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RESEARCH ARTICLE

Cool temperatures interfere with D1 synthesis in tomato by causing ribosomal pausing

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Abstract Photodamage occurs when leaves are exposed to light in excess of what can be used for photosynthesis and in excess of the capacity of ancillary photoprotective as well as repair mechanisms. An important site of photodamage is the chloroplast encoded D1 protein, a component of the photosystem II (PSII) reaction center. Even under optimal growth irradiance, D1 is photodamaged necessitating rapid turnover to prevent the accumulation of photodamaged PSII reaction centers and consequent inhibition of photosynthesis. However, this on-going process of D1 turnover and replacement was impeded in the chilling-sensitive tomato (*Solanum lycopersicum*) plants when exposed to high-growth light at cool temperature. The decrease in D1 turnover and replacement was found not to be due to changes in the steady-state level of the *psbA* message. While the recruitment of ribosomes to *psbA* transcript, initiation of D1 translation, and the association of polysomes with the thylakoid membrane occurred normally, chilling temperatures caused ribosomal pausing during D1 peptide elongation in tomato. The pause locations were non-randomly located on the D1 transcript. The interference with translation caused by ribosomal pausing allowed photodamaged PSII centers to accumulate leading to the consequent inhibition of photosynthesis.

Keywords D1 · Environmental stress · PSII · Low temperature · High light · Translation · Polysomes

Abbreviations

PSII	Photosystem II
Fv/Fm	Ratio of variable maximal fluorescence of PSII, represents the dark-adapted maximum efficiency of PSII photochemistry
PPFD	Photosynthetic photon flux density
RuBP	Ribulose-1,5-bisphosphate
OEC	Oxygen-evolving complex
P680	Reaction center chlorophyll associated with PSII
BBY particles	Oxygen-evolving PSII preparations

Introduction

Plants are routinely exposed to a variety of environmental stresses that can adversely affect both growth and reproductive success. The ability, or lack there of, to adapt to environmental stress exposure can be a driving force for speciation (Lexer and Fay 2005). One of the more common abiotic challenges plants face daily is light in excess

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of what can be used for photochemistry. Exactly what constitutes excess light for a leaf depends on its instantaneous environmental conditions and can vary over an exceedingly wide range of irradiance levels. For example, an irradiance level that is permissible for a C3 plant during the midmorning often becomes excessive in the afternoon as stomatal conductance declines in response to declining leaf water potentials thus restricting CO_2 entry and rendering even moderate irradiances in the top of a crop canopy in excess of photosynthetic capacity.

In order to cope with this environmental stress and avoid photodamage, plants have evolved a variety of responses operating on multiple time scales including strategies to reduce light absorption such as leaf movement (paraheliotrophism), chloroplast movement (Kasahara et al. 2002), the rearrangement of light-harvesting complexes in the thylakoid membrane to optimize the distribution of light received (Melis 1991), and changes in the size of photosynthetic antenna via changes in gene expression (Rochaix 2001). There are also photoprotective mechanisms for the dissipation of absorbed energy, most notably non-photochemical quenching (the thermal dissipation of excess energy) (Holt et al. 2004) and the use of alternative terminal electron acceptors (Ort and Baker 2002) such as O_2 in the Mehler-ascorbate-peroxidase reaction (water–water cycle) (Asada 2000; Rizhsky et al. 2003) and photorespiration (Kozaki and Takeba 1996; Osmond et al. 1997). Although the Mehler reaction and photorespiration both provide a sink for excess electrons, they also produce compounds that are potentially hazardous to the plant. In the Mehler reaction, highly reduced ferredoxin can result in the passage of electrons from PSI directly to oxygen producing superoxide radicals, which are converted by superoxide dismutase into oxygen and another reactive oxygen species hydrogen peroxide. High levels of reactive oxygen species can lead to loss of membrane integrity, damage to proteins, and ultimately to cell death. Additionally, both hydrogen peroxide and singlet oxygen have been implicated in inhibiting the repair of photosystem II (PSII), most notably D1 (reviewed in Nishiyama et al. 2006). Scavenging systems are present in the chloroplast to 'detoxify' reactive oxygen species and mitigate their potential for damage. Hydrogen peroxide can be scavenged by ascorbate/ascorbate-peroxidase and chloroplast membranes can be further protected by carotenoids and tocopherols (Ledford and Niyogi 2005; Asada 2006).

When this aggregate of photoprotective mechanisms is not enough, leaves can become photodamaged, an irreversible or slowly reversible condition characterized by a decrease in the ability to efficiently utilize absorbed light. The PSII reaction center has been consistently identified as a site of photodamage and recent studies suggest that the oxygen-evolving complex (OEC) may be the first site damaged, leading to the release of the manganese cluster (reviewed in Nishiyama et al. 2006). Although the primary site of photodamage is not certain, one important site is the PSII reaction center core protein D1 (Kyle et al. 1984; Melis 1991; Aro et al. 1993), encoded by the chloroplast *psbA* gene.

