The Plant Cell, Vol. 17, 1829–1838, June 2005, www.plantcell.org

Dual Role for Tomato Heat Shock Protein 21: Protecting Photosystem II from Oxidative Stress and Promoting Color Changes during Fruit Maturation

Inbal Neta-Sharir,[a] Tal Isaacson,[b] Susan Lurie,[c] and David Weiss[a,1]

[a] Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture, Faculty of Agricultural, Food, and Environmental Quality Sciences, Hebrew University of Jerusalem, Rehovot 76100, Israel

[b] Department of Genetics, Hebrew University of Jerusalem, Jerusalem 91904, Israel

[c] Department of Postharvest Science, Agricultural Research Organization, Volcani Center, Bet Dagan 50250, Israel

The tomato (*Lycopersicon esculentum*) chloroplast small heat shock protein (sHSP), *HSP21*, is induced by heat treatment in leaves, but also under normal growth conditions in developing fruits during the transition of chloroplasts to chromoplasts. We used transgenic tomato plants constitutively expressing *HSP21* to study the role of the protein under stress conditions and during fruit maturation. Although we did not find any effect for the transgene on photosystem II (PSII) thermotolerance, our results show that the protein protects PSII from temperature-dependent oxidative stress. In addition, we found direct evidence of the protein's role in fruit reddening and the conversion of chloroplasts to chromoplasts. When plants were grown under normal growth temperature, transgenic fruits accumulated carotenoids earlier than controls. Furthermore, when detached mature green fruits were stored for 2 weeks at 2°C and then transferred to room temperature, the natural accumulation of carotenoids was blocked. In a previous study, we showed that preheat treatment, which induces HSP21, allowed fruit color change at room temperature, after a cold treatment. Here, we show that mature green transgenic fruits constitutively expressing HSP21 do not require the heat treatment to maintain the ability to accumulate carotenoids after cold storage. This study demonstrates that a sHSP plays a role in plant development under normal growth conditions, in addition to its protective effect under stress conditions.

INTRODUCTION

All organisms respond to high temperatures by inducing the synthesis of a group of evolutionarily conserved polypeptides known as heat shock proteins (HSPs). Small HSPs (sHSPs) range in size from ~15 to 42 kD and are characterized by a conserved sequence at their C terminus, related to the sHSPs of other organisms and to the α-crystallins of the vertebrate eye lens (Inoglia and Craig, 1982; Sun et al., 2002). Plant sHSPs constitute the most abundant and diverse group of proteins synthesized in response to heat stress. These proteins can also be induced by other environmental stresses, including cold, drought, or salinity, and during various developmental processes, such as embryogenesis, germination, and fruit development (Wehmeyer et al., 1996; Almoguera et al., 1998; Sun et al., 2001). sHSPs have been grouped into six different classes based on sequence alignments, immunological cross-reactivity, and cellular compartmentalization. Three of the classes (CI, CII, and CIII) have been localized to the cytoplasm or the nucleus and the others to the endoplasmic reticulum, mitochondria, and chloroplasts (Sun et al., 2002). The correlations between sHSP synthesis and the stress response led to the assumption that these proteins protect cells from the related destructive effects of high temperature (Sun et al., 2002). Although the mechanism by which sHSPs are involved in cell protection are not fully understood, it has been shown that both plant and mammalian sHSPs possess non-ATP-dependent, chaperone-like activity in vitro (Lee et al., 1997; Lee and Vierling, 2000). It has been suggested that sHSPs bind to partially folded or denatured proteins and prevent their aggregation and facilitate folding by other chaperones (Sun et al., 2002).

The nuclear-encoded chloroplast sHSPs have an apparent molecular mass in the range of 21 to 30 kD. These proteins have two conserved regions found in all sHSPs and a unique amphipathic, Met-rich domain at their N termini, which is highly conserved among all identified chloroplast sHSPs but not found in other sHSPs (Chen and Vierling, 1991). In vitro studies using isolated chloroplasts suggested that the chloroplastic sHSP protects the thermolabile photosystem II (PSII) and is important for heat acclimation (Heckathorn et al., 1998). However, similar experiments performed by others did not confirm these observations (Härndahl and Sundby, 2001). Furthermore, although a correlation between the levels of chloroplastic sHSPs and PSII thermotolerance has been shown in vivo (Heckathorn et al., 2002; Wang and Luthe, 2003), direct evidence of their role in the

[1] To whom correspondence should be addressed. E-mail weiss@agri.huji.ac.il; fax 972-8-9468263.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: David Weiss (weiss@agri.huji.ac.il).

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.105.031914.

protection of PSII in planta has not been provided. Transgenic *Arabidopsis thaliana* plants constitutively expressing the chloroplastic sHSP, HSP21, were slightly more resistant than the wild type to a combination of heat and high-light stresses (Härndahl et al., 1999). These results suggest that HSP21 plays a role in vivo in protecting against temperature-dependent oxidative stress. However, the mechanism of protection is not known. It was speculated that HSP21 could act as an antioxidant under oxidative stress conditions (Levine et al., 1996).

The tomato (*Lycopersicon esculentum*) chloroplast sHSP *HSP21*, also named *TOM111* (Lawrence et al., 1997) and *LeHSP23.8* (Sun et al., 2002), is induced by heat treatment in leaves, flowers, and fruits. It is also induced under normal growth temperature in fruits at the turning stage (i.e., when fruits turn red; Sabehat et al., 1998; Sun et al., 2002). In a previous study, we found that keeping mature green tomato fruit for a long period (2 to 3 weeks) at low temperature (2°C) causes chilling injuries and inhibits normal maturation and carotenoid accumulation after their transfer to room temperature. However, exposing the fruits to high temperature (38°C) for 2 d before the cold storage protects them and allows maturation at room temperature, including reddening (Sabehat et al., 1996). We also found that the heat treatment induces the accumulation of *HSP21* mRNA in the fruits. The transcript was also found after long storage of the heated fruits at low temperature. We thus speculated that HSP21 may play a role in fruit maturation.

Despite the intensive research on Hsp21, the molecular basis of its chaperone activity in vivo and its protected targets remain poorly understood. Furthermore, the role of the protein in fruit development has not been studied. In this work, using transgenic tomato plants constitutively expressing HSP21, we show that the protein protects PSII from temperature-dependent oxidative stress. Furthermore, we present evidence of the protein's role in fruit development, specifically in the accumulation of carotenoids during ripening.

