtHSP82 MA.........DAETFAFQAEINQLLSLIINTFYSNKEIFLRELISNSSD
AtHSP83 MA..DVQMA...DAETFAFQAEINQLLSLIINTFYSNKEIFLRELISNSSD
AtHSP90 MA..DVQMA...DAETFAFQAEINQLLSLIINTFYSNKEIFLRELISNSSD
LeHSP90 ..................................................
NtHSP82 ..................................................
OsHSP82 MAS.........ETETFAFQAEINQLLSLIINTFYSNKEIFLRELISNSSD
PnHSP90 MA..DVQMA...EAETFAFQAEINQLLSLIINTFYSNKEIFLRELISNASD
ZmHSP82 MASADVHMAGGAETETFAFQAEINQLLSLIINTFYSNKEIFLRELISNASD
LeHSC80 MS.........DVETFAFQAEINQLLSLIINTFYSNKEIFLRELISNSSD
                    ETFAFQAEINQLLSLIINTFYSNKEIFLRELISN SD

AtHSP81 ALDKIRFESLTDKSKLDGQPELFIRLVPDKSNKTLSIIDSGIGMTKADLVN
AtHSP82 ALDKIRFESLTDKSKLDGQPELFIHIIPDKTNNTLTIIDSGIGMTKADLVN
AtHSP83 ALDKIRFESLTDKSKLDGQPELFIRLVPDKANKTLSIIDSGIGMTKADLVN
AtHSP90 ALDKIRFESLTDKSKLDGQPELFIRLVPDKPNKTLSIIDSGIGMTKADLVN
LeHSP90 ..................................................
NtHSP82 ..................................................
OsHSP82 ALDKIRFESLTDKSKLDAQPELFIHIVPDKASNTLSIIDSGIGMTKSDLVN
PnHSP90 ALDKIRFESLTDKSKLDAQPELFIRLVPDKTNKTLSIIDSGVGMAKADLVN
ZmHSP82 ALDKIRFESLTDKSKLDAQPELFIRLVPDKASKTLSIIDSGVGMTKSDLVN
LeHSC80 ALDKIRFESLTDKSKLDGQPELFIHIIPDKANNTLTIIDSGIGMTKADLVN
        ALDKIRFESLTDKSKLD QPELFI   PDK   TL IIDSG GM K DLVN

AtHSP81 NLGTIARSGTKEFMEALQAGA.DVSMIGQFGVGFYSAYLVAEKVVVTTKHN
AtHSP82 NLGTIARSGTKEFMEALAAGA.DVSMIGQFGVGFYSAYLVADKVVVTTKHN
AtHSP83 NLGTIARSGTKEFMEALQAGA.DVSMIGQFGVGFYSAYLVAEKVVVTTKHN
AtHSP90 NLGTIARSGTKEFMEALQAGA.DVSMIGQFGVGFYSAYLVAEKVVVTTKHN
LeHSP90 ..................................................
NtHSP82 ..................................................
OsHSP82 NLGTIARSGTKEFMEALAAGA.DVSMIGQFGVGFYSAYLVAERVVVTTKHN
PnHSP90 NLGTIARSGTKEFMEALQAGA.DVSMIGQFGVGFYSAYLVAEKVIVTTKHN
ZmHSP82 NLGTIARSGTKEFMEALAAGATDVSMIGQFGVGFYSAYLVADRVMVTTKHN
LeHSC80 NLGTIARSGTKEFMEALAAGA.DVSMIGQFGVGFYSAYLVAEKVVVTTKHN
        NLGTIARSGTKEFMEAL AGA DVSMIGQFGVGFYSAYLVA  V VTTKHN

AtHSP81 DDEQYVWESQAGGSFTVTRDVDGEPLGRGTKITLFLKDDQLEYLEERRLKD
AtHSP82 DDEQYVWESQAGGSFTVTRDTSGETLGRGTKMVLYLKEDQLEYLEERRLKD
AtHSP83 DDEQYVWESQAGGSFTVTRDVDGEPLGRGTKISLFLKDDQLEYLEERRLKD
AtHSP90 DDEQYVWESQAGGSFTVTRDVDGEPLGRGTKISLFLKDDQLEYLEERRLKD
LeHSP90 ..........................................EY........
NtHSP82 ..................................................
OsHSP82 DDEQYVWESQAGGSFTVTRDTSGEQLGRGTKITLYLKDDQLEYLEERRLKD
PnHSP90 DDEQYIWESQAGGSFTVTRDVDGEQLGRGTKITLFLKEDQLEYLEERRIKD
ZmHSP82 DDEQYVWESQAGGSFTVTHDTTGEQLGRGTKITLFLKDDQLEYLEERRLKD
LeHSC80 DDEQYVWESQAGGSFTVTRDTSGENLGRGTKMVLYLKEDQLEYLEERRLKD
        DDEQYVWESQAGGSFTVTRD  GE LGRGTK  L LK DQ EY EERRLKD
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AtHSP81 LVKKHSEFISYPIYLWIEKTTEKEISDDEDEDEPKKENEGEVEEVDEEKEK
AtHSP82 LVKKHSEFISYPISLWIEKTIEKEISDDEEEEEKKD.EEGKVEEVDEEKEK
AtHSP83 LVKKHSEFISYPIYLWTEKTTEKEISDDEDEDEPKKENEGEVEEVDEEKEK
AtHSP90 LVKKHSEFISYPIYLWTEKTTEKEISDDEDEDEPKKENEGEVEEVDEKKEK
LeHSP90 ..................................................
NtHSP82 ......EFISYPIYLWTEKTTEKEISDDED.DEPKKDEEGAVEEVDEDKEK
OsHSP82 LIKKHSEFISYPISLWTEKTTEKEISDDEDEEEKKDAEEGKVEDVDEEKEE
PnHSP90 LVKKHSEFISYPIYLWTEKTTEKEISDDED.DEPKKEEEGDIEEVDEDKEK
ZmHSP82 LVKKHSEFISYPIYLWTEKTTEKEISDDEEEEDNKKEEEGDVEEVDDEDKD
LeHSC80 LIKKHSEFISYPISLWVEKTIEKEISDDEEEEEKKD.EEGKVEEVDEEKEK
        L KKHSEFISYPI LW EKT EKEISDDE     K   EG  E VD

AtHSP81 D...GKKKKKIKEVSHEWELINKQKPIWLRKPEEITKEEYAAFYKSLTNDW
AtHSP82 E...EKKKKKIKEVSHEWDLVNKQKPIWMRKPEEINKEEYAAFYKSLSNDW
AtHSP83 D...GKKKKKIKEVSHEWELINKQKPIWLRKPEEITKEESAAFYKSLTNDW
AtHSP90 D...GKKKKKIKEVSHEWELINKQKPIWLRKPEEITKEEYAAFYKSLTNDW
LeHSP90 ..................................................
NtHSP82 E...KGKKKKIKEVSHEWQLINKQKPIWLRKPEEITKDEYASFYKSLTNDW
OsHSP82 K...EKKKKKIKEVSHEWSLVNKQKPIWMRKPEEITKEEYAAFYKSLTNDW
PnHSP90 E...GKKKKKIKEVSHEWQLINKQKPIWLRKPEEITKEEYASFYKSLTNDW
ZmHSP82 TKDKSKKKKKVKEVSHEWVQINKQKPIWLRKPEEITRDEYASFYKSLTNDW
LeHSC80 E...EKKKKKVKEVSNEWSLVNKQKPIWMRKPEEITKEEYAAFYKSLTNDW
             KKKKK KEVS EW   NKQKPIW RKPEEI   E A FYKSL NDW

AtHSP81 EDHLAVKHFSVEGQLEFKAILFVPKRAPFDLFDTRKKLNNIKLYVRRVFIM
AtHSP82 EEHLAVKHFSVEGQLEFKAILFVPKRAPFDLFDTKKKPNNIKLYVRRVFIM
AtHSP83 EDHLAVKHFSVEGQLEFKAILFVPKRAPFDLFDTRKKLNNIKLYVRRVFIM
AtHSP90 EDHLAVKHFSVEGQLEFKAILFVPKRAPFDLFDTRKKLNNIKLYVRRVFIM
LeHSP90 ...LAVKHFSVEGQLEFKAILFVPKRAPFDLFDTRKKMNNIKLYVRRVFIM
NtHSP82 EEHLAVKHFSVEGQLEFKAILFVPKRAPFDLFDTRKKMNNIKLYVRRVFIM
OsHSP82 EEHLAVKHFSVEGQLEFKAVLFVPKRAPFDLFDTRKKLNNIKLYVRRVFIM
PnHSP90 EDHLAVKHFSVEGQLEFKAILFVPKRAPFDLFDTRKKMNNIKLYVRRVFIM
ZmHSP82 EDHLAVKHFSVEGQLEFKAILFVPRRAPFDLFDTRKKLNNIKLYVRRVFIM
LeHSC80 EEHLAVKHFSVEGQLEFKAVLFVPKRAPFDLFDTKKKPNNIKLYVRRVFIM
        E HLAVKHFSVEGQLEFKA LFVPKRAPFDLFDT KK NNIKLYVRRVFIM

AtHSP81 DNCEELIPEYLSFVKGVVDSDDLPLNISRETLQQNKILKVIRKNLVKKCIE
AtHSP82 DNCEDIIPEYLGFVKGIVDSEDLPLNISRETLQQNKILKVIRKNLVKKCLE
AtHSP83 DNCEELIPEYLSFVKGVVDSDDLPLNISRETLQQNKILKVIRKNLVKKCIE
AtHSP90 DNCEELIPEYLSFVKGVVDSDDLPLNISRETLQQNKILKVIRKNLVKKCIE
LeHSP90 DNCEELIPEYLGFVKGVVDSDDLPLNISREMLQQNKILKVIRKNLVKKCIE
NtHSP82 DNCEELIPEYLGFVKGVVDSDDLPLNISREMLQQNKILKVIRKNLVKKCIE
OsHSP82 DNCEELIPEWLSFVKGIVDSEDLPLNISREMLQQNKILKVIRKNLVKKCVE