Photosystem II is a large multi-subunit complex, containing proteins encoded by both the nuclear and chloroplast genomes. There are two main subcomponents: a reaction center core, and the peripheral light-harvesting complexes. The chloroplast-encoded proteins D1, D2, and the subunit cytochrome b559, the smallest complex able to perform light-induced charge separation (Nanba and Satoh 1987), which, along with the chlorophyll *a*-binding subunits CP47 and CP43, complete the reaction center core complex. D1 contains the ligands for binding Q_B (mobile quinone electron carrier), P680 (PSII reaction center associated chlorophyll a), and the proteins of the OEC. D1, encoded by the chloroplast *psbA* gene, is synthesized as a larger peptide that undergoes both N-terminal processing and C-terminal cleavage as well as reversible, light-dependent phosphorylation (Elich et al. 1992; Rintamaki et al. 1995). The C-terminal cleavage is necessary for the assembly of the OEC (Hankamer et al. 1997) while reversible phosphorylation is believed to help stabilize the complex (Ebbert and Godde 1996). During chloroplast biogenesis, PSII assembly is a stepwise process and mutant studies suggest coordinated accumulation of D1, D2 and CP47 (Bennoun et al. 1986; Erickson et al. 1986; Jensen et al. 1986; Devitry et al. 1989). A recent study in *Chlamydomonas reinhardtii* identified initiation of translation as a main regulatory step in the biogenesis of the core proteins D1 and apoCP47 (Minai et al. 2006). Repair of photodamaged reaction centers, however, is most likely regulated at elongation of translation (Kim et al. 1994; Kettunen et al. 1997; Minai et al. 2006) as the reaction center core must be partially disassembled to allow the cotranslational insertion of D1 (Adir et al. 1990; van Wijk et al. 1997; Muller and Eichacker 1999; Zhang et al. 1999). Cotranslational insertion explains why D1 is not found free in the membrane and is typically associated with at least D2 (Zhang et al. 1999). D1 is a very labile protein and damage to the protein (Keren et al. 1995) as well as photoinhibition (Tyystjävi and Aro 1996) has been shown to be proportional to light intensity. Under normal growth conditions, D1 damage is compensated by the simultaneous synthesis and replacement of new protein. However, when the rate of damage exceeds the rate of repair an

accumulation of inactive PSII centers results in a consequent decrease in photosynthesis (Vasilikiotis and Melis 1994; Horton et al. 1996).

Plants grown outside the climatic regions in which they have evolved, which include many of the world's most important crop plants, can be particularly vulnerable to photodamage. The combination of high light with low temperature is particularly deleterious to plants with evolutionary origins in tropical and semi-tropical regions such as tomato (Martin and Ort 1985) and maize (Baker et al. 1983). In addition to damaging PSII and thereby the capacity for electron transport, these interacting stresses have also been shown to interfere with the reductive activation of stromal bisphosphatases (Sassenrath et al. 1990; Hutchison et al. 2000) resulting in limited carbon assimilation after the light chill, possibly instigated by a decrease in RuBP (Ribulose-1,5-bisphosphate) regeneration capacity (Hirotsu et al. 2005). It is clear that the primary loss of activity is caused by direct impact at the biochemical level, as opposed to a limitation imposed by stomatal-mediated leaf gas exchange (Martin et al. 1981).

As a primary site of photodamage, D1 has the highest turnover rate of any protein in the chloroplast (Mattoo et al. 1999). When D1 becomes damaged, it is dephosphorylated (Rintamaki et al. 1995) and an initial cleavage between the D and E loops is made by the Clp protease, DegP2 (Haußühl et al. 2001). The cleaved D1, still associated with D2, CP43, CP47, and the OEC, migrates from the appressed region of the grana to the stroma lamella where the subunits disassociate, with the exception of D2 which remains associated with D1 (Zhang et al. 1999, 2000; Baena-Gonzalez and Aro 2002). D1 is then further degraded by a FtsH protease allowing the replacement nascent D1 to associate with D2 (Lindahl et al. 2000; Bailey et al. 2002; Zaltsman et al. 2005). Under permissive conditions, damaged D1 is degraded and replaced (Vasilikiotis and Melis 1994; Mattoo et al. 1999). It has been shown that the rate constant of recovery from photoinactivation is temperature dependent, and is much slower under chilling conditions even in non-chilling sensitive species (Tsonev and Hikosaka 2003, Allakhverdiev and Murata 2004). Additionally, there is a decrease in the de novo synthesis and degradation of D1 (Salonen et al. 1998) under low-temperature conditions.

We were interested in investigating the mechanism of the photodamage characteristic of chilling-sensitive plants when exposed to the combination of high light and cool temperatures. In this work, we have shown that the decrease in D1 synthesis is not due to a change in the steady-state level of the message for D1, but rather due to an event that causes ribosomes to pause at specific sites during the translation of *psbA*, which appears to be the result of the damaged D1 protein not being efficiently removed from the reaction center.

Materials and methods

Plant growth conditions

Solanum lycopersicum var. Mill *Floramerica* seeds were purchased from Tomato Growers (Fort Myers, FL, USA). Plants were grown in growth chambers in Sunshine Mix LC1 (SunGro Horticulture Inc., Bellevue, WA, USA) under a 14 h photoperiod of 350 μmol photons m^{-2} s^{-1} with air temperature of 27/25°C (light/dark) and 60% relative humidity. Plants were fertilized biweekly with 12-31-14 fertilizer (Plant Marvel Laboratories, Inc., Chicago Heights, IL, USA) with a 10 mM KNO_3 supplementation.