RESULTS

Hsp21 Protects PSII from Oxidative Stress

Several studies have shown that Hsp21 protects the thermolabile PSII during heat stress in isolated chloroplasts (Heckathorn et al., 1998). However, its protective effect on PSII in planta was not demonstrated. To analyze the function of HSP21 in vivo, we transformed VF36 tomato plants with a construct containing the *HSP21* (accession number U66300) open reading frame driven by a 35S promoter of *Cauliflower mosaic virus*. Independent transgenic plants exhibiting high *HSP21* expression levels under normal growth conditions (three independent transformants: T0-311, T0-831, and T0-1041) were self-pollinated. T1 progenies were first analyzed by PCR to select for transgenic plants and then analyzed for *HSP21* transcript and protein levels under normal growth conditions (Figure 1). High levels of HSP21 transcript and protein were found in the transgenic lines but not in control, nontransgenic VF36 plants. DNA gel blot analyses confirmed the integration of the transgene to the plant genome and revealed the presence of two copies in T1-311 and single copy in T1-831 and T1-1041 (data not shown). Nevertheless,

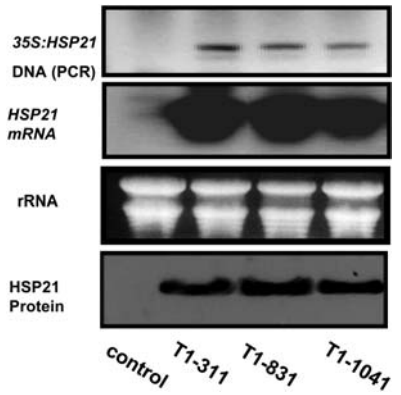

Figure 1. Molecular Analyses (PCR, RNA Gel Blot, and Protein Gel Blot) of Transgenic Tomato Plants Transformed with *35S-HSP21* Construct.

DNA, RNA, and proteins were extracted from a control nontransgenic plant and three T1 plants originating from three independent transformation events.

these differences did not affect the expression levels of *HSP21* in the different lines. Selected T1 progeny (from the three independent transgenic lines) were self-pollinated to produce T2 progenies. T2, T3, and T4 plants used in this study were either homozygous or heterozygous but all were first analyzed for *HSP21* expression to ensure high levels of transcript under normal growth temperature (25°C).

Detached leaves from transgenic (three independent T2 transgenic lines) and control plants were incubated in vials with tap water and exposed for 2.5 h to 40 or 50°C. Quantum yield of PSII was analyzed immediately after the stress treatment by measuring chlorophyll fluorescence (Figure 2A). No change in PSII activity was found in any of the examined leaves. Exposing the detached leaves to 50°C for a longer period (3.5 h) resulted in irreversible damage to PSII in both control and transgenic leaves (data not shown). Further attempts to evaluate whether the transgene promotes PSII thermotolerance, by gradually increasing the temperature and/or reducing the time of exposure, did not reveal any effect: both transgenic and control plants were either not affected or similarly damaged (data not shown).

Previous studies have suggested that HSP21 protects chloroplasts from temperature-dependent oxidative stress but not from heat stress (Härndahl et al., 1999). We therefore exposed leaves to high temperature and then to high light, a combination known to cause oxidative stress (Asada, 1996). Detached leaves (from three independent T2 transgenic lines) were incubated in vials with tap water and exposed to 40, 47, or 50°C for 2 h and then exposed for 2 min to high light (1200 $\mu mol \cdot m^{-2} s^{-1}$). To analyze the effect of the combined stress on PSII activity, chlorophyll fluorescence yield was measured. Leaves that had been exposed to 40°C and then to light did not show any reduction in PSII activity (Figure 2B). After incubation at 50°C and exposure to light, PSII activity in all transgenic lines and control leaves was severely reduced (Figure 2D). Transgenic leaves that were incubated at 47°C and then exposed to light exhibited some, albeit not significant, tolerance (i.e., PSII activity in these leaves was slightly higher than that found in control leaves) (Figure 2C).

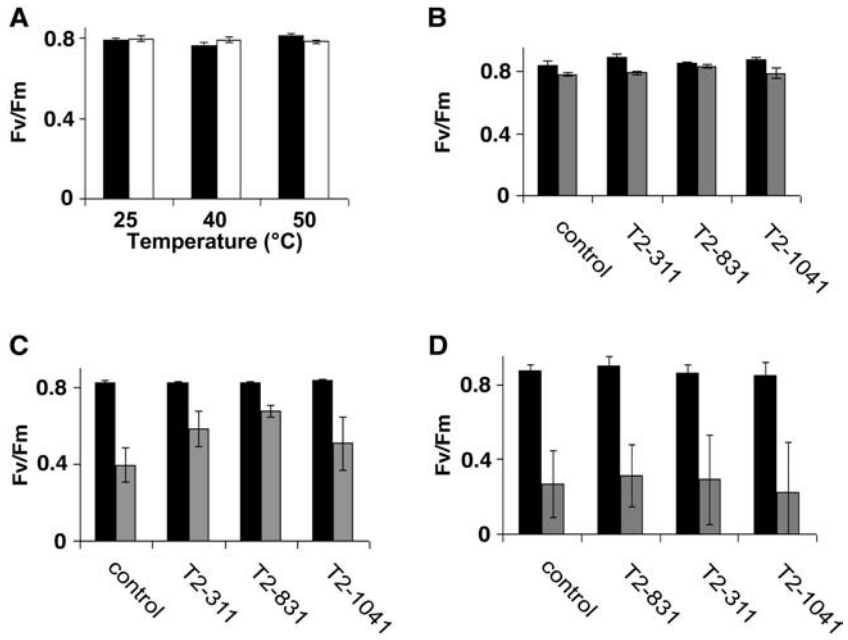

Figure 2. Effect of Heat and High-Light Stresses on PSII Activity in Control and Transgenic Tomato Plants Expressing High Levels of HSP21.

(A) Detached leaves from transgenic line (T2-311, black bars) and control (white bars) were exposed to 40 or 50°C for 2.5 h, and then chlorophyll fluorescence yield (variable fluorescence/maximum fluorescence [Fv/Fm]) was measured.

(B) to **(D)** Detached leaves (from three independent T2 transgenic lines and control) were exposed to 40°C **(B)**, 47°C **(C)**, or 50°C **(D)** for 2 h and then to high white light (1200 μmol·m^{-2}s^{-1}) for 2 min. Chlorophyll fluorescence yield was then measured. Black bars represent chlorophyll fluorescence yield before treatments, and gray bars represent chlorophyll fluorescence yield after stress treatments. Each value represents an average of 10 measurements taken from 10 different leaves ± SE.

Tomato leaves accumulate HSP21 in response to heat stress (Sabehat et al., 1998). It is possible that in the experiment described above, control plants rapidly synthesized HSP21 in response to the high-temperature treatment and, therefore, the differences between transgenic and control plants were not significant. Because oxidative stress can also be induced by combined cold and high-light stresses (Allen and Ort, 2001), neither of which induces the accumulation of *HSP21* (Sabehat et al., 1998), we examined the effect of the transgene on PSII activity in leaves that were first exposed to low temperatures and then to high light. Detached leaves from three independent transgenic lines (T2) and control VF36 were incubated in vials with tap water for 3 d at 4°C and then exposed for 2 min to high light (1200 μmol·m^{-2}s^{-1}). Figure 3A shows a severe reduction in PSII activity in control leaves after the combined stresses. On the other hand, all tested transgenic lines exhibited normal PSII activity. When leaves were incubated for 3 d at 4°C but not exposed to high light, no damage to PSII was observed in either control or transgenic lines (Figure 3B). Furthermore, PSII activity was not affected when leaves were exposed to high light without preincubation at low temperature (data not shown). To test whether the reduction in PSII activity depends on light intensity, cold-treated leaves (3 d at 4°C) detached from control and one representative transgenic line were exposed to low or high light intensity (600 and 1200 μmol·m^{-2}s^{-1}, respectively). Although PSII activity in control leaves was not affected by exposure to low light intensity, a strong reduction was found after illumination with high light (Figure 3C). PSII activity in transgenic leaves, on the other hand, was not affected by low or high light. These results suggest that the accumulation of HSP21 in chloroplasts of transgenic plants protects PSII from light-dependent oxidative stress. These experiments were repeated with T3 plants and similar results were found.