PnHSP90 DNCEELIPEYLGFVKGVVDSDDLPLNISREMLQQNKILKVIRKNLVKKCIE
ZmHSP82 DNCEELIPEWLGFVKGVVDSDDLPLNISRETLQQNKILKVIRKNLVKKCIE
LeHSC80 DNCDELIPEYLSFVKGIVDSEDLPLNISRETLQQNKILKVIRKNLVKKCVE
        DNC  LIPE L FVKG VDS DLPLNISRE LQQNKILKVIRKNLVKKC
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*At*HSP81 MFNEIAENKEDYTKFYEAFSKNLKLGIHEDSQNRGKIADLLRYHSTKSGDE
*At*HSP82 LFFEIAENKEDYNKFYEAFSKNLKLGIHEDSQNRTKIAELLRYHSTKSGDE
*At*HSP83 MFNEIAENKEDYTKFYEAFSKNLKLGIHEDSQNRGKIADLLRYHSTKSGDE
*At*HSP90 MFNEIAENKEDYTKFYEAFSKNLKLGIHEDSQNRGKIADLLRYHSTKSGDE
*Le*HSP90 MFNEIAENKEDYNKFYEAFSKNLKLGIHEDSQNRAKLADFLRYQSTQECDE
*Nt*HSP82 MFNEIAENKEDYNKFYEAFSKNLKLGIHEDSQNRAKLADLLRYHSTKSGDE
*Os*HSP82 LFFEIAENKEDYNKFYEAFSKNLKLGIHEDSTNRNKIAELLRYHSTKSGDE
*Pn*HSP90 MFNEIAENKDDYNKFYEAFSKNLKLGIHEDSQNRAKLADLLRYYSTKSGDE
*Zm*HSP82 MFFEIAENKDDYAKFYDAFSKNIKLGIHEDSQNRAKLADLLRYHSTKSGDE
*Le*HSC80 LFFEIAENKEDYNKFYEAFSKNLKLGIHEDSQNRAKFAELLRYHSTKSGDE
F EIAENK DY KFYEAFSKNLKLGIHEDS NR K A LRY ST DE

*At*HSP81 MTSFKDYVTRMKEGQKDIFYITGESKKAVENSPFLERLKKRGYEVLYMVDA
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*At*HSP83 MTSFKDYVTRMKEGQKDIFYITGESKKAVENSPFLERLKKRGYEVLYMVDA
*At*HSP90 MTSFKDYVTRMKEGQKDIFYITGESKKAVENS.FLERLKKRGYEVLYMVDA
*Le*HSP90 LTSLKDYVTRMKEVQKDIYYITGESKKAVENSPFLERLKKKGYEVLFMVDA
*Nt*HSP82 MTSLKDYVTRMKEGQKDIYYITGESKKAVENSPFLERLKKKGYEVLYMVDA
*Os*HSP82 LTSLKDYVTRMKEGQNDIYYITGESKKAVENSPFLEKLKKKGYEVLYMVDA
*Pn*HSP90 LTSLKDYVTRMKEGQKDIYYITGESKKAVENSPFLERLKKKGYEVLFMVDA
*Zm*HSP82 TTSLKDYVTRMKEGQKDIYYITGESRKAVENSPFLERLKKKGYEVLFMVDA
*Le*HSC80 MTSLKDYVTRMKEGQNDIYYITGESKKAVENSPFLEKLKKKGYEVLYMVDA
S KDYVTRMKE Q DI YITGES KAVENSPFLE LKKKG EVL MVDA

*At*HSP81 IDEYAVGQLKEYDGKKLVSATKEGLKLEDETEEE.KKKREEKKKSFENLCK
*At*HSP82 IDEYAIGQLKEFEGKKLVSATKEGLKLDETEDE..KKKKEELKEKFEGLCK
*At*HSP83 IDEYAVGQLKEYDGKKLVSATKEGLKLEDETEEE.KKKREEKKKSFENLCK
*At*HSP90 IDEYAVGQLKEYDGKKLVSATKEGLKLEDETEEE.KKKREEKKKSFENLCK
*Le*HSP90 IDEYAIGQLKEYDGKKLVSVTKEGLKLDDESEEE.KKKKEEKKQSFESLCK
*Nt*HSP82 IDEYAVGQLKEYDGKKLVSATKEGLKLDDDSEEE.KKKKEEKKKSFENLCK
*Os*HSP82 IDEYAVGQLKEFEGKKLVSATKEGLKLDESEDE..KKRKEELKEKFEGLCK
*Pn*HSP90 IDEYAVGQLKEYDGKKLVSATKEGLKLEDDDEEE.KKKREEKKKSFENLCK
*Zm*HSP82 IDEYAVGQLKEYDGKKLVSATKEGLKLDDEDDEEAKKRREERKKRFEELCK
*Le*HSC80 IDEYSIGQLKEFEGKKLVSATKEGLKLDESEDE..KKKQEELKEKFEGLCK
IDEY GQLKE GKKLVSATKEGLKL E KK EE K FE LCK

*At*HSP81 TIKEILGDKVEKVVVSDRIVDSPCCLVTGEYGWTANMERIMKAQALRDSSM
*At*HSP82 VIKDVLGDKVEKVIVSDRVVDSPCCLVTGEYGWTANMERIMKAQALRDSSM
*At*HSP83 TIKEILGDKVEKVVVSDRIVDSPCCLVTGEYGWTANMERIMKAQALRDSSM
*At*HSP90 TIKEILGDKVEKVVVSDRIVDSPCCLVTGEYGWTANMERIMKAQALRDSSM
*Le*HSP90 VIKDILGDKVEKVVVSDRIVDSPCCLVTGEYGWTANMERIMKAQALKDNSM
*Nt*HSP82 IIKDILGDKVEKVVVSDRIVDSPCCLVTGEYGWTANMERIMKAQALRDSSM
*Os*HSP82 VIKEVLGDKVEKVVVSDRVVDSPCCLVTGEYGWTANMERIMKAQALRDSSM
*Pn*HSP90 IIKDILGDKVEKVVVSDRIVDSPCCLVTGEYGWTANMERIMKAQALRDSSM
*Zm*HSP82 VIKDILGDRVEKVVVSDRIVDSPCCLVTGEYGWTANMERIMKAQALRDSSM
*Le*HSC80 VMKDVLGDKVEKVIVSDRVVDSPCCLVTGEYGWTANMERIMKAQALRDSSM
K LGD VEKV VSDR VDSPCCLVTGEYGWTANMERIMKAQAL D SM

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AtHSP81 SGYMSSKKTMEINPDNGIMEELRKRAEADKNDKSVKDLVMLLYETALLTS
AtHSP82 AGYMSSKKTMEINPENSIMDELRKRADADKNDKSVKDLVLLLFETALLTS
AtHSP83 SGYMSSKKTMEINPDNGIMEELRKRAEADKNDKSVKDLVMLLYETALLTS
AtHSP90 SGYMSSKKTMEINPDNGIMEDLRKRAEADKNDKSVKDLVMLLYETALLTS
LeHSP90 SSYMSSEKTMEINPDNGIVEELRKRAEVDKNDKSVKDLVLLLFETALLTS
NtHSP82 SSYMSSKKTMEINPDNGIMEELRKRAEADKNDKSVKDLVLLLFETALLTS
OsHSP82 AGYMSSKKTMEINPENAIMEELRKRADADKNDKSVKDLVLLLFETALLTS
PnHSP90 SSYMSSKKTMEINPDNGIMEELRKRAEADKNDKSVKDLVLLLFETALLTS
ZmHSP82 SAYMSSKKTMEINPDNGIMEELRKRAEADRNDKSVKDLVLLLFETALLTS
LeHSC80 AGYMSSKKTMEINPENSIMDELRKRADADKNDKSVKDLVLLLFETALLTS
        YMSS KTMEINP N I  LRKRA  D NDKSVKDLV LL ETALLTS

AtHSP81 GFSLDEPNTFAARIHRMLKLGLSIDEDENVE.EDGDMPELEE..DAAEES
AtHSP82 GFSLDEPNTFGSRIHRMLKLGLSIDDDDAVEAD.AEMPPLED.DADAEGS
AtHSP83 GFSLDEPNTFAARIHRMLKLGLSIDEDENVE.EDGDMPELEE..DAAEES
AtHSP90 GFSLDEPNTFAARIHRMLKLGLSIDEDENVE.EDGDMPELEE..DAAEES
LeHSP90 GFSLDDPNTFAARIHRMLKLGLSIDEEEEAGVDVDDMPPLED...VGEES
NtHSP82 GFSLDDPNTFAARIHRMLKLGLSIDEEEE.AVEDADMPALEE...TGEES
OsHSP82 GFSLDDPNTFGSRIHRMLKLGLSIDEDETAEAD.TDMPPLED...DAGES
PnHSP90 GFSLDDPNTFGARIHRMLKLGLSIDEEEA.G.DDADMPALEE..EAGEES
ZmHSP82 GFSLDDPNTFAARIHRMLKLGLNIDEDAAAD.EDADMPALDE..GAAEES
LeHSC80 GFSLEEPNTFGNRIHRMLKLGLSIDEESG.DAD.ADMPALEDPEADAEGS
        GFSL  PNTF  RIHRMLKLGL ID          DMP L        S

AtHSP81 KMEEVD
AtHSP82 KMEEVD
AtHSP83 KMEEVD
AtHSP90 KMEEVD
LeHSP90 KMEEVD
NtHSP82 KMEEVD
OsHSP82 KMEEVD
PnHSP90 KMEEVD
ZmHSP82 KMEEVD
LeHSC80 KMEEVD
        KMEEVD
```

domain, is involved in HSP90 dimerization (Minami et al., 1994; Nemoto et al., 1995; Wearch and Nicchita, 1996). Recently, the C-terminal domain of the HSP90 was demonstrated to be necessary and sufficient for interaction with the tetratricopeptide repeat (TPR)-containing cofactor proteins, including the immunophilins FKBP52 and CyP-40 (Young et al., 1998). Finally, a typical carboxyl-terminal pentapeptide MEEVD, common in cytosolic HSP90 homologues from both plant and animals, was observed in the *Le*HSP90.

Expression Patterns of the smHSPs, HSP90s and Ripening Related Genes.