In vivo pulse labeling of proteins

Treatment conditions were as follows: Control: 350 μmol photons m^{-2} s^{-1} with air temperature of 26°C; High light: 1000 μmol photons m^{-2} s^{-1} with air temperature of 26°C; High light at low temperature: 1000 μmol photons m^{-2} s^{-1} with air temperature of 4°C; Growth light a low temperature: 350 μmol photons m^{-2} s^{-1} with air temperature of 4°C. High-light conditions were reached using supplemental lighting and chilling treatments were performed in a low-temperature growth chamber at 70% relative humidity. Treatments were for 6 h. The plants were then returned to growth conditions where two fully expanded leaflets (from the fourth leaf) were lightly abraded and 200 μCi of [^{35}S]methionine was applied (Martino-Catt et al. 1993). Incorporation was allowed to continue for 15 min, at which time the leaves were rinsed, harvested, and the thylakoid proteins were isolated.

Isolation, separation of thylakoid proteins, and Western blot analysis

Thylakoid proteins were isolated essentially as described in Cooper and Ort (1988). Proteins were denatured by heating at 70°C for 10 min in sample buffer (1:1; Laemelli sample buffer, 0.062 M Tris (pH 6.8), 2% SDS, 0.2% glycerol, 0.71 M β-mercaptoethanol, 0.001% (w/v) bromophenol blue) prior to separation by 10–20% gradient SDS-PAGE (Bio-Rad, Hercules, CA, USA). Molecular weights were estimated by running prestained standards along side the samples (Bio-Rad, Hercules, CA, USA). Proteins were electroblotted to Immobilon-P PVDF membrane (Millipore, Bedford, MA, USA) using a Bio-Rad Transblot Cell (Bio-Rad, Hercules, CA, USA) operated at constant

voltage (100 V) for 1 h with cooling according to the Bio-Rad protocol. The western blot was performed essentially according to the Bio-Rad Immuno-Blot assay kit protocol. The BCIP/NBT liquid substrate system from Sigma (St. Louis, MO, USA) was used for detection. Polyclonal D1 antibody, raised against full length D1 in rabbits, was obtained from Anastasios Melis, University of California, Berkley, CA, USA.

BBY subchloroplast particle isolation

BBY particles (oxygen-evolving PSII preparations) were isolated using the method of Schiller and Dau (2000). Thylakoids were placed in incubation buffer (1 M glycine-betaine, 15 mM NaCl, 10 mM $MgCl_2$, 25 mM Hepes (pH 6.0) in an ice bath in the dark with slow stirring at a final chlorophyll concentration of 22 mM. About 25% Triton X-100 (v/v) (Sigma, St. Louis, MO, USA) was added dropwise to the thylakoid solution to attain a detergent concentration of 25 mg/mg chlorophyll and incubated for 20 min. The suspension was then centrifuged for 20 min at 48,000 × *g* at 4°C. The pellet consisted of a bottom white starch layer and an upper green layer, which contained the BBY particles. The upper green layer was carefully resuspended in BBY storage buffer (0.4 M sucrose, 5 mM $MgCl_2$, 0.015 M NaCl, 0.02 M Hepes (pH 7.5) and stored at –20°C.

Total RNA isolation, gel electrophoresis and northern blot analysis

Fully expanded leaflets (from the fourth leaf) were ground in liquid nitrogen and total RNA was extracted using Trizol reagent (GibcoBRL, Life Technologies, Inc., Rockville, MD, USA) as in Chung et al. (2006). RNA quality was checked on 1% TAE agarose gel and absorbance at 260 nm was determined. RNA was separated on denaturing formaldehyde gels containing 2.5% 40× gel buffer (1.3 M triethanolamine, 0.08 M $Na_2EDTA–2H_2O$, adjusted to pH 7.5 with 85% H_2PO_4), 1.5% agarose, 8.4% formaldehyde and adjusted to the desired volume with sterile purified H_2O. The gels were prerun for at least 15 min at 70 V before the samples were loaded. The samples were prepared by combining 1 µl RNA (2 µg/µl) with 4 µl sample buffer (3.125% 40× gel buffer, 20.625% formaldehyde, 62.5% formamide, 75% glycerol, 0.02 g bromophenol blue) heated to 60°C for 10 min and then chilled on ice. The gel was run at 60 V until the dye front had progressed 75% of the length of the gel. RNA was transferred from the agarose gel to nylon membrane using passive downward transfer as described in Ambion (Austin, TX, USA) Technical Bulletin #169. Tomato *psbA* was randomly labeled using MegaPrime kit (Amersham, Piscataway, NJ, USA). Probe hybridization was done using the ULTRAhyb protocol from Ambion.

Polysome isolation

A modification of a protocol from Barkan (1993) was used to isolate total leaf polysomes. Frozen leaf tissue was ground in a chilled mortar and pestle under liquid nitrogen in 0.2 M Tris (pH 9.0), 0.2 M KCl, 0.025 M EGTA, 0.035 M $MgCl_2$, 0.2 M sucrose, 2% PTE, 1% Triton X-100, 0.5–1.0 mg/ml heparin, 0.1 M β-mercaptoethanol, 100 µg/ml chloramphenicol, 25 µg/ml cycloheximide until it was a slurry. The sample was centrifuged for 2 min at 2000 × *g* to remove large particles. The slurry was filtered through glass wool and the flow-through was collected and incubated on ice for 15 min. The samples were then microcentrifuged for 15 min at 12,000 × *g* in order to remove membranes. The chlorophyll concentration was determined using the method of Graan and Ort (1984).