HSP21 Promotes Carotenoid Accumulation during Fruit Maturation

HSP21 is induced in tomato fruit during maturation; its transcript is first detected at the turning stage, when carotenoid pigments start to accumulate (Figure 4). To study the possible role of HSP21 in fruit maturation, we compared fruit development of the control VF36 cultivar with that of transgenic plants. The transgenic fruits accumulated high levels of HSP21 transcript throughout their development (Figure 4). We labeled flowers from control and transgenic (three independent T2 transgenic lines) plants at anthesis and followed fruit development and maturation. Figure 5A shows that fruits in all three transgenic lines reached the turning stage earlier than control fruits (i.e., color change in transgenic fruits was observed ~7 to 10 d earlier than in the control). We repeated the analyses using T3 and T4 plants and similar results were obtained (data not shown). We also analyzed carotenoid content in fruits of control and transgenic lines (T4-311) at three different time points after anthesis. Figure 5B shows that carotenoids are accumulated in transgenic

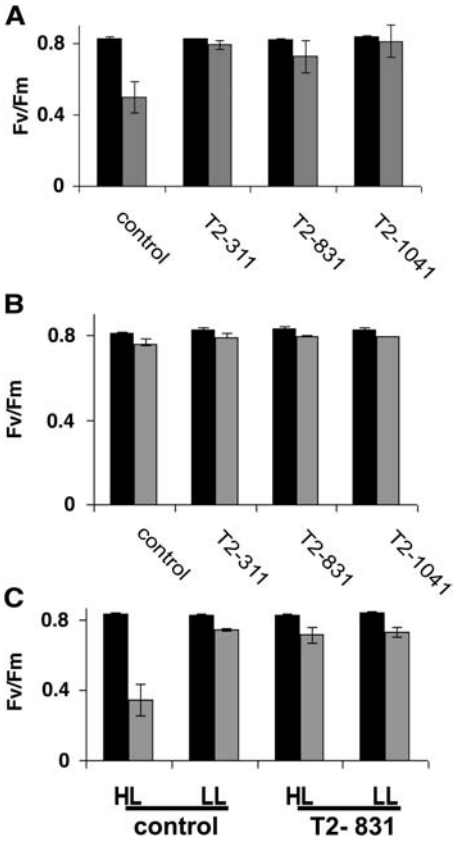

Figure 3. Effect of Cold and High-Light Stresses on PSII Activity in Control and Transgenic Tomato Plants Expressing High Levels of HSP21.

(A) Detached leaves from a control and transgenic lines (T2) were incubated in vials with tap water for 3 d at 4°C and then exposed for 2 min to high light (1200 μmol·m^{-2} s^{-1}). Chlorophyll fluorescence yield (Fv/Fm) was then measured.

(B) Detached leaves from a control and transgenic lines (T2) were incubated for 3 d at 4°C and then chlorophyll fluorescence yield was measured.

(C) Detached leaves from a control and transgenic line (T2-831) were incubated for 3 d at 4°C and then exposed for 2 min to low (600 μmol·m^{-2}s^{-1}) or high white light (1200 μmol·m^{-2} s^{-1}). Chlorophyll fluorescence yield was then measured. HL, high light; LL, low light. Black bars represent chlorophyll fluorescence yield before treatments, and gray bars represent chlorophyll fluorescence yield after stress treatments. Each value represents an average of at least 10 measurements taken from 10 different leaves ± SE.

fruits before control. The level of carotenoids found 50 d after anthesis in transgenic fruits was ~10 times higher than that found in control (Table 1). The final levels, however, were similar. HPLC analyses (Table 1) revealed a similar profile of carotenoids in control and transgenic fruits: 40 d after anthesis the major carotenoid in both was lutein (~85%) and 10 d later, lycopene (the major pigment of ripe fruit, 60 to 80%). The red color of control and transgenic fruits is determined exclusively by carotenoids because we did not detect any anthocyanin in the tissue (data not shown). We also examined other processes related to fruit maturation, such as changes in sugar and organic acid levels, but no significant differences were found (data not shown). It should be noted however, that sugar and organic acid levels do not change dramatically at turning, but slightly increase throughout the process of ripening. Thus, it would be difficult, if not impossible, to observe significant differences in these parameters between transgenic and control fruits when only slight advance in fruit maturation occurs.

In our previous study (Sabehat et al., 1998), we found that long storage (2 to 3 weeks) of mature green tomato fruits at 2°C inhibits fruit maturation and carotenoid accumulation after transfer to room temperature. The long cold storage also led to other chilling injuries, such as surface blemishes on the fruits and their rapid decay. However, if fruits were first exposed to high temperatures and then stored at low temperature, they reddened almost normally, and blemish and decay were inhibited. We found that *HSP21* transcript accumulates as a result of the heat shock and is also expressed during the cold treatment (Sabehat et al., 1998). Thus, the presence of HSP21 protein in the plastids may be required for carotenoid accumulation. The cold treatment may inhibit normal HSP21 accumulation and therefore fruit color change. To test this hypothesis, we stored mature green control fruits and mature green transgenic fruits (T3-311 and T3-1041) expressing high levels of *HSP21* transcript at 2°C. After 2 weeks, fruits were transferred to room temperature (25°C) for observation. RNA gel blot analyses for RNA extracted before and during the cold treatment, and 48 h after transferal to room temperature, revealed high levels of *HSP21* transcript in transgenic but not control fruits (Figure 6A). As expected, control fruits did not redden and remained at the mature green stage during the first 9 d at room temperature. On the other hand, most transgenic fruits changed their color and after 9 d had reached the orange-red stage (stage 3; Figures 6B and 6C). We could not follow pigment accumulation for a longer period because most

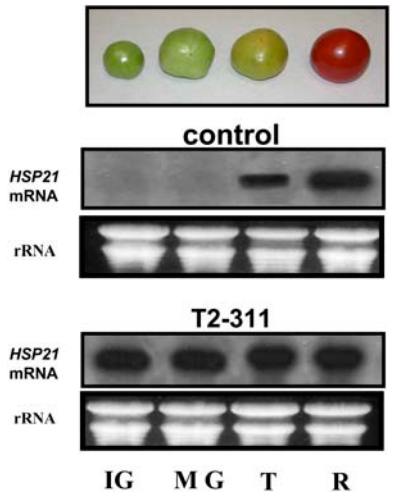

Figure 4. Expression of HSP21 during Tomato Fruit Development in Control and Transgenic Plants (T2-311).