Mature green tomato fruits heat-treated at 42°C for 2 days were transferred to 2°C for 14 days, and then were subsequently raised to 20°C. Heat shock at 42°C for 2 days caused an increase in the expression of the cytosolic smHSPs members but had little or no effect on the expression of the chloroplastic smHSP (*LeHCT5*) and the *LeHSP90* (Figure 11). Interestingly, the developmentally regulated *LeHSC80* transcript and the fruit ripening *LeE8* transcript were decreased to low or undetectable levels, respectively. Storing the heat-treated fruits at 2°C for 2 weeks caused a strong induction of the cytosolic class I smHSPs, the chloroplastic smHSP, the *LeHSP90* as well as the *LeHSC80* transcripts; mRNA levels of the cytosolic class II smHSPs were unaffected. In contrast, the mRNA levels of *LeE8* were increased slowly indicating that low temperature stimulated its expression. Increasing the temperature from 2 to 20°C resulted in a decrease of steady-state levels of the smHSP (cytosolic class I and II as well as chloroplastic) and the *LeHSP90* after 3 days. However, the levels of the *LeHSC80* were slightly decreased

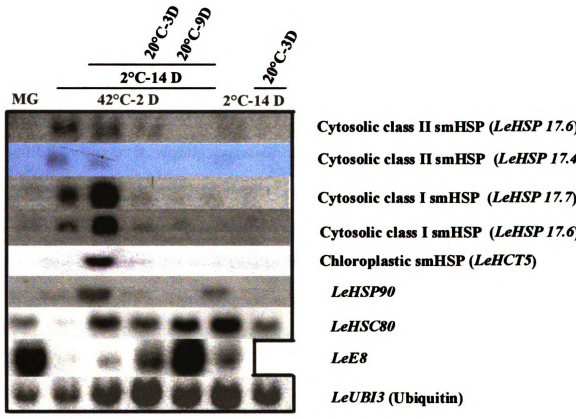


Figure 11. The expression patterns of the tomato smHSPs, HSP90s and E8 are affected by heat treatment and low temperature storage of the fruits. RNA was extracted from fruits harvested at mature green (MG) stage. These fruits were heat-treated at 42°C for 2 days and then the temperature was lowered to 2°C for 14 days. After the low temperature storage, the fruits were transferred to 20°C for 3 or 9 days. Another set of mature green fruits was transferred directly to 2°C for 14 days and then to 20°C for 3 days. Dr. Comai (University of Washington, Seattle) and Dr. Fischer (University of California, Berkley) kindly provided the *LeHSC80* and the *LeE8* specific probes, respectively.

after 3 days at 20°C but recovered after 9 days. The steady-state levels of *LeE8* increased when the fruits were transferred to a permissive ripening temperature and after 9 days at 20°C and were higher than those at the mature green stage indicating that the ripening process had recovered from the imposed stress.

When the mature green tomato fruits were transferred to 2°C, the expression of the smHSPs differed from that of the heat-treated fruits. For instance, low temperature did not stimulate the expression of the cytosolic class I smHSP and the chloroplastic smHSP, whereas it slightly increased the levels of cytosolic class II smHSP. Interestingly, low temperature elevated the expression of the HSP90s members. However, the low temperature induced expression of the *LeHSP90* mRNA was lower than the expression of the same gene in the heat-treated tomatoes stored at the same temperature and period. The *E8* expression was down-regulated by low temperatures, but at a lower rate than observed in fruits from the combination of heat- and low temperature treatments.

The sustained strong induction of the smHSPs and HSP90s at low temperature after the heat treatment strongly suggests that chaperone participation is needed to prevent chilling injury at reduced temperatures.

Immunodetection of the smHSP and HSP90 in Heat-Treated Cold Stored Tomato Fruit.

To test whether the cytosolic class I and II smHSP as well as the HSP90 proteins accumulated at low temperatures after the heat treatment, we used antibodies for cytosolic class I HSP17.6 from *Arabidopsis thaliana* (Helm and Vierling, 1989), for cytosolic class II HSP17.8 from wheat (Vierling, unpublished) and for HSP83 from *Pharbitis nil* (Krishna et al., 1997). Proteins were extracted from mature green fruits that have been heated at 42°C for 2 days, from heat and nonheated fruits stored at 2°C for 2 weeks or transferred at 20°C for 3 days and from fruits kept at 20°C for 9 days.

Immunodetection analyses indicate that the cytosolic class I and II smHSP as well as the HSP90 proteins are present in the heat-treated fruits at low temperatures (Figure 12). More importantly, these proteins were still detectable when the fruits were transferred to room temperature for 3 days after the cold period. Low temperatures had no effect on the expression of the smHSP and the HSP90. During fruit ripening the expression of these were low or undetectable.

The cytosolic class I antiserum from *Arabidopsis thaliana* cross-reacted with at least two tomato homologues in one-dimensional SDS-PAGE. The size was determined to be 18.3 and 17.6kD, respectively. The cytosolic class II antibody from wheat recognized a tomato protein with molecular mass of 17.8kD, but the cross-reactivity was poor, suggesting that an antibody raised for the *Le*HSP17.6 is necessary to accurately determine the expression of this protein. The antibody raised against the C-terminus of the *Pn*HSP83a (Felsheim and Das, 1992) detected a tomato homologue with expected size of 83.5kD.

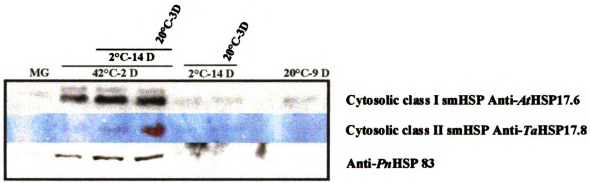


Figure 12. Immunodetection of cytosolic small HSPs and HSP90 protein levels in heat-treated/cold stored tomato fruits. Total soluble proteins were extracted from mature green (MG) tomato fruits heat-treated at 42°C for 2 days. The temperature was then lowered to 2°C and fruits were held for 14 days. After the low temperature storage, the fruits were transferred to 20°C for 3 days. Another set of mature green fruits was transferred directly to 2°C for 14 days and then to 20°C for 3 days. Fruits kept at 20°C for 9 days were the ripening controls. Western blots were performed using the cytosolic class I and II smHSP antibodies from *Arabidopsis* and wheat, respectively, as well as the *Pharbitis nil* HSP83 antiserun. *At*HSP17.6 and *Ta*HSP17.8 antibodies were kindly provided by Dr. Vierling (University of Arizona, Tuscon) and the *Pn*HSP83 antibody was provided by Dr. Krishna (University of Western Ontario, London, Canada).

Expression Patterns of the Tomato smHSPs and HSP90s Genes under Different Heat treatment Regimes.

To examine further the heat-shock proteins transcript accumulation during different regimes of heat shock and subsequent low temperature storage, mature green fruits were kept at 42°C for 12, 24, 36 and 48 h. The fruits were then transferred to 2°C for 14 days or room temperature for 1 to 3 days. RNA was extracted at the specific times and analyzed for the heat-shock proteins transcript accumulation.

Northern analyses indicate that the cytosolic class I, cytosolic class II, the *Le*HSP90 and the *Le*HSC80 mRNA are rapidly induced after 12 h exposure to 42°C (Figure 13). Similarly, the chloroplastic smHSP is induced by heat shock (Figure 14B). The RNA levels for the cytosolic smHSPs declined slowly as the heat shock continued. In contrast, the HSP90s and the chloroplastic smHSP mRNA decreased rapidly to almost undetectable levels after 36 h at 42°C. The differentiation of the cytosolic smHSP versus the HSP90 mRNA accumulation during heat shock suggests that the cytosolic smHSPs mRNA is more stable at prolonged high temperatures than the chloroplastic smHSP and the HSP90s mRNAs.

After holding fruits at 20°C for 1 or 3 days after heat shock, the cytosolic class II and the *LeHSP90* mRNA levels declined rapidly to insignificant levels regardless the heat shock period. The cytosolic class I smHSP transcript decreased slowly and it was present after 3 days at 20°C. The decline of the cytosolic class I transcript at 20°C was more rapid for the moderate heat shock than for severe heat shock, indicating that the mRNA was more stable under prolonged heat shock. Contrary to the *LeHSP90* pattern, the

developmentally regulated *LeHSC80* mRNA recovered from the heat shock and reached the same levels as the control fruits after 3 days at 20°C. The recovery of the *LeHSC80* mRNA was independent from the duration of the heat shock. Surprisingly, the putative mitochondrial smHSP (*HCT6*) was constitutively expressed at very low levels. The fruit ripening related gene E8 mRNA virtually disappeared after 36 h of high temperatures

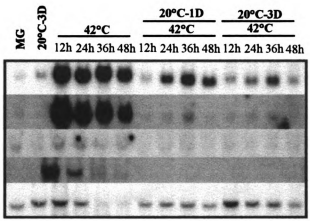
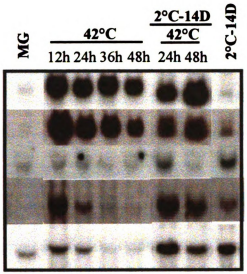


Figure 13. The effect of heat shock on the level of transcripts for the smHSPs and HSP90s in tomato fruits. Mature green fruits were heat-treated at 42°C for 12, 24, 36 and 48 h and then were transferred to 20°C for 1 or 3 days. Mature green tomatoes were also kept at 20°C for 3 days. Total RNA was prepared from the pericarp tissue and the transcript level for cytosolic class I *LeHSP17.7*, cytosolic class II *LeHSP17.4*, mitochondrial *LeHSP22*, *LeHSP90* and *LeHSC80* were analyzed as described in the text.

Figure 14. The effect of heat treatment on smHSPs, HSP90 and E8 mRNA levels in tomato fruits subsequently stored at 2°C for 14 days. A. Mature-green (MG) fruits were heat-treated at 42°C for 12, 24, 36 and 48 h. The 24 and 48 h heat-treated and the nonheated fruits were transferred to 2°C for 14 days. Total RNA was extracted and analyzed for the cytosolic class I *LeHSP17.7*, cytosolic class II *LeHSP17.4*, mitochondrial *LeHCT6*, *LeHSP90* and *LeHSC80* gene expression. B. Mature green fruits were kept at 20°C for 3, 6, and 9 days or heat-treated at 42°C for 12, 24, 36 and 48 h. The heat-treated and the nonheated fruits were transferred to 2°C for 14 days. After the low temperature storage, the heat-treated and nonheated fruits were shifted to 20°C for 3 days. Total RNA was extracted and analyzed for cytosolic class I smHSP (*LeHSP17.7*), chloroplastic smHSP (*HCT5*), *LeHSP90* and *LeE8* gene expression.

A



Cytosolic class I smHSP *LeHSP 17.7*

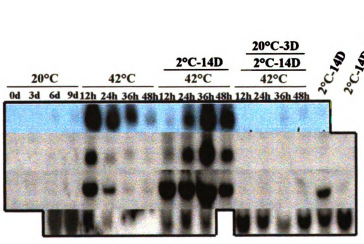
Cytosolic class II smHSP *LeHSP 17.4*

Mitochondrial smHSP *LeHSP 22*

LeHSP 90

LeHSC 80

B



Cytosolic class I smHSP *Le HSP 17.7*

Chloroplastic smHSP *Le HSP 21*

Le HSP 90

Le E8

(Figure 14B), suggesting that the fruit ripening and especially the ethylene-dependent responses were down-regulated.

In contrast to fruits held at ambient temperature, when the heat-treated fruits were exposed to low temperatures, the mRNA for the cytosolic classes I and II smHSPs, *Le*HSP90, *Le*HSC80 (Figure 14A) and chloroplastic smHSP (Figure 14B) were reinduced. However, low temperature exposure of nonheated fruit, had no effects on the expression of the cytosolic class I smHSP (Figure 14A) and chloroplastic smHSP (Figure 14B) mRNA while the cytosolic class II smHSP was slightly induced. Interestingly, low temperatures regulated the putative mitochondrial smHSP (Figure 14A).