For polysome fractionation, the supernatant was layered onto a linear sucrose gradient (15–55% sucrose, 0.04 M Tris (pH 8.0), 0.02 M KCl, 10 mM MgCl, 0.5–1.0 mg/ml heparin) and centrifuged for 205 min at 32,000 rpm (SW 40Ti rotor, Beckman, Fullerton, CA, USA). Fractions of 0.5 ml were collected and absorbance at 254 nm was determined using ISCO type 6 optical unit (Lincoln, NE, USA). In the case of total polysomes, the supernatant was layered onto sucrose cushions (1.5 M sucrose, 0.04 M Tris (pH 8.0), 0.02 M KCl, 10 mM MgCl, 0.5–1.0 mg/ml heparin) and centrifuged for 220 min at 40,000 rpm (type 50Ti rotor Beckman, Fullerton, CA, USA). Pellets were resuspended in purified H_2O.

Fractions and pellets were diluted with 0.05 M Tris (pH 8.0), 0.02 M EDTA, 0.5% SDS, and phenol-extracted once. Total RNA was precipitated with 1/10 volume 3 M Na-acetate pH 5.2 and 2.5 volume 100% EtOH at –20°C for 2 h. The RNA was resuspended in 10 mM Tris (pH 8.5) and separated on formaldehyde denaturing gels, as described above.

Chloroplast isolation and chloroplast fractionation

Chloroplast isolation was performed by a modified method of Klein and Mullet (1986). All procedures were performed at 4°C or on ice. Fully expanded tomato leaflets (from fourth leaf) were diced and briefly blended in a Warring blender in isolation buffer (0.33 M sorbitol, 0.05 M Tricine (pH 7.6), 10 mM NaCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM EDTA, 1% PVP-40, 0.5% BSA, 0.2% sodium ascorbate, 0.1% DTT). The homogenate was filtered through four layers of Miracloth (Calbiochem, La Jolla,

CA, USA) and one layer of cheesecloth and centrifuged for 2 min at 12,000 × *g*. The pellet was resuspended in 1 ml resuspension buffer (0.33 M sorbitol, 0.05 M Hepes (pH 7.6), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5% BSA) using a soft brush. The resuspended chloroplasts were layered over a discontinuous Percoll (Sigma, St. Louis, MO, USA) gradient (40–80% Percoll in resuspension buffer, 8 ml to 4 ml) and centrifuged for 2 min at 12,000 × *g*. Chloroplasts were removed from the interface and resuspended in one volume resuspension buffer and centrifuged for 1 min at 2000 × *g* to remove Percoll. Chloroplasts were fractionated into membrane and soluble fractions by the method of Klein et al. (1988).

Fluorescence measurements

Chlorophyll fluorescence was measured with a PAM-2000 portable chlorophyll fluorometer (Walz, Effeltrich, Germany). Plants were dark adapted for 20 min prior to measurement. All values are an average of six samples.

Results

Photodamage was accentuated under conditions of high light and low temperature

Exposure of tomato plants to either high light or cool temperatures resulted in a decrease in variable chlorophyll fluorescence indicative of damage to PSII (Fig. 1). Tomato plants were exposed to the different light and temperature conditions for 6 h under controlled environmental conditions and then dark adapted for 20 min at room temperature before chlorophyll fluorescence measurements were taken. The combination of high light and low-temperature invoked the largest decrease in Fv/Fm of nearly 30% indicating that de novo synthesis and turnover of D1 was inhibited by high light at low temperature exposure. However, after 2 h of recovery at low light and growth temperature, Fv/Fm in high light and low temperature exposed plants returned to levels comparable to control showing that the damage could be repaired under permissive conditions.

After exposure to high light, low temperature or a combination of both, leaves were pulsed with [^{35}S]-translabeled methionine to monitor changes in D1 protein synthesis in response to the stress treatment (Fig. 2A). The pulse labeling was done at growth temperature to avoid any direct thermodynamic effects of temperature on the rate of protein synthesis. D1 synthesis was suppressed 25% by high light conditions and 50% by low temperature at growth light (Fig. 2A). However, when combined, the

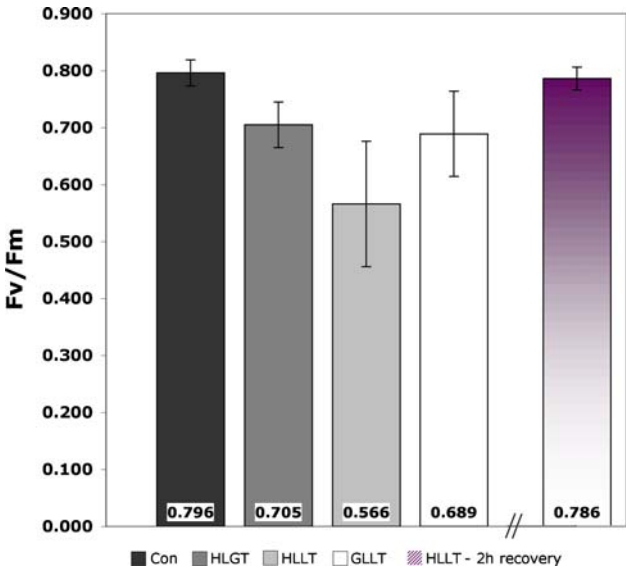

Fig. 1 Chlorophyll fluorescence measurements of tomato leaves. Leaf discs were taken from plants exposed to control (Con; 350 µmol photons m^{-2} s^{-1} with air temperature of 26°C), high light at growth temperature (HLGT; 1000 µmol photons m^{-2} s^{-1} with air temperature of 26°C), high light at low temperature (HLLT; 1000 µmol photons m^{-2} s^{-1} with air temperature of 4°C) or growth light at low temperature (GLLT; 350 µmol photons m^{-2} s^{-1} with air temperature of 4°C) for 6 h and dark adapted for 30 min prior to variable fluorescence measurements (Fv/Fm). For the 2 h recovery sample (after break in x axis), HLLT plants were allowed to recover for 2 h at low light (150 µmol photons m^{-2} s^{-1}) at growth temperature (26°C) prior to sampling. The numbers are the average of four independent experiments

stresses of high light and low temperature depressed the de novo synthesis of D1 by >80%. Experiments indicate that although there was a 20–25% drop in the incorporation of methionine into D1 in the high-light growth temperature samples compared to control, this resulted in a somewhat smaller 12% decrease in light-energy transfer efficiency. We are uncertain about the cause of this small difference.