Developmental stages: IG, immature green; MG, mature green; T, turning; R, red ripe. The top panel shows control fruits at the four different developmental stages.

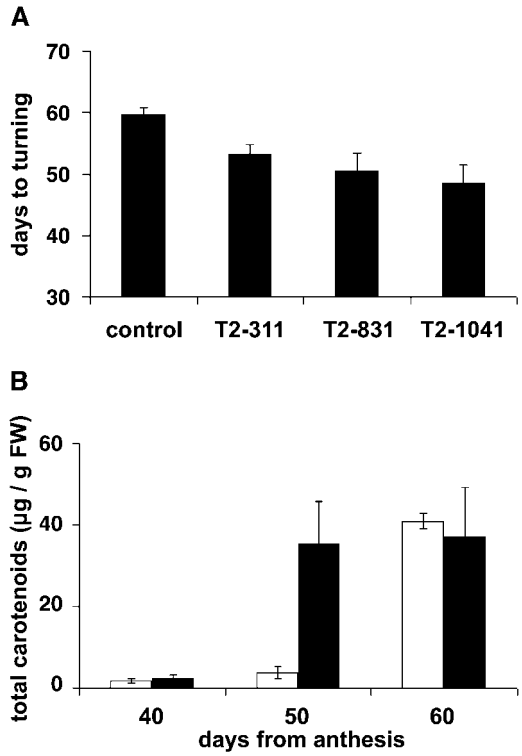

Figure 5. Effect of *HSP21* Overexpression on Tomato Fruit Reddening.

(A) Flowers of the control VF36 cultivar and three independent T2 transgenic lines were labeled at anthesis, and the time for fruits to reach turning stage was monitored. Each value represents an average of at least 50 different fruits from at least five different plants (control and three independent T2 transgenic lines) ± SE.

(B) Carotenoids were extracted from control and transgenic (T4-311) plants at different time point after anthesis (40, 50, and 60 d) and measured with a spectrophotometer. Results are average values of five different fruits. FW, fresh weight.

fruits developed chilling injuries (blemish and decay) and rotted soon after (data not shown). It should be noted that control and transgenic fruits exhibited similar levels of blemish and decay, suggesting that HSP21 is not involved in protection against all chilling injuries. We also measured changes in carotenoids in the cold-treated fruits. The level of the pigment in the transgenic fruits (T4-311) 7 d after transferal to room temperature was ~8 times higher than that measured in control fruits (Table 2). HPLC analyses revealed similar distribution of carotenoids before the cold storage, with lutein being the major one (~85% of total). Seven days after transferal from the cold treatment (Figure 6D), the transgenic fruits accumulated mainly lycopene (~70% of total), whereas control fruits contained relatively high levels of lutein (~40%) and β-carotene (~35%) and lower levels of lycopene (~17%).

DISCUSSION

The function of sHSPs has been the subject of many studies. Nevertheless, their biological functions under stress conditions and during plant development are largely unknown. In this work, we studied the role of the chloroplastic sHSP, HSP21, under stress conditions and during fruit development. The results presented here provide evidence of a dual role: HSP21 protects PSII in leaves from oxidative stress and promotes carotenoid accumulation in the developing fruits.

HSP21 and Light-Dependent Oxidative Stress

The function of HSP21 under stress conditions has been studied previously in isolated chloroplasts, and the results suggested a role in PSII thermotolerance (Heckathorn et al., 1998). Other studies suggested that HSP21 (Härndahl et al., 1999), as well as other sHSPs (Sun et al., 2002; Jofre et al., 2003), protects plants and bacteria from oxidative stress. We found that constitutive expression of *HSP21* in transgenic tomato plants did not increase PSII thermotolerance. It also did not promote the protection of PSII against oxidative stress created by high temperature and high light. Nevertheless, this could be due to the rapid accumulation of the endogenous HSP21 in control plants in response to the high-temperature treatment (Sabehat et al., 1998), which may have masked the effect of the transgene. An oxidative environment can also be created in the chloroplasts by combined cold and high-light stresses (Allen and Ort, 2001), conditions which do not induce endogenous *HSP21*. Indeed, under these conditions, the effect of the transgene was clear: whereas the activity of PSII in control plants was severely reduced, it was almost unaffected in transgenic plants.

The mechanism of protection from oxidative stress is unknown. HSP21 may prevent aggregation and facilitate refolding of components of PSII. Alternatively, it has been speculated that the unique Met-rich domain of the chloroplast sHSPs plays a role as an antioxidant, shielding other oxidation-sensitive proteins

Table 1. HSP21 Promotes Carotenoid Accumulation in Developing Tomato Fruit

Type of Carotenoid	Distribution of Carotenoid (% of Total) Days from Anthesis			
	40		50	
	Control	T4-311	Control	T4-311
Phytoene	n.d.	n.d.	12.6	12.4
Phytofluene	n.d.	n.d.	7.4	6.4
β-Carotene	n.d.	n.d.	9.5	1.0
ζ-Carotene	n.d.	n.d.	1.6	1.2
γ-Carotene	n.d.	n.d.	0.3	n.d.
Neurosporene	n.d.	n.d.	2.5	0.9
Lycopene	n.d.	n.d.	61.6	77.4
Lutein	86.8	87.2	4.3	0.7
Violaxanthin	7.2	7.0	0.2	n.d.
Neoxanthin	6.0	5.8	n.d.	n.d.
Total carotenoid (µg/g FW)	1.73 ± 0.49	2.21 ± 0.29	3.81 ± 1.52	44.4 ± 1.0

Flowers of the control VF36 and one T4 transgenic line (T4-311) were labeled at anthesis, and after 40 and 50 d, carotenoids were extracted, measured with a spectrophotometer, and analyzed by HPLC. Results are average values of four different fruits. n.d., not detected.

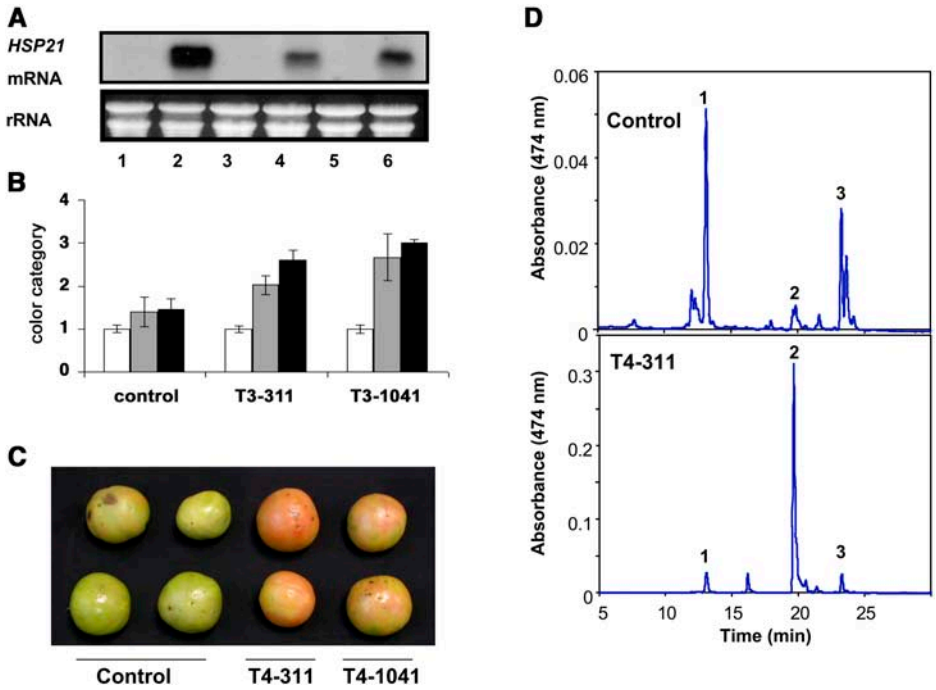

Figure 6. HSP21 Promotes Carotenoid Accumulation in Cold-Treated Tomato Fruits.