Collectively, the results suggest that some of the HSP genes, which are not cold inducible, are regulated by low temperatures only if they have been induced by heat shock. Surprisingly, the reinduction of the smHSP mRNAs at low temperatures was dependent on the length of the heat shock prior to cold storage. For instance, fruits that received 36 h or 48 h heat shock accumulate more smHSPs mRNA than fruits that received high temperatures for 12 or 24 h.

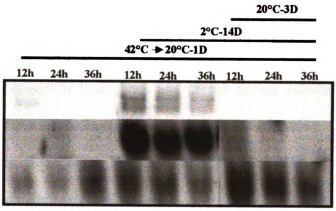
Although the *LeHSP90* mRNA was initially induced by heat shock (Figure 13), the *LeHSP90* was also regulated by low temperatures (Figure 14A and 14B). Interestingly, the heat-treated fruits had a higher *LeHSP90* transcript level than the nonheated fruits at low temperature storage. Similarly to *LeHSP90*, low temperatures stimulated *LeHSC80* mRNA accumulation. The cold-induced expression of HSP genes

in the heat-treated fruits raises the possibility that both high- and low molecular weight HSPs may play critical roles in resistance to chilling stress.

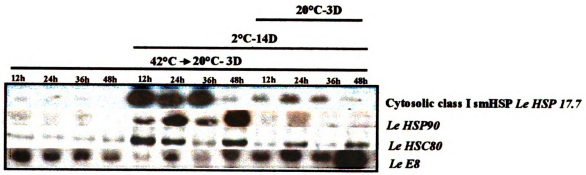
In addition, we examined whether exposure of heat-treated fruits to ambient temperature affects the subsequent accumulation of the HSP transcripts under low temperature. Mature green fruits were exposed to 42°C for 12, 24, 36 or 48 hours, transferred to 20°C for 1 or 3 days, then shifted to 2°C for 14 days and moved back to ambient temperatures for 3 days. Unexpectedly, exposure of heat-treated fruits for 1 or 3 days at 20°C did not prevent the reinduction of HSPs at low temperature (Figure 15A and B). Nevertheless, these fruits developed chilling injury. This was particularly the case for the fruits which were kept at ambient temperature for 3 days. These data suggest that heat-shock proteins are not the only factors that contribute to the chilling tolerant phenotype of tomatoes. Holding the tomatoes at 20°C for 3 days after heat shock and before storage allowed ripening to recover and proceed. During the recovery period, the fruit produced ethylene (Lurie and Klein, 1992; Whitaker, 1994) and the ripening related genes, like *LeE8*, were restored (Figure 15A and B).

Figure 15. The effect of heat treatment and post-treatment at 20°C on smHSPs, HSP90 and E8 mRNA levels in tomato fruits subsequently stored at 2°C for 14 days. A. Heat-treated fruits at 42°C for 12, 24, and 36 h kept at 20°C for 1 day and then transferred to 2°C for 14 days. The fruits were shifted to 20°C for 3 days after the low temperature storage. Total RNA was extracted and analyzed for the cytosolic class II *LeHSP17.4*, *LeHSP90* and *LeE8* gene expression. B. Heat-treated fruits at 42°C for 12, 24, 36, and 48 h were kept at 20°C for 3 days and then transferred to 2°C for 14 days. After the low temperature storage the fruits were shifted to 20°C for 3 days. Total RNA was extracted and analyzed for the cytosolic class I *LeHSP17.7*, *LeHSP90*, *LeHSC80* and *LeE8* gene expression.

A


20°C-3D
2°C-14D
42°C → 20°C-1D
12h 24h 36h 12h 24h 36h 12h 24h 36h
Cytosolic class II smHSP Le HSP17.4
Le HSP 90
Le E8

B


20°C-3D
2°C-14D
42°C → 20°C- 3D
12h 24h 36h 48h 12h 24h 36h 48h 12h 24h 36h 48h
Cytosolic class I smHSP Le HSP 17.7
Le HSP90
Le HSC80
Le E8

DISCUSSION

We hypothesized that heat-shock proteins (HSP) may be involved in increasing tolerance to chilling injury following heat treatment. This was based on using differential display of mRNA, to clone and characterize a cytosolic class II small heat-shock protein (*Le*HSP17.6) (Kadyrzhanova et al., 1998). To elucidate if other heat-shock proteins are induced by heat shock and persist during subsequent storage at low temperatures, we cloned a second member of the cytosolic class II smHSP, three cDNAs encoding a cytosolic class I smHSP and partial cDNA clones for a chloroplastic smHSP, a mitochondrial smHSP and a heat-inducible HSP90 member.

*Le*HSP17.4 is similar to the previous cytosolic class II HSP17.6 isolated from tomato fruit (Kadyrzhanova et al., 1998). There was no difference in the expression pattern of the two cytosolic class II smHSPs in Northern analysis conducted with gene-specific probes. Both genes are induced by heat shock and the mRNAs are more stable as the heat persists. Interestingly, the transcripts were detected after the heat-treated fruits were stored at 2°C for 2 weeks. However, when the fruits were chilled at 2°C without prior heat treatment, the mRNA accumulation was low but still detectable, indicating a weak stimulation by cold. Cytosolic class II smHSPs have been induced in response to several conditions including, ozone (Eckey-Kaltenbach, et al., 1997), gamma irradiation and H_2O_2 application (Banzet et al., 1998), different light regimes (Krishna et al., 1992), water stress (Almoguera et al., 1993), treatment with amino acid analogues (Lee et al., 1996), heavy-metal stress (Czarnecka et al., 1984), embryogenesis (Coca et al., 1994; DeRocher and Vierling, 1994; Dong and Dunstan, 1996) and pollen

development (Bouchard, 1990; Dietrich et al., 1991; Atkinson et al., 1993; Kobayashi et al., 1994). Fruit ripening appears to have no role in the expression of cytosolic class II smHSP.

Polyclonal antibodies raised against *Ta*HSP17.8 recognized tomato cytosolic class II homologues. The pattern of the protein expression followed the mRNA patterns and the proteins were still detectable at 20°C 3 days after the cessation of the low temperature storage. This suggests that these proteins may be important in the repair of damage incurred during the heat stress or subsequent cold stress. The pea cytosolic class II smHSP are components of large complexes *in vitro* and *in vivo*, and are larger than those of the class I smHSP complexes (Helm et al., 1997). It has been suggested that the quaternary structure of class II complexes are different from those of the class I complexes (Helm et al., 1997). Secondary structure protein prediction suggests that the two classes might have similar structure with a few differences. Although it has been proposed that cytosolic class II functions as a molecular chaperone (Lee et al., 1995a; Helm et al., 1997), we do not have any evidence of chaperone activity *in vivo* or *in vitro*, particularly at low temperatures.

In addition to the cytosolic class II smHSP, we have isolated and characterized three cDNAs that encode members of the cytosolic class I smHSP family. The first gene (*LeHSP17.7*) was identical to the *pTOM66* gene previously isolated as a fruit ripening related gene (Fray et al., 1990; Sabehat et al., 1998a). The other two have 97% similarity at the amino acid level. The mRNA levels of the *LeHSP17.7* and *LeHSP17.6* were induced by high temperature and the transcripts were detected in fruit during subsequent low temperature storage. Interestingly, the expression of these genes was not detected in

fruits exposed to low temperatures only. These data are in agreement with the results reported by (Sabehat et al., 1996; 1998a) where the induction of HSP17 mRNA during the heat treatment and translation of HSP17 protein persisted during subsequent storage of the fruit at 2°C.

The expression of the cytosolic class I smHSPs were distinguished from the cytosolic class II smHSPs only in the fact that they are induced during fruit ripening in tomatoes (Picton and Grierson, 1988; Rothan et al., 1997; Sabehat et al., 1998a) and strawberries (Medina-Escobar et al., 1998). Cytosolic class I smHSP was also induced in the CO_2-treated tomato fruit (Rothan et al., 1997) and perhaps may be required to protect proteins against cellular acidosis induced by CO_2 solubilization. In many plants, the cytosolic class I smHSP is expressed during development. For example, cytosolic class I mRNA and its protein accumulated during embryo development (Vierling and Sun, 1989; Almoguera and Jordano, 1992; Coca et al., 1994; DeRocher and Vierling, 1994; zurNieden et al., 1995; Wehmeyer et al., 1996; Carrango et al., 1997), seed germination (Vierling and Sun, 1989; Kaukinen et al., 1996; Wehmeyer et al., 1996), somatic embryogenesis (Gyorgyey et al., 1991; Zarky et al., 1995).

Western analysis indicates that the *At*HSP17.6 polyclonal antibody cross-react with at least two cytosolic class I smHSP from tomato fruit. The expression pattern of proteins mimics the transcript pattern and the results obtained with cytosolic class II homologues. Interestingly the protein presence in fruits at 20°C after cold storage suggesting that the class I smHSP may protect other proteins during recovery from the stress. Cytosolic class I smHSP from pea has been shown to have chaperone activity *in vitro* (Lee et al., 1995a; Lee et al., 1997) by protecting citrate synthase against thermal

aggregation in an ATP-independent fashion. Interestingly, Forreiter et al. (1997), using a stable transformed *Arabidopsis* cell suspension culture overexpressing luciferase as a reporter gene, demonstrated that the cytosolic class I *At*HSP17.6 showed chaperone activity *in vivo* by preventing the thermal inactivation of the reporter protein. Moreover the crystal structure of the *Mj*HSP16.5 has been reported (Kim et al., 1998) and it forms a hollow spherical complex, where certain protein or RNAs critical for the cell survival may reside in under stress conditions. Several observations, suggests that the consensus region II of the "heat shock domain" are not necessary only for oligomerization but also for chaperone activity (Merck et al., 1993; Plater et al., 1996; Leroux et al., 1997; Yeh et al., 1997).

Another cDNA isolated was a chloroplastic smHSP identical to pTOM111 (Lawrence et al., 1997; Sabehat et al., 1998a). The mRNA accumulation profile was similar to cytosolic class I smHSP. In addition, similar results by Sabehat et al. (1998a) indicate that the protein was detected at low temperatures only in the heat-treated fruit.