Western blot analysis with polyclonal antibodies raised against D1 showed that even though the combined stress of high light and low temperature severely inhibited D1 synthesis, there was no detectable change in the amount of full length D1 protein (Fig. 2B).

There was no change in steady-state *psbA* message level associated with the decline D1 synthesis

In order to determine if the decrease in D1 de novo synthesis in response to high light and low temperature was caused by a decrease in *psbA* transcript, total RNA was isolated from control as well as tomato plants exposed to low temperature and high light. Northern blot analysis of *psbA* illustrated that there was little change in the steady-

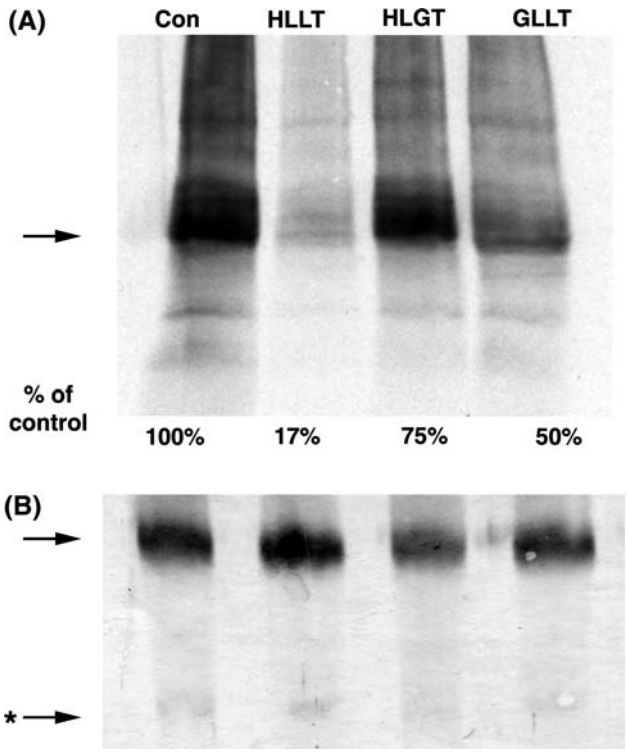

Fig. 2 In vivo pulse labeling of proteins with [^{35}S]methionine (**A**) and western blots (**B**) of thylakoid proteins after exposure to 6 h high light at growth temperature (HLGT), high light and low temperature (HLLT), growth light at low temperature (GLLT) and control (con). Labeling was done at room temperature to minimize any low temperatures effect on translation. Thylakoid proteins were isolated and separated by SDS-PAGE. Arrow indicates the full length D1 protein. Proteins were quantified by densitometry from three independent experiments and are expressed as percent of control. There was a decrease in the de novo synthesis of D1 under high light and low temperature exposure compared to control conditions but there was no change in the total amount of protein as measured by Western blot

state message levels under these conditions (i.e., minor increase of 4%, Fig. 3).

The association of *psbA* mRNA with chloroplast ribosomes increased following high light, low-temperature exposure

Although no change in the steady-state *psbA* level under the condition of high light and low temperature was observed, this cannot be taken to mean that the message was recruited to ribosomes and subsequently translated. Total polysomes were isolated to determine if the light and/or chilling treatment affected the association of *psbA* transcripts with ribosomes (Fig. 4A). Somewhat surprisingly there was no change in *psbA* association with ribosomes after a 6 h high light and chilling exposure even though the rate of D1 synthesis was depressed >80% relative to control conditions (Fig. 2A). In order to ensure that the RNA was truly associated with ribosomes, EDTA was added to disperse the polysomes by removal of magnesium. After EDTA treatment, minimal *psbA* mRNA was found in the pellet (Supplemental Fig. 1) indicating that the message was bound to ribosomes even under the stress condition.

D1 is a membrane-spanning protein, and as such is translated on membrane-bound ribosomes, thereby allowing for co-translational insertion (Zerges and Rochaix 1998). A potential reason for the decrease in de novo synthesis of D1 during light exposure at low temperature is that the ribosome-nascent chain complex may not be targeted to the thylakoid membrane. To address this possibility, intact chloroplasts were isolated and fractionated into membrane and soluble fractions. Polysomes were isolated from each of these fractions and the associated RNA was used for northern blot analysis. Under the conditions of high light at low temperature we observed a 42% increase in the association of *psbA* polysomes with chloroplast membranes compared to control (Fig. 4B).

psbA polysomes are larger following high light at low temperature exposure

Isolation of polysomes through a sucrose gradient allows separation based on the numbers of ribosomes associated with the transcript (Barkan 1998). Under control conditions, the majority of *psbA* message was found near the top of the gradient with smaller numbers of ribosomes (Fig. 5). With the combination of high light and low temperature, there were on average larger numbers of ribosomes associated with the *psbA* message (Fig. 5).