(A) Expression of *HSP21* in a control VF36 cultivar and transgenic T3 plant (T3-311) before and during storage at 2°C and 48 h after transferring to room temperature (25°C). Lanes 1 and 2, control (1) and transgenic (2) mature green fruits at harvest; lanes 3 and 4, control (3) and transgenic (4) fruits after 10 d at 2°C; lanes 5 and 6, control (5) and transgenic (6) fruits 48 h after transferring from cold to room temperature.

(B) Evaluation of color change (from green to red) in fruits from control VF36 and two T3 transgenic lines, stored for 2 weeks at 2°C and then transferred to 25°C. Color was evaluated at the end of the cold storage (white bars), 7 (gray bars) and 9 (black bars) d after transferring to room temperature. Each value represents an average of at least 30 different fruits from at least four different plants ± SE.

(C) Control and transgenic fruits 7 d after the end of the 2-week cold storage.

(D) HPLC analysis of carotenoids extracted from cold-treated control and transgenic (T4-311) fruits 7 d after transferal to room temperature. 1, lutein; 2, lycopene; 3, β-carotene.

(Levine et al., 1996). Hamilton and Heckathorn (2001) provided evidence of plastid sHSPs protecting cellular proteins through a mechanism involving reactive oxygen species scavenging, similar to that of antioxidants, and suggested that the mechanism involves scavenging by the Met residues. A secondary-structure prediction of HSP21 indicates that the Met-rich domain can form an amphipathic α-helix, with all Met residues exposed on one side (Chen and Vierling, 1991). Under oxidative stress conditions, the Met residues can oxidize to Met sulfoxide and thus shield other oxidation-sensitive proteins (Levine et al., 1996). Sulfoxidation of the Met residues causes conformational changes and a complete loss of the protein's chaperone-like activity (Härndahl et al., 2001). The sulfoxidized Met residues are then reduced by a specific Met sulfoxide reductase, which restores the protein's chaperone-like activity (Gustavsson et al., 2002). On the other hand, substitution of the conserved Met residues in the HSP21 protein by oxidation-resistant Leu residues did not abolish its chaperone-like activity in vitro (Gustavsson et al., 2001); thus, the function of this domain is still not clear.

An HSP21-protected protein(s) has also not yet been identified. Because the most sensitive element of PSII to oxidation is the D1 protein (Andersson and Aro, 2001), it may be one of HSP21's protected targets. However, we did not detect any changes in the stability of D1 under oxidative stress in the transgenic plants (data not shown). Furthermore, a previous study has shown that *Chenopodium album* chloroplastic sHSPs physically interact with the oxygen-evolving complex of PSII but not with D1 (Heckathorn et al., 2002). Alternately, it is possible that HSP21 does not interact directly with PSII, but affects other components required for PSII activity. For example, HSP21 may stabilize thylakoid membranes under extreme temperatures. Osteryoung and Vierling (1994) have shown that after heat stress, HSP21 localization in the chloroplast is changed from soluble to nonsoluble fraction, and Tsvetkova et al. (2002) have shown that sHSPs protect membrane fluidity. They suggested that such an activity may serve as a general mechanism in the protection of membrane integrity under fluctuating temperature.

HSP21 and Fruit Ripening

The expression of various tomato sHSPs, including *HSP21* and the cytosolic *LpHsp17.7*, *LpHsp17.3*, and *TOM66*, is induced in fruits during maturation at the turning stage (Lawrence et al., 1997; Sabehat et al., 1998; Low et al., 2000). Similarly, transcript of the *NJJS4* gene, coding for class I sHSP, is accumulated in strawberry fruit (*Fragaria* spp) (receptacle) during ripening

Table 2. HSP21 Promotes Carotenoid Accumulation in Tomato Fruits after Cold Storage

	Distribution of Carotenoid (% of Total)			
	At Harvest (Mature Green)		7 d after Cold Storage	
Type of Carotenoid	Control	T4-311	Control	T4-311
Phytoene	n.d.	n.d.	4.8	12.5
Phytofluene	n.d.	n.d.	2.7	5.9
β-Carotene	n.d.	n.d.	34.7	5.9
ζ-Carotene	n.d.	n.d.	n.d.	n.d.
γ-Carotene	n.d.	n.d.	n.d.	n.d.
Neurosporene	n.d.	n.d.	n.d.	n.d.
Lycopene	n.d.	n.d.	16.6	68.3
Lutein	86.3	86.1	41.2	7.4
Violaxanthin	7.5	7.6	n.d.	n.d.
Neoxanthin	6.2	6.3	n.d.	n.d.
Total carotenoid (μg/g FW)	1.76 ± 0.52	2.21 ± 0.31	1.53 ± 0.3	11.89 ± 1.1

Carotenoids were extracted from mature green control and transgenic (T4-311) fruits at harvest and 7 d after cold storage. Total carotenoids were measured with a spectrophotometer and analyzed by HPLC. Results are average values of four different fruits. n.d., not detected.

(Medina-Escobar et al., 1998). These observations suggest a role for sHSPs in fruit development. However, in contrast with the intensive research on the role of sHSPs in stress tolerance, their role in fruit maturation has been neglected.

During tomato fruit ripening, chloroplasts are transformed into chromoplasts. This process is characterized by a breakdown of the photosynthetic apparatus and massive synthesis and deposition of carotenoids (Cheung et al., 1993). Because HSP21 accumulates in the plastids at the same stage (Lawrence et al., 1997), it may play a role in the conversion of chloroplasts to chromoplasts during fruit maturation. Our data support such a role because constitutive expression of HSP21 promoted carotenoid (lycopene) accumulation in the developing fruits. The fact that the transgene did not induce pigment accumulation at the mature green stage, but advanced it only by several days, indicates that HSP21 plays a secondary role in this process while other developmentally regulated factors (e.g., carotenoid biosynthetic enzymes) are the key factors.