In parallel with the identification of small HSPs, which were induced by heat shock and persisted at low temperatures, we isolated a partial cDNA that encodes a member of HSP90 family. The HSP90 family is composed of genes encoding structurally related proteins ranging from 80 to 90kD (Scheibel and Buchner, 1997). HSP90 genes have been isolated from several plant species, including *Arabidopsis* (Conner et al., 1990; Yabe et al., 1994, Milioni and Hatzopoulos, 1997), barley (Walther-Larsen et al., 1993), *Brassica napus* (Krishna et al., 1995), *Catharanthus roseus* (Schroder et al., 1993), maize (Marrs et al., 1993), *Pharbitis nil* (Felsheim and Das, 1992), rye (Schmitz et al., 1996), and tomato (Koning et al., 1992). *Le*HSP90 is

predicted to be in the cytosol and has 82% similarity with the *Le*HSC80 at the amino acid level. The *LeHSC80* is expressed constitutively at high levels at physiological temperatures (Koning et al., 1992) and induced only slightly by heat shock in tomato fruits. The *LeHSP90* however is not expressed at ambient temperatures, but the expression is enhanced strongly by heat shock. The induction of both genes appears to be transient. The maximum level of HSP90 mRNA is achieved after 12 h of HS followed by a gradual decline, a phenomenon called autoregulation of the heat shock response (Schoffl and Key, 1982; Gurley and Key, 1991). The inhibition of the HSP90 expression by prolonged heat shock is distinguished from smHSP autoregulation; it appears that HSP90 mRNAs are less stable at high temperatures (Gallie et al., 1995). Another explanation of the negative regulation of the HSP90s is the observation that HSP90 is a major repressor of the HSF in mammalians cells (Ali et al., 1998; Zou et al., 1998).

In addition, we found that both HSP90s are induced by low temperatures. Similar results have been reported by Krishna et al. (1995). Using a polyclonal antibody raised against *Pn*HSP83 (Krishna et al., 1997) we detected a cross-reacting protein from tomato. The protein expression pattern indicates that the HSP90s are detectable after 2 days at 42°C. The level of the protein in the heat-treated fruit is high at low temperatures and continues to be expressed at 20°C for 3 days. In contrast, cold had little effect in HSP90 expression of nonheated fruit. The role of the HSP90 at low temperatures is unknown.

HSP90 is the most abundant chaperone (Jakob and Buchner, 1994). HSP90 is referred to as "molecular glue" in the cytoplasm of mammalian cells (Csermely et al., 1998). HSP90 binds to a wide range of proteins including, kinases (Miyata and Yahara, 1992), phosphatases (Chen et al., 1996), nuclear hormone receptors (Pratt, 1997), actin

(Czar et al., 1997), tubulin (Fostinis et al., 1992), calmodulin (Minami et al., 1993), proteasome (Tsubuki et al., 1994) and heat shock transcription factor (Nadeau et al., 1993). HSP90 also forms a large cytosolic complex, named as foldosome, with other molecular chaperones such as HSC70, immunophilins, CDC37 and p23 (Pratt, 1993).

Recent studies (Young et al., 1997; Scheibel et al., 1998) demonstrated that HSP90 has two independent chaperone sites, the N-terminal and the C-terminal site. The N-terminal chaperone site can be inhibited by geldanamycin (Whiteshell et al., 1994). HSP90 displays a heat-induced chaperone activity at 46°C (Yonehara et al., 1996). However, recent studies in yeast suggest that HSP90 is required for a specific subset of proteins having difficulties reaching their native conformation (Nathan et al., 1997). Moreover, under stress conditions, HSP90 enhances the rate at which a heat-damaged protein is reactivated (Forreiter et al., 1997; Nathan et al., 1997) and does not protect the proteins from thermal inactivation.

One of the possible models for the mechanism by which heat treatment attenuates chilling injury may be attributed to molecular chaperone activities of HSPs (Boston et al., 1996). Molecular chaperones are a group of intracellular proteins that ensures correct folding, oligomeric assembly, transport across membranes or disposal by degradation of other conformer unstable proteins by binding to them and releasing them. Additionally, molecular chaperones prevent incorrect interaction within and between non-native polypeptides which result in their irreversible aggregation (Hartl, 1996). The cytosolic class I smHSP prevent thermal aggregation by selectively binding to non-native proteins forming soluble high molecular weight complexes (Lee et al., 1997). These complexes were stable even after storage at 4°C (Lee et al., 1997). However, we do not know if the

complexes are formed at low temperatures. If so, expression of smHSP during low temperature will be beneficial to chilling-sensitive plants like tomatoes. Therefore, demonstration of chaperone activity of heat-shock proteins at low temperatures is necessary.

The combination of the heat treatment and low temperature storage revealed an interesting observation. The expression of the smHSPs mainly was induced at low temperatures only if the fruits have been exposed to heat shock. The reinduction of the transcripts in the cold was more favorable after prolonged exposure to high temperatures. To our knowledge similar phenomena have not been reported in plant or other organisms. Perhaps the HSFs are able to bind to the promoter regulatory elements and switch on the transcription of the smHSP in the cold. This is consistent with the observation that the *Drosophila* heat shock factor had little trimer dissociation and loss of DNA binding activity when incubated at 4°C after a heat shock period (Zhong et al., 1998). Furthermore, exposure to low temperature may activate new cold-inducible transcription factors that reactivate smHSP gene transcription. Transcription factors that bind to the putative STRE element (Marchler et al., 1993), found in the promoter of the cytosolic class II HSP17.6, might be a good candidates.

The heat shock induced protection against chilling injury was transient. Fruits transferred to ripening temperatures after exposure to heat shock and before chilling lost the heat shock induced protection within several days (Lurie and Sabehat, 1997). Unpredictably, exposure of heat-treated fruits for 1 or 3 days at 20°C did not prevent the reinduction of HSPs at low temperature. Collectively, these data suggest that heat-shock proteins are not the only factors that contribute to the chilling tolerant phenotype of

tomatoes. In agreement, Guy et al. (1986) suggested that the heat-induced freezing tolerance of *Neurospora crassa* spores did not involve synthesis of heat-shock proteins, since the presence of cycloheximide during heat shock did not diminish the protection that heat shock afforded against freezing injury.

Indeed, heat treatments induce the synthesis of heat-shock proteins and interrupts or down-regulates the expression of genes involved in fruit ripening metabolism. The latter is likely to be responsible for the cross-protection, whereas the former is mainly involved in thermotolerance. The difference in the behavior to chilling sensitivity between heat-treated fruit and fruit that recovered from heat shock lies on the ability of the latter to synthesize and perceive ethylene the ripening hormone (Theologis, 1992). The notion that heat-induced chilling tolerance is due to discontinuation of the fruit ripening metabolism is supported by the following observation. Antisense-ACC oxidase melon fruits were resistant to chilling injury (JC Pech, personal communication). When the same fruits were treated with ethylene, the susceptibility to chilling injury was restored indicating that ethylene insensitivity is important for chilling tolerance. Thus, in order to promote chilling insensitivity not only heat-shock proteins but also ethylene insensitivity may be required.

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CHAPTER IV

IDENTIFICATION OF THE GENOMIC SEQUENCE OF *LeHSP17.6* AND ITS *CIS*-ACTING ELEMENTS

ABSTRACT

The *LeHSP17.6* gene encodes a cytosolic class II smHSP of tomato. *LeHSP17.6* is accumulated massively in tomato fruits exposed to heat shock and slightly by low temperatures. The combination of the heat treatment and low temperature storage revealed an interesting observation. The expression of the *LeHSP17.6* was induced at low temperatures only if the fruits have been exposed to heat shock. The reinduction of the transcripts in the cold was more favorable after continued exposure to high temperatures. We have cloned an extended 5' flanking region of the *LeHSP17.6* in order to identify cis-acting elements involved in the regulation of this gene, particularly during the reinduction in the cold after the heat shock treatment. A detailed analysis of the *LeHSP17.6* promoter region revealed multiple heat shock elements, stress response elements, GATA elements and long tracts of repetitive sequences with high AT content. The possible function of these sequences is discussed.

INTRODUCTION

Plants are bound to their habitat, they can not run away from many threatening environmental and anthropogenic stressors, and therefore need special mechanism to avoid stress or to adapt to stress (Lichtenthaler, 1998). Cells pre-exposed to mild non-damaging stress conditions will induce resistance/tolerance not only to the factor used, but also tolerance against severe stress caused by other agents. This is referred as the cross-protection or cross-resistance mechanism. For example, a mild heat shock will protect the plants against severe heat shock, a process called thermotolerance (Nover, 1991; Vierling, 1991). In addition, it will induce resistance against other environmental stresses (Bonham-Smith et al, 1987; Orzeck and Burke, 1988; Kuznetsov et al., 1997; Sabehat et al., 1998b). The induction of the heat shock response can protect cells against a variety of other toxic insults, such as ethanol and hydrogen peroxide (Ruis and Shuller, 1995; Storz and Polla, 1996).

Heat and anoxia stress treatments are being used in postharvest to induce resistance to chilling injury (Lurie and Klein, 1991; Pesis et al., 1994). Protection of tomatoes from chilling injury afforded by prestorage heat treatment has been correlated with the induction of transcription of smHSPs mRNA during the heat treatment and translation of the smHSPs which persisted during subsequent storage of the fruit at 2°C (Sabehat et al., 1996; 1998a; Kadyrzhanova et al., 1998). Using differential display of mRNA we cloned a full-length *HCT*1 cDNA that encodes a 17.6 kD cytosolic class II smHSP (Kadyrzhanova et al., 1998). This gene is expressed in tomato fruits exposed to high temperatures. Further, this gene is expressed in massive amounts at low

temperatures only if the fruits have been exposed to heat shock (see chapter III). The same expression pattern has been reported for the other tomato smHSP (Sabehat et al., 1998a; Chapter III).

The purpose of this study was to isolate and characterize the genomic DNA sequence of the cytosolic class II smHSP *LeHSP17.6* and investigate the role of the upstream sequence element in the heat required cold expression of this gene.

MATERIAL AND METHODS

Genomic Library Screening

The tomato genomic library, provided by Dr. J. Giovanonni of Texas A&M Univ., was used. This library harbors approximately ten genomic equivalents and was constructed in lambda DASHII (Stratagene) from *L. esculentum* (cv. Alisa Craig) genomic DNA. Approximately $5x10^5$ phages from a tomato genomic library were screened with a ^{32}P-labeled probe corresponding to the *LeHSP17.6* coding region using standard procedures (Sambrook et al., 1989). Single hybridizing phages containing a 7-kb tomato DNA insert were isolated, subcloned and approximately 3kb was sequenced.

DNA Sequencing

DNA sequence determination was performed on Applied Biosystems 373A (Foster City, CA) at the MSU Instrumentation Facility. Nucleotide sequence data were assembled and analyzed using DNA STAR (DNA STAR, Madison, WI).

Promoter Analysis

Analysis of transcription factor-binding sites on the *LeHSP17.6* promoter was performed using the TESS transcription element search software of J. Schug and G.C. Overton (http://agave.humgen.upenn.edu/tess/index.html, University of Pennsylvania, Philadelphia) or by screening the databases on transcriptional regulation TRANSFAC, TRRD, and COMPEL (Heinemeyer et al., 1998).