An increase in specific D1 translation intermediates after high light at low-temperature indicated non-random ribosomal pause sites

The increase in *psbA* polysome size caused by the chilling and light treatment indicated that ribosomes paused during translation, most likely during elongation. If the chilling and light exposure caused pausing at specific sites along the *psbA* transcript, translation intermediates associated with the polysome pellets would be anticipated. Polyclonal D1 antibodies were used to probe proteins isolated from polysome pellets for the presence of intermediates. The translation intermediates of the same size were found in all treatments (Fig. 6), but under the high light at low-temperature condition there was an increase in specific bands suggesting the progression of ribosomes along the *psbA* transcript stalled or paused at specific sites during translation. The predominant D1 fragments in the polysome

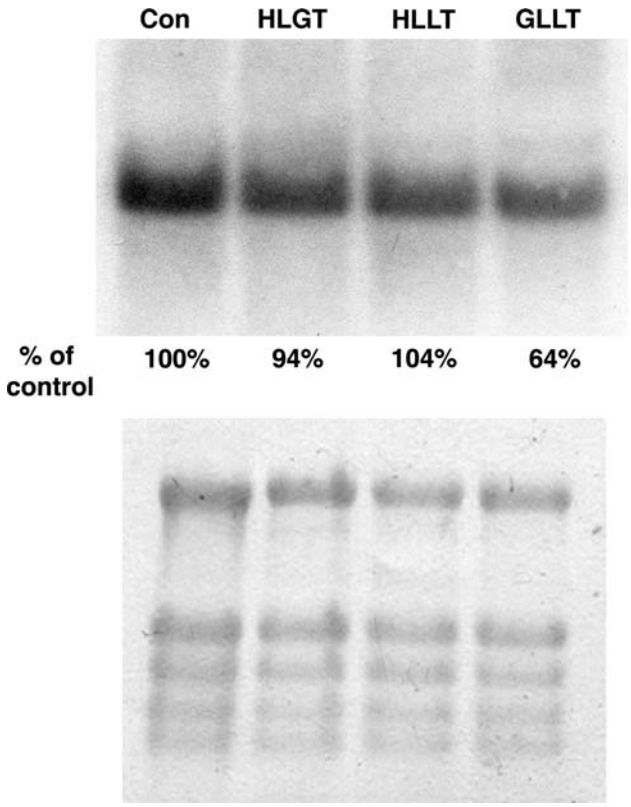

Fig. 3 Total RNA from tomato leaves probed with *psbA*. Top image is autoradiograph of blot probed with *psbA*, bottom image is methylene blue staining of the blot to demonstrate equal RNA loading. Con is control, HLGT is high light at growth temperature, HLLT is high light at low temperature and GLLT is growth light at low temperature. Quantification was done using a phosphorimager and is an average of three independent experiments

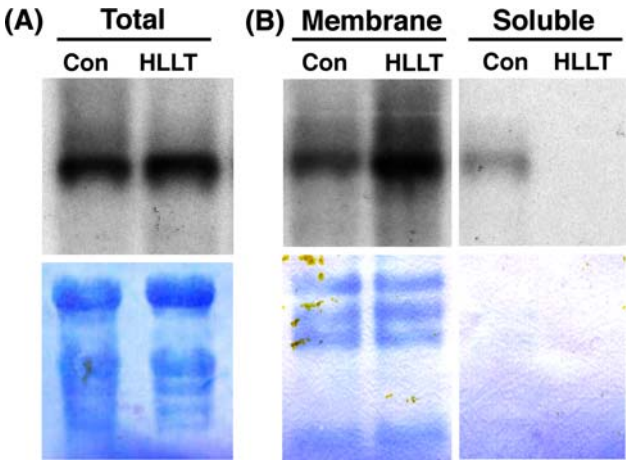

Fig. 4 Northern blots of *psbA* isolated from total polysomes from control (Con) and high light, low temperature (HLLT) exposed plants. (**A**) Total polysomes were isolated from whole leaves through a sucrose cushion. In HLLT exposed plants, *psbA* levels are 5% less than control. (**B**) Chloroplasts were isolated from leaf tissue, the sample was fractionated into membrane and soluble fractions. Total polysomes were isolated from both fractions by centrifugation through a sucrose cushion. There is a 90% decrease in *psbA* associated with polysomes found in the soluble fraction and a 42% increase in *psbA* associated with polysomes in the membrane fraction. Quantification was done using a phosphorimager and is expressed by percent of control. Quantification is an average of three independent experiments. The methylene blue stained blot demonstrating equal loading is presented below each Northern blot

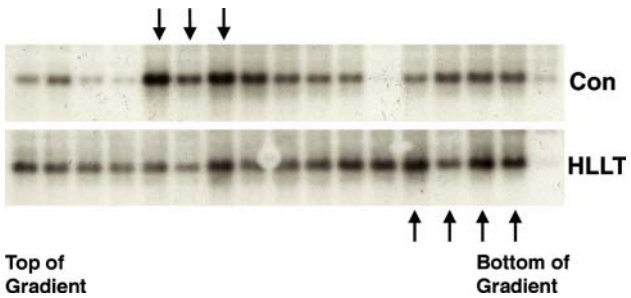

Fig. 5 Profiles of *psbA* in tomato leaf polysomes probed from control (Con) and high light, low temperature (HLLT) exposed plants. Total leaf polysomes were isolated on sucrose density gradients to separate by the amount of ribosomes. The greater the number of ribosomes on a message the further down it will be found in the gradient. Arrows point to fractions where there is a difference in *psbA* levels between the two treatments. ''Top'' and ''Bottom'' refer to the locations on the gradient

pellets are between 15 and 19 kDa. These fragments correspond to the translation intermediates that were observed in barley where the major sizes observed were 15 and 23 kDa (Kim et al. 1991).