Tomato fruits detached at the mature green stage and stored at room temperature ripen normally. During this process, *HSP21* is induced and expressed as in developing attached fruits (data not shown). However, if the detached fruits are first stored at low temperature for 2 to 3 weeks and only then transferred to room temperature, further reddening is inhibited and the fruit remain green. This effect can be prevented by a preheat treatment (Lurie and Klein, 1991). The results of this study and our previous work (Sabehat et al., 1998) show that the cold treatment inhibits the natural activation of *HSP21* in the developing fruits. On the other hand, the preheat treatment induces the gene and allows its expression throughout the cold treatment as well as later, after transfer to room temperature. These results show a clear correlation between *HSP21* transcript level and carotenoid accumulation in the fruit. The results of this study provide direct evidence for HSP21's role in this process: transgenic mature green fruits constitutively expressing *HSP21* can be stored at low temperature for 2 weeks without preheat treatment and still accumulate lycopene and turn red later at room temperature.

The mechanism by which HSP21 promotes lycopene accumulation in the developing fruits is yet unknown. During tomato fruit ripening, the nuclear genes coding for enzymes involved in lycopene biosynthesis (phytoene synthase, phytoene desaturase, and ζ-carotene desaturases) are upregulated, whereas those coding for downstream enzymes (lycopene cyclases) are suppressed (Pecker et al., 1996; Ronen et al., 1999). Our data show that lycopene accumulates also in the absence of HSP21 (i.e., in cold-treated control fruits after transferal to room temperature). This suggests that HSP21 is not required for the induction of lycopene biosynthetic enzymes during fruit ripening or for the suppression of later steps in the pathway (i.e., lycopene cyclases). However, both the activation of the early steps and the suppression of the later steps were incomplete in the absence of HSP21 in cold-treated control fruits, as evidenced by the low levels of lycopene and the relatively high levels of lycopene cyclization product, β-carotene. Thus, HSP21 is necessary for obtaining maximal lycopene accumulation. This effect of HSP21 could be at the level of gene expression or protein activity. Because HSP21 accumulates in plastids, at the site of lycopene synthesis, it may promote the activity of the relevant enzymes (i.e., phytoene synthase, phytoene desaturase, and ζ-carotene desaturases). The chaperone/antioxidant-like activity of HSP21 may be required for optimal activities of these enzymes. As already mentioned, during the transition of chloroplasts to chromoplasts, there is massive degradation of membranes and proteins. At the same time and the same place, other proteins, involved in carotenoid synthesis and sequestration, are active (Gillaspy et al., 1993). Thus, HSP21 may protect these proteins from the destructive environment, which might include reactive oxygen species (Römer et al., 1992; Lawrence et al., 1997). Several studies have shown that chromoplast development associates with oxidative stress. Livne and Gepstein (1988) reported on the accumulation of the tomato chloroplastic superoxide dismutase at turning stage, and Kuntz et al. (1998) have shown the induction of oxidative stress-induced promoters during chromoplast development in tomato fruits. Moreover, Römer et al. (1992) have shown that differentiation of chloroplast to chromoplast in pepper fruits (*Capsicum annuum*) involves an active synthesis of potential antioxidants and redox modulators. Although the plastid localization of HSP21 supports a role for the protein in the promotion of carotenoid enzyme activity, it can also affect gene expression through crosstalk with nuclear elements. The incomplete suppression of lycopene cyclization in the absence of HSP21 in cold-treated control fruits supports this possibility.

In conclusion, this study provides evidence that the chloroplastic sHSP, HSP21, protects PSII under oxidative stress conditions but is also involved in plastid development (i.e., the conversion of chloroplasts to chromoplasts during tomato fruit maturation). Because the expression of other nonchloroplastic sHSPs also correlates with tomato fruit maturation (Sabehat et al., 1998; Low et al., 2000; Sun et al., 2002), they might also

play a role in this process. Elucidating their role in fruit development would provide a significant contribution to our understanding of the molecular machinery controlling fruit ripening.

METHODS

Plant Material

Lycopersicon esculentum line VF36 was used throughout the study. Seedlings at the six-leaf stage were transplanted into 18-cm pots filled with a peat:volcanic gravel mixture (1:1, v/v) and grown in a greenhouse under controlled temperature (26°C/18°C day/night) and natural daylength. Fruit development was determined as by Gillaspy et al. (1993), and color categories were defined accordingly (i.e., immature green [0], mature green [1], turning [2], orange [3], and red ripe [4]).

Construction of Binary Vectors and Plant Transformation

A 1-kb fragment (cDNA), containing the *L. esculentun Hsp21* open reading frame clone, was inserted into *Eco*RI-*PST*I in pCD (Broido et al., 1991), behind the 35S promoter and upstream of the octopin synthase terminator. The resultant plasmid was then digested with *Kpn*I-*Xba*I, and the fragment (1.65 kb) containing *HSP21* was inserted into the binary pCGN 1559 vector (Comai et al., 1990).

The chimeric construct was transferred via *Agrobacterium tumefaciens* to *L. esculentum* variety VF36 cotyledons using the transformation and regeneration methods described by McCormick (1991). To confirm stable transformation, kanamycin-resistant T0 plants were analyzed by PCR using specific 35S-F primer (5′-CTATCCTTCGCAAGACCCTTCC-3′) and HSP21-R primer (5′-ATTTCCCCTGTTC-CTGATGCT-3′). Plants containing the construct were grown in the greenhouse, and RNA gel blot analyses were performed to confirm the gene's expression in leaves under normal growth conditions.

RNA Gel Blot Analyses

Total RNA was isolated from leaves according to van Tunen et al. (1988) and from fruits using Tri Reagent, according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). Subsequently, 10 μg of total RNA were fractionated in a 1% (w/v) agarose gel containing formaldehyde and blotted onto Hybond N^+ membranes (Amersham Biosciences, Buckinghamshire, UK). The blots were hybridized in 0.263 M Na_2HPO_4, 7% (w/v) SDS, 1 mM EDTA, and 1% (w/v) BSA at 60°C with ^{32}P-labeled cDNA probe (Rediprime; Amersham Biosciences) for *HSP21* (*tom111*, Sabehat et al., 1998). The membranes were washed twice in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 60°C for 20 min each and exposed to x-ray film (Fuji, Tokyo, Japan) with two intensifying screens at −70°C.

Protein Extraction Gel Electrophoresis and Protein Gel Blot Analysis

Protein extraction was performed according to Hurkman and Tanaka (1986). Tissues were ground in liquid nitrogen and then in extraction buffer (0.1 M Tris-HCl, pH 8, 5% [w/v] sucrose, 5% [w/v] β-mercaptoethanol, 2% SDS, and 2 mM PMSF). After centrifugation, proteins were back-extracted from the supernatant with Tris-buffered phenol and washed three times in the same extraction buffer. The phenol phase was precipitated with five volumes of 100 mM ammonium acetate in methanol at −20°C. The precipitate was washed three times with 100 mM ammonium acetate in methanol, then with 80% acetone and then solubilized in Tricine sample buffer (Bio-Rad, Hercules, CA) for gel electrophoresis. Protein concentration was determined using the detergent-compatible protein assay (Bio-Rad).