RESULTS

The tomato genomic library, was screened to obtain the genomic sequence of *LeHSP17.6*. A single hybridizing phage containing a 7kb pair tomato DNA insert was isolated. The DNA sequence of the ~3kb from the genomic clone was determined. The genomic clone of the *LeHSP17.6* exactly matches the sequence of *LeHSP17.6* cDNA, indicating that is an intronless gene. In addition, it contains a 1.095kb fragment of the promoter region as well as 1.0kb of the 3' end region.

The promoter sequence of *LeHSP17.6* is presented in Figure 1. The transcription-starting site was predicted to be 78 nucleotide upstream of the start codon ATG. The sequence at this site, CTCACTG closely resembles the consensus plant transcription initiation sequence, CTCATCA (Joshi, 1987). A putative TATA box, TATATAA, about 30 bp upstream from this site (position -110). Table I summarizes the potential transcription binding sites and cis-acting elements in the *LeHSP17.6* promoter. Several

copies of the HSE (heat shock element), AGAAn or nTTCT, are found at position -120 to -129, -153 to -174, -279 to -287, -301 to -317, -880 to -891 and -905 to -915. In addition there are three copies of the putative STRE (stress response element), CCCCT, at positions -92 to 96, -254 to 259, and -615 to 620. Moreover there are six NIT2 motifs, TTGATA or its complement TATCAA, at the positions -257 to -263, -271 to -277, -326 to -332, -337 to -343, -722 to -728 and -985 to -991. Furthermore, the promoter contains a G-box (-181 to -185), a Cd responsive element (-693 to -698), a salicylic acid inducible element (-487 to -495), a GAGA element (-174 to -178), a CCAAT or Y-box element (-291 to -297) and two REa element, the first overlaps with the Y-box at position -294 to -299 and the second is located in the 5' UTR of the gene at position -56 to -62. In addition, the 5' flanking region of the tomato cytosolic class II smHSP include runs of simple sequences, $(A)_n$, $(T)_n$ and $(TA)_n$ preceeding the HSEs and an AT-rich upstream region.

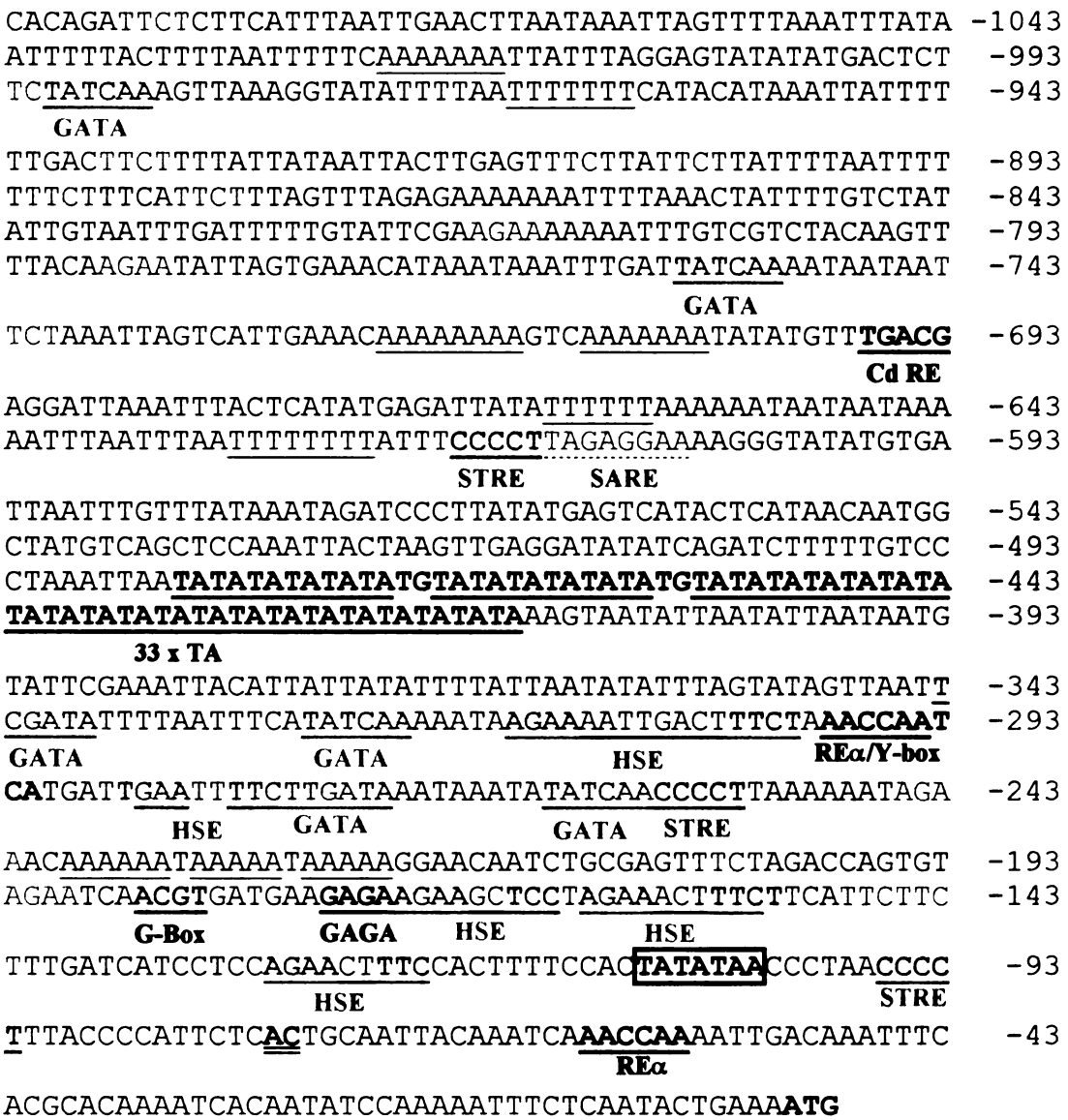

Figure 1. Nucleotide sequence of tomato cytosolic class II smHSP *LeHSP17.6* gene. Upstream sequences from the start codon are assigned negative numbers. The TATA sequence is bold boxed. Important sequences are bold underlined: HSE-like promoter sequences (in red), STRE (in blue), GATA-1 like elements (in magenta), G-box (in green), TA-runs, REa-like elements, CCAAT or Y-box and the GAGA factor. The start of the 5' untranslated region is double underlined.

Table I. Potential binding sites for transcription factors and *cis*-acting elements in the *LeHSP17.6* gene promoter. The nucleotides identical to those reported sequences are shown in bold face

Abbreviation	Function	Sequence	Position	Reference
HSE	Heat shock response	**AGAAn**	-120/129, -153/174, -279/287, -301/317, -880/891, -905/915	Barros et al. (1992)
G-box	ABA response, environmental stimuli	**ACGT**	-181/185	Marcotte et al. (1989)
STRE	Stress response element	**CCCCT**	-92/96, -254/259, -615/620	Marchler et al. (1993)
AT- Box		**poly (AT)**	-416/484	Czarnecka et al. (1992)
REa	Phytochrome regulation, repression of transcription in the dark	**AACCAA**	-56/62 -294/299	Degenhardt and Tobin (1996)
GATA (I-box, NIT2)	Light regulation, Nitrogen regulation	**TTGATA**	-257/263 -271/277 -326/332 -337/343 -722/728 -985/991	Terzaghi and Cashore (1995); Fu and Marzluf (1990)
TGACG	Cd responsive element		-693/698	Kusaba et al. (1996)
GAGA element		**GAGA**	-174/178	
SARE	SA inducible element	**CTTAGAGGAA**	-607/617	Goldsbrough et al. (1993)

DISCUSSION

The genomic sequence of the cytosolic class II smHSP *LeHSP17.6* has been determined; it is an intronless gene. One common feature that the cytosolic small HSPs genes have, is the absence of an intron within the protein-coding region (Gurley and Key, 1991). These heat shock genes may have lost the introns in the optimization of expression under temperature stress conditions. A brief and severe heat shock has been shown to interrupt the splicing and translation of *Drosophila hsp83* mRNA (Yost and Lindquist, 1986), suggesting a selective advantage for intronless heat shock genes under extreme temperature stress conditions (Lindquist, 1986).

Promoter regions of cytosolic class II smHSPs genes have been reported from maize (Dietrich et al., 1991), *Pharbitis nil* (Krishna et al., 1992) and soybean (Raschke et al., 1988). The conserved features within the cytosolic class II smHSP gene promoters are the occurrence and the relatively strict spacing of the TATA box (20 to 30 bps from the transcription start site), the transcription start site and the first proximal HSE. Next, I discuss the most important elements observed in the *LeHSP17.6* promoter.

HSE (Heat Shock Element)

The expression of the heat shock genes in plants is regulated primarily at the transcription level. Induction of the heat shock genes requires a conserved *cis*-acting element, termed as heat shock element (HSE), located within the promoter region of these genes (Pelham, 1982). HSEs consists of repeated units of the 5-bp sequence

nGAAn arranged in alternating orientation (Amin et al, 1988; Xiao and Lis, 1988) and recently refined as AGAAn (Fernandes et al., 1994). The number of pentameric units in an HSE can vary from three to eight (Fernandes et al., 1994). Furthermore, the degree of homology of each pentameric unit to the consensus AGAAn motif can vary (Bonner et al., 1994). The nature of the initial pentamer, GAA or its complement TTC, is also varied. The nTTCT pentamer displayed higher biological activity in yeast and it was capable of binding two HSF trimers instead of one (Bonner et al., 1994). In addition, HSE can tolerate 5 bp insertion between repeating units, provided that the spacing and the orientation of the pentameric units are maintained (Amin et al., 1988).

The first demonstration that the plants shared the highly conserved mode of promoter activation was seen in the heat-inducible transcription of the *Drosophila HSP70* promoter in transgenic tobacco callus and plants (Spena et al., 1985; Spena and Schell, 1987). In plants the optimal HSE core consensus was shown to be 5'-aGAAg-3' (Barros et al., 1992). The promoter regions of the small heat-shock proteins genes have multiple copies of HSE-like elements within 150 bp upstream from the TATA box sequence (Schoffl et al., 1984; Nagao et al., 1985; Raschke et al., 1988). Analyses of 5' and 3' promoter deletions of the cytosolic class I *GmHSP17-3B* in transgenic tobacco demonstrated the functional significance of HSEs for the heat shock dependent transcription of the native genes or fused reporter genes (Baumann et al., 1987; Schoffl et al., 1989). Similar results have been observed by deletion mutagenesis of the cytosolic class I *Gmhsp17.5*-E promoter indicating that the TATA-proximal HSEs contribute to the abundance of the thermal induction (Nagao et al., 1986; Czarnecka et al., 1989).