The migration of PSII centers with photodamaged D1 was impaired by high-light chilling exposure

In chloroplasts, PSII is predominantly found in grana stacks of the thylakoid. However, in order for damaged D1 to be replaced, the complex needs to migrate from the grana to the stroma lamellae, the site of cotranslational D1 insertion. If damaged D1 is not removed from the reaction center, the new D1 cannot be properly inserted (Baena-Gonzalez and Aro 2002; Aro et al. 2005; Nixon et al. 2005). To explore the possibility that PSII was not migrating from the grana after the high light at low-temperature exposure, the amount of D1 in the grana stacks was compared between the control and stress condition (Fig. 7). Thylakoids, isolated from leaves after 6 h of high light at low temperature or from control leaves, were partially solubilized with Triton X-100 (Schiller and Dau 2000) allowing the isolation of the grana stacks as BBY particles (Berthold et al. 1981). Western blot analysis revealed a distinct band at 32.5 kDa, the full-length D1 protein. An immuno reactive band at 22 kDa was observed in the BBY particles isolated from the high light, low-temperature exposed leaves, corresponding to the size of the D1 degradation fragment believed to result from the

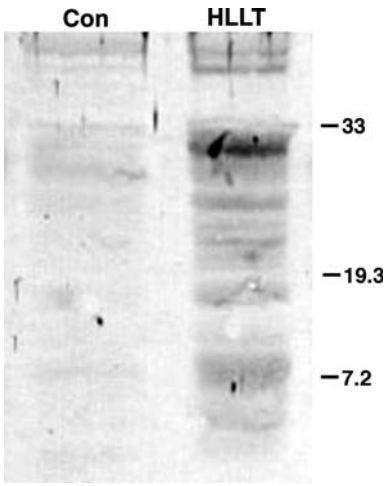

Fig. 6 Leaf polysome pellets probed with D1 antibody isolated from control (Con) and high light, low temperature exposed (HLLT) plants. Proteins from total leaf polysome pellets were separated by SDS-PAGE, transferred to membranes and probed with D1 antibody. The image is representative of five independent experiments. After the high light, low temperature exposure, there was an increase in the number of D1 fragments found in the polysome pellet

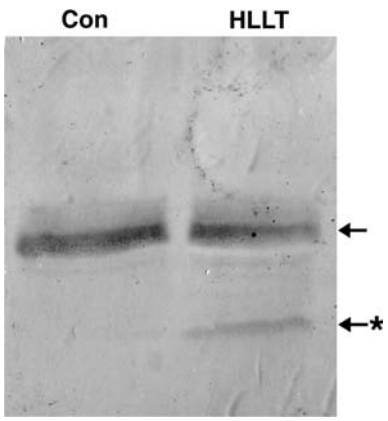

Fig. 7 D1 protein present in appressed thylakoids (grana stacks) of tomato leaf chloroplasts from control and high light, low temperature exposed plants. Grana proteins were isolated, separated by SDS-PAGE and probed with D1 antibody. Top arrow is full length D1 while bottom arrow with the star corresponds to the 22 kDa degradation fragment. The image is representative of three independent experiments. There was a greater amount of the D1 degradation product after exposure to high light at low temperature

initial cleavage of the protein (Haußühl et al. 2001), suggesting a disruption to the D1 degradation process. Under control conditions, the 22 kDa band was not observed, an indication that the damaged PSII centers migrated from the grana stacks allowing D1 to be further degraded by the FtsH protease (Lindahl et al. 2000; Bailey et al. 2002; Zaltsman et al. 2005). The appearance of the degradation product in the stress-exposed leaves suggest impairment in the migration of the damaged protein to the stroma lamellae.

Discussion

A central focus of this study was to investigate the mechanistic basis for the decrease in de novo D1 synthesis following damage to D1 by treatment of tomato plants at low temperature in high light. The basis for the observed decrease in net D1 de novo synthesis (Fig. 2) could thus be narrowed to interference with one or more processes in the overall protein expression pathway. Treatment-induced changes in message translatability, initiation or elongation of translation, post-translational assembly of PSII complexes, protein stability or degradation of D1 could all potentially contribute to the observed decrease in D1 accumulation in tomato following exposure to high light at low temperature.

Changes in mRNA availability were eliminated as the cause of the decrease in D1 synthesis, as no decrease in the steady-state level of *psbA* mRNA under these stress conditions was observed (Fig. 3). Since the majority of gene expression in mature chloroplasts is post-transcriptionally controlled (Rochaix 1992; Tonkyn et al. 1992), numerous possibilities remained including message recruitment to ribosomes.

To investigate the possibility that *psbA* mRNA might not be properly recruited to ribosomes, polysome profiles were compared. A greater proportion of D1 transcript was shown to be associated with ribosomes following high light at low-temperature conditions than under control conditions (Fig. 4A). High light alone did not elicit this response as D1 message association with ribosomes was about the same as control, but growth light at low-temperature resulted in lower *psbA* mRNA/ribosome association (Supplemental Fig. 2). Certain stress conditions (e.g., anaerobiosis) are known to cause an increase in message found in the ''ribosome pellet'' which is due to an increase in ribonucleoprotein complexes that cannot be translated rather than an increase in translation complexes (Stuger et al. 1999). The difference between message association with ribosomes versus ribonucleoprotein complexes can be determined by treating the samples with EDTA before centrifugation to chelate magnesium. The removal of magnesium causes ribosomes to dissociate and release the message from the ribosomal pellet whereas ribonucleoprotein complexes are not affected by magnesium chelation and message persists in the pellet. The EDTA treatment released *psbA* message from the pellet of the sample corresponding to the high light at low-temperature treatment just as it did in control samples (Supplemental Fig. 1), ruling out an increase in message association with ribonucleoprotein instead of ribosomes.