Protein samples were boiled for 5 min with 5% β-mercaptoethanol and centrifuged at 13,500*g* for 5 min. Samples containing 50 μg of protein were loaded onto a 15% SDS polyacrylamide gel and run at 35 mA per gel. For immunoblot analyses, proteins were transferred to nitrocellulose membranes using a gel blotter (Bio-Rad). Membranes were blocked with 5% (w/v) nonfat milk powder in TBS/T (137 mM NaCl, 20 mM Tris-HCl, pH 7.6, and 0.5% [v/v] Tween) and then incubated for 3 h with anti-pea Hsp21 (a gift from E. Vierling's laboratory, University of Arizona, Tucson, AZ). A chemiluminescence detection kit (Amersham Biosciences) was used for HSP21 protein detection.

Stress Treatments to Leaves

Detached young leaves were placed in tap water in either an incubator for heat treatment or a cold room (4°C) for chilling treatment. All treatments were performed in the dark. Heat- or cold-treated leaves were exposed to white light supplied by halogen lamps (300 W). Temperature (25°C) at the leaf surface was controlled using a bottle filled with cold water, which was placed between the light source and the leaves.

Chlorophyll Fluorescence Measurements

Measurements of modulated chlorophyll fluorescence emission from the upper surface of the leaf were made using a pulse amplitude modulation fluorometer (PAM-101; H. Waltz, Effeltrich, Germany). The light guide that delivers the measuring and saturating light was held 5 mm from the upper surface of the leaves. The intensity of the measuring, modulated red light was ~0.1 $\mu mol \cdot m^{-2} s^{-1}$. Leaves were dark-adapted for 10 min before measuring the induction of fluorescence. The measuring beam was used to induce the minimum fluorescence (F_0). Saturating flashes were provided to completely reduce the PSII acceptor site Q_A^- and to measure the maximum fluorescence yield (F_m). The intensity of the saturating light flash (1 s) used for the measurements of F_m was 3000 $\mu mol \cdot m^{-2} s^{-1}$. The variable fluorescence (F_v) was calculated as $F_m - F_0$. The ratio F_v:F_m reflects the potential yield of the photochemical reaction of PSII (Krause and Weis, 1991).

Carotenoid Extraction, Measurement, and Analysis by HPLC

Fruit pigments were extracted from 0.2 to 0.6 g of fresh tissues. The tissues were ground in 1 mL acetone, the solvent was collected and filtered, the grinding was then repeated with 1 mL of dichloromethane, and the solvent was filtered and pooled with the acetone filtrate. Grinding and collecting of solvents were repeated until tissue lost all pigmentation. Pigments were then extracted by partitioning the solvent mixture against equal volume of diethyl ether and 0.2 volume of 12% (w/v) NaCl/H_2O. Ether fraction (upper phase) was collected and dried under a stream of nitrogen, and the dried lipid extract was redissolved in acetone. Total carotenoid content was determined spectroscopically according to Lichtenthaler (1987).

Carotenoids were separated by HPLC using a Waters system and a Spherisorb ODS2 C18 (5 μm, 4.6 × 250 mm) reverse-phase column (Waters, Milford, MA). Samples of 25 μL were injected to a Waters 600 pump. A gradient of acetonitrile:water (9:1) and ethylacetate at a constant flow rate of 1.6 mL/min was used as previously described (Isaacson et al., 2004). Light absorption peaks were detected in the range of 250 to 600 nm using a Waters 996 photodiode array detector. Carotenes were identified by their absorption spectra, retention time, and comparison with authentic reference substances. Quantification was performed by integrating the peak areas using Millennium chromatography software (Waters).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number U66300.

ACKNOWLEDGMENTS

This research was supported by Research Grant 610/98-17.1 from the Israel Science Foundation. This work was also supported by the Pearlstein Fund for research in floriculture at the Hebrew University. We would like to thank Victor Raskin, Izhak Ohad, and Joseph Hirschberg for helpful discussions.

Received February 17, 2005; revised April 12, 2005; accepted April 13, 2005; published May 6, 2005.

REFERENCES

Allen, D.J., and Ort, D.R. (2001). Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends Plant Sci. **6,** 36–42.

Almoguera, C., Prieto-Dapena, P., and Jordano, J. (1998). Dual regulation of a heat shock promoter during embryogenesis: Stage-dependent role of heat shock elements. Plant J. **13,** 437–446.

Andersson, B., and Aro, E.-M. (2001). Photodamage and D1 protein turnover in photosystem II. In Advances in Photosynthesis and Respiration, Regulation of Photosynthesis, Vol. 11, E.-M. Aro and B. Andersson, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 377–393.

Asada, K. (1996). Radical production and scavenging in the chloroplasts. In Molecular Biology of Free Radical Scavenging Systems, Vol. 5, N.R. Baker, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 123–150.

Broido, S., Loyter, A., and Vainstein, A. (1991). Expression of plant genes in transfected mammalian cells: Accumulation of recombinant preLHCIIb proteins within cytoplasmic inclusion bodies. Exp. Cell Res. **192,** 248–255.

Chen, Q., and Vierling, E. (1991). Analysis of conserved domains identifies a unique structural feature of a chloroplast heat shock protein. Mol. Gen. Genet. **226,** 425–431.

Cheung, A.Y., McNellis, T., and Piekos, B. (1993). Maintenance of chloroplast components during chromoplast differentiation in the tomato mutant *green flesh*. Plant Physiol. **101,** 1223–1229.

Comai, L., Moran, P., and Maslyar, D. (1990). Novel and useful properties of a chimeric plant promoter combining CaMV 35S and MAS elements. Plant Mol. Biol. **15,** 373–381.

Gillaspy, G., Ben-David, H., and Gruissem, W. (1993). Fruits: A developmental perspective. Plant Cell **5,** 1439–1451.

Gustavsson, N., Kokke, B.P.A., Anzelius, B., Boelens, W.C., and Sundby, C. (2001). Substitution of conserved methionines by leucines in chloroplast small heat shock protein results in loss of redox-response but retained chaperone-like activity. Protein Sci. **10,** 1785–1793.

Gustavsson, N., Kokke, B.P.A., Härndahl, U., Silow, M., Bechtold, U., Poghosyan, Z., Murphy, D., Boelens, W.C., and Sundby, C. (2002). A peptide methionine sulfoxide reductase highly expressed in photosynthetic tissue in *Arabidopsis thaliana* can protect the chaperone-like activity of a chloroplast-localized small heat shock protein. Plant J. **29,** 545–553.

Hamilton, E.W., and Heckathorn, S.A. (2001). Mitochondrial adaptation to NaCl. Complex I is protected by anti-oxidants and small heat shock proteins, whereas complex II is protected by proline and betain. Plant Physiol. **126,** 1266–1274.

Härndahl, U., Hall, R.B., Osteryoung, K.W., Vierling, E., Bornman, J.F., and Sundby, C. (1999). The chloroplast small heat shock protein undergoes oxidation-dependent conformational changes and may protect plants from oxidative stress. Cell Stress Chaperones **4,** 129–138.

Härndahl, U., Kokke, B.P.A., Gustavsson, N., Linse, S., Berggern, K., Tjerneld, F., Boelens, W.C., and Sundby, C. (2001). The chaperone-like activity of a small heat shock protein is lost after sulfoxidation of conserved methionines in a surface-exposed amphipathic α-helix. Biochim. Biophys. Acta **1545,** 227–237.