HSEs are the binding sites for the *trans*-active heat shock factor (HSF), and the efficient binding requires at least three pentameric units (Perisic et al., 1989; Sorger and Nelson, 1989). Tomato HSFs are able to trimerize and translocate to the nucleus (Lyck et al., 1997, Scharf et al., 1998). The *Arabidopsis thaliana* HSF1 has been shown to bind consensus tripartite HSE sequences and the HSE-containing region of *Drosophila HSP70* promoter (Hubel and Schoffl, 1994; Hubel et al., 1995).

Prior to a heat signal the promoter of the heat shock proteins are in open configuration as evidenced by nuclease hypersensitivity experiments (Wu, 1980). At least three different transcription factors are bound to the uninduced promoter, the GAGA factor, the CCAAT factor and the TBP (TATA binding protein) (Wu, 1984; Giardina et al., 1992; Landsberger and Wolffe, 1995). The GAGA factor, which occupies the GAGA elements, act at the early step of the assembly of the promoter by keeping the HSE clear from repressive nucleosomes (Lis and Wu, 1994). The potentiation of heat shock promoters *in vivo* is characterized by an initiated, paused polymerase, waiting to receive an activating signal from HSF in order to resume transcription (Sandaltzopoulos and Berger, 1998). Recently, TBP was found to interact with the HSF at the *Drosophila hsp70* promoter (Mason and Lis, 1997) and with *At*HSF1 *in vitro* and *in vivo* (Reindl and Schoffl, 1998). The HSF have been reported to increase transcription from heat shock promoter by affecting the rate of reinitiation (Sandaltzopoulos and Becker, 1998).

When cells are stressed, HSF homotrimerizes, acquires DNA-binding activity, translocates to the nucleus, is hyperphosphorylated and becomes transcriptional competent (Morimoto et al., 1996; Voellmy, 1996). Prolonged heat shock or recovery from heat shock results in attenuation of the heat shock response. It has been suggested

that the attenuation of the heat shock response is autoregulated by heat shock proteins, particularly the HSP70 and the HSP90 (DiDomenico et al., 1982; Abravaya et al., 1991; Baler et al., 1996; Shi et al., 1998; Zou et al., 1998). In plants, certain HSFs in subclass B (Nover et al., 1996) may work as repressors that counteract transcriptional activation (Czarnecka-Verner et al., 1998). Negative regulation of the HSFs also occurs by phosphorylation (Kline and Morimoto, 1997; Schoffl et al., 1998). However, when amino acids analogs are incorporated into the naked polypeptides, the heat-shock proteins are constitutively up-regulated and attenuation does not occur (DiDomenico et al., 1982, Mosser et al., 1988).

AT-rich Promoter Element

The 5' flanking region of the *LeHSP17.6* is comprised of 33 contiguous A-T bp positioned 300 bp upstream of the TATA box (Figure 1). The smHSPs genes often have blocks of AT-rich sequences located within the promoter region (Nagao et al., 1985; Baumann et al., 1987; Raschke et al., 1988; Czarnecka et al., 1989; Krishna et al., 1992). Deletion of an AT-rich region from the *Gmhsp17.3*B promoter resulted in a substantial loss of transcriptional activity, suggesting that this region may function as enhancer-like elements (Baumann et al., 1987). The best evidence that the AT-rich sequences may act as enhancer-like elements is the observation that synthetic AT-rich oligonucleotide are able to stimulate transcription when placed prior to a truncated maize *Adh1* promoter (Czarnecka et al., 1992). However, the function of the AT-rich domains depends on additional *cis*-elements (Rieping and Schoffl, 1992).

The AT-rich blocks from the *Gmhsp17.5E* promoter were able to bind nuclear proteins *in vitro* (Czarnecka et al., 1990; 1992). Proteins that bind with high affinity to AT-rich sequences belong to the HMGs (high mobility group proteins) family (Pedersen et al., 1991; Czarnecka et al., 1992). HMG may help to maintain a chromatin structure accessible to specific transcription factors. AT-rich region are very similar to scaffold or matrix attachment regions reported in *Drosophila* (Jackson, 1986). A scaffold attachment sequence in the 3' flanking region of the *GmHSP17.6*-L, mediated enhanced and gene-dosage expression of a chimeric heat shock gene in transgenic tobacco plants (Schoffl et al., 1993).

CCAAT (Y-box) and REa Elements

In addition to the preceeding elements (HSE and AT-rich), a perfect CCAAT box is located 188 bp upstream the TATA box (Figure 1). The CCAAT boxes act as functional *cis* elements in the soybean *hsp17.3-B* promoter (Rieping and Schoffl, 1992). The CCAAT boxes and the HSE act synergisticly but not additively to increase heat shock inducibity (Rieping and Schoffl, 1992). In other organisms, CCAAT (or Y-box) elements are required for oocyte expression of the *Xenopus HSP70* (Bienz, 1986). The CCAAT-binding protein may interact with HSE to enhance its binding to HSF, especially in the absence of heat stress (Bienz, 1986). In the *Xenopus HSP70* promoter, the Y-box elements are important for disrupting local chromatin structure and for facilitating the association of HSF and transcriptional activation in chromatin (Landsberger and Wolffe, 1995). Recently, it has been reported that the CCAAT-binding protein, in the *Xenopus*

HSP70 promoter, establishes pre-set chromatin architecture that can modulate transcription by recruiting the p300 acetyltransferase (Li et al., 1998).

The CCAAT element found in the *LeHSP17.6 promoter* is overlapped by another element, the AACCAA (designated as REa), which is required for phytochrome regulation of the *Lemna gibba Lhcb2*1* (Degenhardt and Tobin, 1996). This element appears also in 5' UTR region. The REa element was proposed to function as a repressor of transcription in the dark (Degenhardt and Tobin, 1996).

STRE (Stress Response Element)

The 5' flanking region of the *LeHSP17.6* contains three motifs that closely resemble the stress response element (STRE) present in the promoter of stress-induced genes in yeast. STREs have never been reported in plant gene promoters. However, the cytosolic class II smHSP gene promoter from maize has two STREs, one near the TATA box and the other 320 nucleotides upstream of the transcription site (Dietrich et al., 1991). The cytosolic class II smHSP gene promoter from *Pharbitis nil* and from soybean does not have this. The only plant promoters that have the same number and orientation pattern of the STREs, observed in the *LeHSP17.6* promoter, are the *HVA22* gene, an ABA-induced gene (Shen et al., 1993) and *HVA1* (Straub et al., 1994) a ABA, cold, salt and heat-induced gene. The STREs are not the essential elements for the ABA-induction of the *HVA22* (Shen and Ho, 1995). Therefore, the role of the STREs in plants is unknown. Below I summarized the role of the STREs in yeast stress-inducible genes.

In yeast there are at least three positive transcriptional elements activated by stresses (Attfield, 1997). These are heat shock elements (HSEs), stress-response

elements (STREs) and AP-1 responsive elements (AREs). Kobayashi and McEntree (1990) have demonstrated that a nonpalidromic sequence from *DDR2* promoter was able to confer heat shock inducibity of a reporter gene, independent of HSF. This element (also called STRE), which has the consensus core sequence CCCCT or AGGGG, has been reported to mediate stress induction (Marchler et al., 1993).

STRE-dependent response involves diverse genes such as *CTT1*, a cytosolic catalase T (Wieser et al., 1991), *DDR2*, a DNA damage repair protein (Kobayashi and McEntree, 1990; 1993), *HSP12* (Praekelt and Meacock, 1990; Varela et al., 1995), *HSP26* (Petko and Lindquist, 1986), *HSP104* (Mager and De Kruijff, 1995), *SSA1*, *SSA4* (HSP 70), *UBI4* (Kobayashi and McEntree, 1993), *GPD1*, a glycerol-3-phosphate dehydrogenase (Blomberg and Adler, 1989), *PTP2* (Mager and De Kruijff, 1995), *GAC1*, a phosphatase type-1 subunit (Francois et al., 1992), *TPS1* (trehalose-6-phosphate synthase), *TPS2*, a trehalose phosphate phosphatase (Gounalaki and Thireos, 1994), *CYC7* (iso-2-cytochrome C), *ALD2* (cytosolic aldehyde dehydrogenase), *GSY2*, a glycogen synthase (Ni and LaPorte, 1995) and *SOD2* (Flattery-O'Brein et al., 1997). The diversity of these gene functions indicates the generality of the STRE-dependent response (Attfield, 1997). Genes with STREs are crucial to survival of cells that have been exposed to severe (lethal) stress (Ruis and Schuller, 1995).

Basal and induced levels of STRE-dependent transcription are enhanced in *ras2* mutants with low protein kinase A activity and dramatically reduced in *bcy1* mutants with high constitutively protein kinase A (Belazzi et al., 1991; Boorstein and Craig, 1990; Marchler et al., 1993; Engelberg et al., 1994). It appears that protein kinase A represses the activity of STRE elements (Ruis and Schuller, 1995). Moreover, Schuller et al.

(1994) identified STREs as specific targets of the HOG (high osmolarity glycerol) pathway. Defects in this pathway completely abolished the transcription of a STRE-*lacZ* reporter gene construct by osmotic stress. Therefore, two signaling pathways identified as STRE mediators, the HOG pathway (a MAP kinase cascade) and the RAS-cAMP pathway as a negative regulator.

The transcription factors that recognize the STRE have been identified as multicopy suppressors of the Snf1p protein kinase mutant (Estruch and Carlson, 1993). STRE-transcriptional factors (designated as Msn2 and Msn4) are C_2H_2 zinc finger DNA-binding proteins and function as transcription activators (Estruch and Carlson, 1993; Schmitt and McEntee, 1996). MSN2 and MSN4 have been shown to bind to STRE in the gel mobility-shift assays (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). Moreover, yeast strains with deleted Msn2/4 function are hypersensitive to carbon starvation, DNA damage and other stresses such as heat, oxidative and osmotic stress (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). The stress sensitivity of those mutants was restricted to severe stress. Expression of *CTT1*, *HSP12* and *DDR2* induced by heat shock, high salt concentration, sorbic acid or ethanol was abolished in the double *msn2msn4* mutant (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). In addition the *TPS2*, encoding trehalose phosphate phosphatase, is regulated through the MSN2/STRE pathway (Schmitt and McEntee, 1996).