A plausible explanation for the increase in ribosome-associated D1 message is that the nascent peptide is not properly targeted to the thylakoid membrane. In higher

plants, targeting of D1 to the thylakoid occurs during elongation, possibly via the chloroplast equivalent of the signal recognition pathway (Nilsson et al. 1999), and is co-translationally inserted into the membrane (Zhang et al. 1999). High light and low temperature in tomato clearly do not interfere with proper polysome targeting to the membrane (Fig. 4B). Polysomes isolated from both membrane and soluble fractions of chloroplasts from control conditions contained *psbA* mRNA, although the majority of the *psbA* transcript containing polysome fraction was in the membrane fraction (Fig. 4B). The thylakoid targeting was even stronger under conditions of combined high light and low temperature, in which case very few of the *psbA* polysomes were found in the soluble fraction but instead found almost exclusively associated with the thylakoid membrane.

Exposure of tomato to high light at low temperature increased the number of ribosomes associated with *psbA* (Fig. 5), an indicator of ribosomal pausing during the elongation phase of translation (Barkan 1998). Soon after a ribosome attaches to a message and starts to translate down the message, another ribosome is able to attach to the 5′-end and initiate translation. If a ribosome pauses during elongation, the ribosomes that attached to the message afterward cannot progress beyond that position, usually leading to an increase in the number of ribosomes associated with the message (Wollin and Walter 1988). Translational pausing has been previously observed in messages of the chloroplast encoded genes *atpA* (Kim and Hollingsworth 1992) and *psbA* (Kim et al. 1991) during growth conditions and in the case of D1, was hypothesized to facilitate the insertion of the protein into the membrane as well as the cotranslational binding of cofactors.

The presence of D1 translation intermediates in the ribosome pellets confirmed ribosome pausing during elongation (Fig. 6). Total proteins isolated from polysome pellets were probed with polyclonal antisera raised against D1. Under both stress and control conditions the translation intermediates are of the same size distribution, similar to what has been previously observed in barley where ribosomes pausing was demonstrated to occur at discrete locations (Kim et al. 1991). However, under the combined stress conditions an increase in the abundance of four translation intermediates was seen compared to the control plus the appearance of three new bands, as would be expected if pausing were occurring during elongation.

In the case of developing chloroplasts, the synthesis of D1 is part of the de novo formation of the reaction centers and the observed pausing was hypothesized to facilitate the binding of pigment molecules to the apo-protein (Kim et al. 1991). Under our conditions of high light at low temperature in tomato, the leaves are fully expanded and the chloroplasts are mature. Thus, the synthesis of D1 is predominantly in a repair capacity, and not the de novo formation of reaction centers, and as such is under different regulatory control (Choquet and Vallon 2000; Choquet et al. 2001; Choquet and Wollman 2002; Ossenbuhl et al. 2004; Minai et al. 2006; Peng et al. 2006). The most likely explanation for the ribosome pausing during translation of D1 is that the high light, low-temperature condition interferes with the orderly degradation and removal of damaged D1 from the reaction center during the repair process. Western blot analysis of the polysome pellets from control and high light at low temperature exposed plants indicated that D1 translation intermediates under both conditions were of the same size distribution (Fig. 6) and further showed the appearance of a 22 kDa band in the grana stacks (Fig. 7) that corresponds to the size of the primary cleavage product of D1 (Haußühl et al. 2001). This observation indicates that the pausing observed under the combined stress conditions occurs at regular pause sites during translation elongation which would not be expected if the pausing were due to a low-temperature effect on translation. In that instance pausing would be expected at random locations where the low temperature ‘‘stalled’’ the ribosomes.

Functional PSII is normally found nearly exclusively in the grana stacks of the thylakoid. When PSII is rendered inoperable, the PSII center migrates from the grana to the stroma lamellae where the damaged D1 is removed and the new protein inserted (Choquet and Vallon 2000). Studies of D1 synthesis in intact chloroplast translation systems (Zhang et al. 1999) have demonstrated that D1 associates with a D2-cytochrome b559 complex during translation (Adir et al. 1990; van Wijk et al. 1997; Muller and Eichacker 1999; Zhang et al. 1999), which is thought to stabilize the nascent protein. A previous study by Salonen et al. (1998) also observed a decrease in the turnover of D1 in leaf discs of chilling-sensitive pumpkin exposed to low temperature and high light. In that study the authors noted a correlation between the phosphorylation state of D1 and its degradation. Although there is a relationship between the phosphorylation state of D1 and the repair cycle (Ebbert and Godde 1996; Baena-Gonzalez et al. 1999), the dephosphorylation of damaged D1 does not appear to be adversely affect by low temperature since dephosphorylated D1 was observed in pumpkin leaf discs that had been low temperature photoinhibited (Salonen et al. 1998). The translational pausing and subsequent decrease in de novo D1 synthesis in tomato under the condition of high light at low temperature is very likely due to the D1 insertion site not being vacated and damaged PSII centers still in the grana stacks.

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