Härndahl, U., and Sundby, C. (2001). Does the chloroplast small heat shock protein protect photosystem II during heat stress in vitro? Physiol. Plant. **111,** 273–275.

Heckathorn, S.A., Downs, C.A., Sharkey, T.D., and Coleman, J.S. (1998). The small, methionine-rich chloroplast heat-shock protein protects photosystem II electron transport during heat stress. Plant Physiol. **116,** 439–444.

Heckathorn, S.A., Ryan, S.L., Baylis, J.A., Wang, D., Hamilton, E.W., Cundiff, L., and Dawn, S.L. (2002). In vitro evidence from an *Agrostis stolonifera* selection genotype that chloroplast small heat-shock protein can protect photosystem II during heat stress. Funct. Plant Biol. **29,** 933–944.

Hurkman, W.J., and Tanaka, C.K. (1986). Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol. **81,** 802–806.

Inoglia, T., and Craig, E. (1982). Four small *Drosophila* heat shock proteins are related to each other and to mammalian α-crystallin. Proc. Natl. Acad. Sci. USA **79,** 2360–2364.

Isaacson, T., Ohad, I., Beyer, P., and Hirschberg, J. (2004). Analysis in vitro of the enzyme CRTISO establishes a poly-cis-carotenoid biosynthesis pathway in plants. Plant Physiol. **136,** 4246–4255.

Jofre, A., Molinas, M., and Pla, M. (2003). A 10-kDa class-CI sHsp protects *E. coli* from oxidative and high-temperature stress. Planta **217,** 813–819.

Krause, G.H., and Weis, E. (1991). Chlorophyll fluorescence and photosynthesis—The basics. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42,** 313–349.

Kuntz, M., Chen, H.C., Simkin, A.J., Romer, S., Shipton, C.A., Drake, R., Schuch, W., and Bramley, P.M. (1998). Upregulating of two ripening-related genes from a nonclimacteric plant (pepper) in a transgenic climacteric plant (tomato). Plant J. **13,** 351–361.

Lawrence, S.D., Cline, K., and Moore, G.A. (1997). Chromoplast development in ripening tomato fruit: Identification of cDNAs for chromoplast-targeted proteins and characterization of a cDNA encoding a plastid-localized low-molecular-weight heat shock protein. Plant Mol. Biol. **33,** 483–492.

Lee, G.J., Roseman, A.M., Saibil, H.R., and Vierling, E. (1997). A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. EMBO J. **16,** 659–671.

Lee, G.J., and Vierling, E. (2000). A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. Plant Physiol. **122,** 189–198.

Levine, R.L., Mosoni, L., Berlett, B.S., and Stadtman, E.R. (1996). Methionine residues as endogenous antioxidants in proteins. Proc. Natl. Acad. Sci. USA **93,** 15036–15040.

Lichtenthaler, H.K. (1987). Chlorophylls and carotenoids: Pigments of photosynthetic membranes. Methods Enzymol. **148,** 350–382.

Livne, A., and Gepstein, S. (1988). Abundance of the major chloroplast polypeptides during development and ripening of tomato fruits. Plant Physiol. **87,** 239–243.

Low, D., Brandle, K., Nover, L., and Forreiter, C. (2000). Cytosolic heat-stress proteins Hsp17.7 class I and Hsp17.3 class II of tomato act as molecular chaperones in vivo. Planta **211,** 575–582.

Lurie, S., and Klein, J.D. (1991). Acquisition of low-temperature tolerance in tomatoes by exposure to high temperature stress. J. Am. Soc. Hortic. Sci. **116,** 1007–1012.

McCormick, S. (1991). Transformation of tomato with *Agrobacterium tumefaciens*. In Plant Tissue Culture Manual, H. Linclsey, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1–9.

Medina-Escobar, N., Cardenas, J., Munoz-Blanco, J., and Caballero, J.L. (1998). Cloning and molecular characterization of a strawberry fruit ripening-related cDNA corresponding a mRNA for a low-molecular-weight heat-shock protein. Plant Mol. Biol. **36,** 33–42.

Osteryoung, K.W., and Vierling, E. (1994). Dynamics of small heat shock protein distribution within the chloroplasts of higher plants. J. Biol. Chem. **269,** 28676–28682.

Pecker, I., Gabbay, R., Cunningham, F.X., Jr., and Hirschberg, J. (1996). Cloning and characterization of the cDNA for lycopene β-cyclase from tomato reveals decrease in its expression during fruit ripening. Plant Mol. Biol. **30,** 807–819.

Römer, S., D'Harlingue, A., Camera, B., Schantz, R., and Kuntz, M. (1992). Cysteine synthase from *Capicum annuum* chloroplasts characterization and cDNA cloning and up-regulated enzyme during fruit development. J. Biol. Chem. **267,** 17966–17970.

Ronen, G., Cohen, M., Zamir, D., and Hirschberg, J. (1999). Regulation of carotenoid biosynthesis during tomato fruit development: Expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant *Delta*. Plant J. **17,** 341–351.

Sabehat, A., Lurie, S., and Weiss, D. (1998). Expression of small heat-shock proteins at low temperatures: A possible role in protecting against chilling injuries. Plant Physiol. **117,** 651–658.

Sabehat, A., Weiss, D., and Lurie, S. (1996). The correlation between heat shock protein accumulation and persistence and chilling tolerance in tomato fruit. Plant Physiol. **110,** 531–537.

Sun, W., Bernard, C., van de Cotte, B., van Montagu, M., and Verbruggen, N. (2001). At-HSP17.6A, encoding a small heat-shock protein in Arabidopsis, can enhance osmotolerance upon overexpression. Plant J. **27,** 407–415.

Sun, W., van Montagu, M., and Verbruggen, N. (2002). Small heat shock proteins and stress tolerance in plants. Biochim. Biophys. Acta **1577,** 1–9.

Tsvetkova, N.M., Horvath, I., Torok, Z., Wolkers, W.F., Balogi, Z., Shigapova, N., Crowe, L.M., Tablin, F., Vierling, E., Crowe, J.H., and Vigh, L. (2002). Small heat-shock proteins regulate membrane lipid polymorphism. Proc. Natl. Acad. Sci. USA **99,** 13504–13509.

van Tunen, A.J., Koes, R.E., Spelt, C.E., van der Krol, A.R., Stuitje, A.R., and Mol, J.N.M. (1988). Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*: Coordinate, light-regulated and differential expression of flavonoid genes. EMBO J. **7,** 1257–1263.

Wang, D., and Luthe, D.S. (2003). Heat sensitivity in a bentgrass varient. Failure to accumulate a chloroplast heat shock protein isoform implicated in heat tolerance. Plant Physiol. **133,** 319–327.

Wehmeyer, N., Hernandez, L.D., Finkelstein, R.R., and Vierling, E. (1996). Synthesis of small heat-shock proteins is part of the developmental program of late seed maturation. Plant Physiol. **112,** 747–757.