MSN2/4p controls a lot of genes induced at the diauxic transition (Boy-Marcotte et al., 1998), including alcohol dehydrogenase-like (*ALD3/5*), transketolase (*TKL2*), hexokinase 1, HSP70, trehalose-6-phosphate synthase (*TPS1*), glutamate dehydrogenase, glycerol phosphate phosphatase (*GPP*), aldehyde dehydrogenase-like, HSP104,

glucokinase (*GLK1*), phosphoglucomutase (*PGM2*), and *SOD2*. Diauxic transition is the transient growth arrest of yeast grown on glucose media, when the glucose is exhausted. Then, the cells are grown on ethanol as a carbon source. In addition, STRE-driven *lacZ* reporter gene was induced during diauxic transition and cAMP had a negative effect on this induction (Boy-Marcotte et al., 1998).

Genes like *TPS1* and *PGM2* are involved in carbohydrate storage which is a cellular response to various stresses (Parrou, 1997). Interestingly, a lot of genes encoding metabolic enzymes are dependent on Msn2/4 STRE response (DeRisi et al., 1997; Boy-Marcotte et al., 1998; Moskvina et al., 1998).

In yeast five genes encoding HSPs (HSP12, HSP26, HSP42, HSP78, HSP104) were identified to have several STREs in their promoter (Moskvina et al., 1998; Treger et al., 1998). The promoters of these genes contain HSEs, which have been shown to be required for heat shock activation. Treger et al. (1998) evaluated the contribution of the STREs in the heat shock regulation of these genes. The HSC 70 showed no requirement for Msn2/4p. The HSP12 was predominadly regulated by the STRE, while the HSP26 expression occurred mainly via HSF/HSE pathway. Both transcripts were abolished after heat shock exposure in the triple mutant (*msn2/msn4/hsf1*). Similarly the *HSP78* and the *HSP104* expression were regulated by the two pathways. Surprisingly, the level of *HSP82* gene was slightly affected by inactivation of both pathways, suggesting that a third independent stress regulatory pathway might occur for the heat shock induced expression of these genes. The role of the STRE element in the regulation of *LeHSP17.6* upon heat shock and heat-required cold induction it remains to be elucidated.

NIT2 or GATA Core Elements

The promoter sequence of *LeHSP17.6* contains six NIT2 consensus core sequences, TTGATA or its complement TATCAA. The arrangement of the NIT2 elements is analogous to *cis*-regulatory sequences found in the promoters of nitrate-inducible genes in *Neurospora crassa*, regulated by the NIT2 Zn-finger protein (Fu and Marzluf, 1990; Chiang and Marzluf, 1994). The NIT2 binding sites contain two GATA elements, which have varied spacing but must be less than 30 bp of each other (Chiang and Marzluf, 1994). In *Neurospora* the NIT2, a member of the GATA family of transcription factors, is a globally acting nitrogen regulatory protein that positively activates the expression of many nitrogen catabolic genes, including nitrate reductase (Marzluf, 1997). In spinach, nitrite reductase, the second enzyme in the nitrate assimilation pathway reducing nitrite to ammonium, is induced upon addition of nitrate. This expression was mediated by nitrate-specific binding of the NIT2 factor to a TTGATA sequence, observed in the promoter of the nitrite reductase (Rastogi et al., 1997). NIT2-like protein (NTL1) has been cloned and characterized from tobacco (Daniel-Vedele and Caboche, 1993). Although the promoter of the tomato cytosolic class II small *HSP17.6* has 6 NIT2-related *cis*-acting elements, there is no report that this gene is involved in nitrate assimilation pathway or during nitrogen limitation.

The core sequence of the NIT2 element is the "GATA" which is recognized by other GATA factors. This motif, called GATA box or I-box, is present in the light regulated *CAB* genes of different species (Gidoni et al., 1989). Therefore, the I-box has been suggested to be involved in light-regulated gene expression of photosynthetic genes (Manzara et al., 1991; Terzaghi and Cashore, 1995). The *Pharbitis nil* cytosolic class II

smHSP *shp-1* is induced by photoperiod and by light treatment of dark grown seedling (Krishna et al., 1992). However, the light induction may be mediated by GT-1-like elements found in the promoter of the *shsp-1* (Krishna et al., 1992). These GATA factors regulate distinct metabolic pathways. How these factors specifically regulate their own target genes is unknown. Perhaps, the flanking region of the GATA core binding sites might determine the DNA-binding specificity of each GATA factor (Ravagnani et al., 1997).

In conclusion, detailed analyses of the *LeHSP17.6* promoter region revealed multiple heat shock elements, stress response elements, GATA elements and long tracts of repetitive sequences with high AT content. The *LeHSP17.6* is accumulated in tomato fruits exposed to heat shock and slightly by low temperatures. The combination of the heat treatment and low temperature storage revealed an interesting observation. The expression of the *LeHSP17.6* was induced at low temperatures only if the fruits have been exposed to heat shock. The reinduction of the transcripts in the cold was more favorable after continued exposure to high temperatures. These *cis*-acting elements may be useful to further understand the novel expression pattern of the *Le*HSP17.6 during the combination of the high and low temperatures.

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CONCLUSIONS AND FUTURE RESEARCH

The specific goals of this study were a) to develop, optimize and characterize a commercially applicable postharvest heat treatment for cold storage of tomato and b) to investigate the involvement of heat shock genes and heat-shock proteins in heat treatment-induced resistance to chilling injury.

To achieve the first goal, heat treatments (warm air) were investigated. Treatment of tomatoes in 42°C in humidified air for 36 h or 48 h before storage at chilling temperatures (2°C) for 14 days reduced CI and decay during subsequent ripening at 20°C. This protection was lost if the tomatoes were transferred from 42°C to 20°C for 1 to 3 days before low temperature storage, however the CI was less than that of control fruit. Ripening of the heat-treated fruits was normal although slower than that of the control fruits. The next step is to evaluate the effectiveness of heat treatments on a commercial scale. Hot water application appears to be promising as part of the packinghouse line. Thus, in the future this work may have very practical application to postharvest treatment of fruits and vegetables. Heat treatments can decrease fungal rots, both by killing the fungal spores and by activating defense mechanism in the fruit tissue. Further research on the defense mechanisms activated by heat shock may yield benefits to agriculture; heat treatment may slow ripening, decrease sensitivity to chilling injury, and decrease fungal infections.

The second goal, to investigate the involvement of HSP in the resistance to chilling injury, has been well documented in this study. Using differential display of mRNA, a full-length cDNA that encodes a cytosolic class II smHSP (*Le*HSP17.6). To elucidate if

other heat-shock proteins are induced by heat shock and persist during subsequent storage at low temperatures, we cloned a second member of the cytosolic class II smHSP, three cDNAs encoding a cytosolic class I smHSP and partial cDNA clones for a chloroplastic smHSP, a mitochondrial smHSP and a heat-inducible HSP90 member.

The cytosolic class I and II smHSPs and the chloroplastic smHSP were up regulated by heat treatment, and this was slightly increased during subsequent storage at cold temperatures. Non-heated fruits stored at chilling temperatures displayed low levels of cytosolic class II smHSP mRNA. On the other hand, although the HSP90 mRNA was induced during heat treatment, its expression was regulated by cold; the heat-treated fruits had a higher transcript level when stored cold than non-heated fruits. Cytosolic class I and II smHSPs transcripts were more stable than those of *Le*HSP90s and chloroplastic smHSP if heat shock was prolonged. The expression pattern of proteins encoded by mRNAs for the smHSPs and HSP90s followed a similar trend. More importantly, the cytosolic class I and II smHSP as well as the HSP90 were still detectable when the fruits were transferred to 20°C for 3 days.

Collectively, correlation of accumulation of HSPs with resistance of the tissue to chilling injury was established. The cold-inducible expression of HSP genes in the heat-treated fruits raised the possibility that both high- and low-molecular-weight HSPs may play a critical role in resistance to chilling stress. Therefore, it would be interesting to test the *in vivo* role of the HSP in the attenuation of chilling injury in tomatoes by using transgenic plants that over- or underexpress each gene (cytosolic class I or II or HSP90) under control by CaMV 35S promoter, a fruit specific promoter(s) and a cold- inducible promoter. It would also be interesting to study the role of HSPs on chilling tolerance,

fruit development and ripening by expressing the tomato HSFs in sense and antisense orientation under control of a fruit-specific promoter (E8 or 2A11) or a cold-inducible promoter such as cor15a. Perhaps the induction of the whole battery of HSPs will be more beneficial than the overexpression of a single gene. In addition, demonstration that the smHSP have chaperone activity at low temperatures *in vitro* and *in vivo* is needed.

Interestingly, the carboxyl end of the *Le*HSP17.6 cytosolic class II smHSP contains a polyproline motif PPPEPKKP. The PxxP motif is known to be involved in protein/protein interactions among proteins with SH3 (Src homology)-binding domain. The SH3-domain is important for intra- as well as intermolecular interaction that regulates Src catalytic activity, localization and recruitment of substrates. The SH3-binding pocket has two hydrophobic grooves that contact the core xPxxP sequence. However, in plants there is no report on SH3 domain-containing proteins. To test the possibility that the polyproline motif of the *Le*HSP17.6 cytosolic class II smHSP binds to an SH3 domain, a yeast two-hybrid analysis is recommended utilizing the SH3 domain from known proteins. If the *Le*HSP17.6 interacts with the SH3 domain proteins, then the next step is to screen a tomato yeast two-hybrid library to find proteins with a SH3 domain from plants. It would be interesting to determine the biological significance of SH3 domain-containing proteins in plant development and responses to the environment.

The combination of the heat treatment followed by low temperature storage enhanced the expression of the smHSPs and this was correlated with chilling injury tolerance. An intervening return to 20°C prior to returning the fruits to a chilling temperature re-induced expression of the smHSPs but the fruits were subject to chilling injury. The re-induction of the transcripts in the cold was more favorable after continued

exposure to high temperatures. To our knowledge similar phenomena have not been reported in plants or other organisms. Detailed analyses of the *LeHSP17.6* promoter region revealed multiple heat shock elements (HSE), stress response elements (STRE), GATA elements and long tracts of repetitive sequences with high AT content. The expression of the *LeHSP17.6* was mainly induced at low temperatures only if the fruits had been exposed to heat shock. These *cis*-acting elements may be useful to further investigate the novel expression pattern of the cytosolic smHSPs employing high and low temperature regimes.

The heat shock induced protection against CI was lost if the fruits were transferred to 20°C after heat shock and before cold storage. Surprisingly, the re-induction of the HSPs at low temperatures was not prevented. These data suggest that heat-shock proteins are not the only factor(s) that contribute to the chilling-tolerant phenotype of tomatoes. The data suggests that ethylene insensitivity as a consequence of the prolonged heat treatment may be important. Thus, transgenic plants that overexpress smHSPs and in parallel are ethylene insensitive will increase the chilling tolerance of tomatoes and other subtropical and tropical fruits.

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