Association of Rubisco activase with chaperonin-60β:
a possible mechanism for protecting photosynthesis
during heat stress

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Received 24 September 2007; Revised 14 November 2007; Accepted 12 December 2007

Abstract
Previous studies have shown that inhibition of photosynthesis by moderate heat stress is a consequence of Rubisco deactivation, caused in part by the thermal instability of Rubisco activase. This involvement of Rubisco activase was confirmed in heat stress and recovery experiments using transgenic Arabidopsis plants. Compared with wild-type plants, photosynthesis, the effective quantum yield of photosystem II, and Rubisco activation were less thermotolerant and recovered more slowly in transgenic Arabidopsis plants with reduced levels of Rubisco activase. Immunoblots showed that 65% of the Rubisco activase was recovered in the insoluble fraction after heat stress in leaf extracts of transgenic but not wild-type plants, evidence that deactivation of Rubisco was a consequence of thermal denaturation of Rubisco activase. The transgenic Arabidopsis plants used in this study contained a modified form of Rubisco activase that facilitated affinity purification of Rubisco activase and proteins that potentially interact with Rubisco activase during heat stress. Sequence analysis and immunoblotting identified the β-subunit of chaperonin-60 (cpn60β), the chloroplast GroEL homologue, as a protein that was bound to Rubisco activase from leaf extracts prepared from heat-stressed, but not control plants. Analysis of the proteins by non-denaturing gel electrophoresis showed that cpn60β was associated with Rubisco activase in a high molecular mass complex. Immunoblot analysis established that the apparent association of cpn60β with Rubisco activase was dynamic, increasing with the duration and intensity of the heat stress and decreasing following recovery. Taken together, these data suggest that cpn60β plays a role in acclimating photosynthesis to heat stress, possibly by protecting Rubisco activase from thermal denaturation.

Key words: Carbon metabolism, heat shock, molecular chaperone, photosynthesis, Rubisco, temperature stress.

Introduction
Heat stress is a major abiotic factor limiting the growth, development, and distribution of plants (Berry and Björkman, 1980). Over the 17 year period from 1982 to 1998, decreases in agricultural yields were linked to increases in growing season temperatures (Lobel and Asner, 2003), suggesting that the prevalence of heat stress has increased over the last two decades. Among the myriad of physiological processes in plants, photosynthesis is one of the most sensitive to inhibition by elevated temperatures, with inhibition occurring at temperatures only slightly higher than those optimal for growth. While the rate of carboxylation by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) increases with temperature up to at least 50 °C (Salvucci et al., 2001), the solubility of CO2, particularly relative to O2, and the specificity of Rubisco for CO2 decrease. These changes favour oxygenation of RuBP by Rubisco over carboxylation, increasing flux through photorespiration, which, together with increased rates of dark respiration, reduces the potential increase in CO2 fixation at high temperature (Berry and Björkman, 1980; Jordan and Ogren, 1984; Kobza and Edwards, 1987).
In addition to stimulating photorespiratory activity, high temperatures have also been shown to have a direct inhibitory effect on photosynthesis (Kobza and Edwards, 1987). While photosystem II (PSII) has often been regarded as the primary target of heat stress, evidence was presented >20 years ago showing that Rubisco was even more sensitive to inactivation by moderate heat stress (Weis, 1981; Kobza and Edwards, 1987). To be active in vivo, Rubisco requires the continued action of Rubisco activase, an AAA⁺ protein that converts Rubisco sites from a closed, inactive conformation to an open and potentially active conformation (Andrews et al., 1995; Spreitzer and Salvucci, 2002; Portis, 2003). The closed conformation is stabilized and continually formed by the binding of certain sugar-phosphates that act as inhibitors of the enzyme (Andrews et al., 1995). Conversion from the closed to the open conformation not only frees Rubisco sites of bound inhibitors, but is also required for spontaneous ‘activation’ by carbamylation and Mg²⁺ binding, prerequisites for productive RuBP binding and subsequent catalysis (Spreitzer and Salvucci, 2002). Thus, by promoting conformational changes that are essential for activity, Rubisco activase regulates the fraction of Rubisco sites that are catalytically competent, i.e. the activation state of Rubisco.

Measurements of the activation state of Rubisco in leaves, determined from the ratio of initial extractable activity to the activity after incubation under conditions that fully carbamylate the enzyme, show that the activation state of Rubisco decreases when net photosynthesis is inhibited by moderate heat stress (Feller et al., 1998; Crafts-Brandner and Salvucci, 2000; Sharkey et al., 2001; Salvucci and Crafts-Brandner, 2004a; Haldimann and Feller, 2004; Kim and Portis, 2005; Yamori et al., 2006). Renewed interest in the mechanistic basis for heat inactivation of Rubisco and its association with inhibition of photosynthesis has occurred recently, spurred by the realization that Rubisco activase is acutely sensitive to inactivation by heat, while processes that inactivate Rubisco, such as catalytic misfire, increase with temperature (Crafts-Brandner and Salvucci, 2000; Salvucci et al., 2001; Portis, 2003; Salvucci and Crafts-Brandner, 2004a). Consistent with this idea is the observation that isolated Rubisco activase exhibits a relatively low temperature optimum of between 22 °C and 35 °C, depending upon the species, with the variation relating to the natural thermal environment of the individual plant species (Salvucci and Crafts-Brandner, 2004a, b). In an elegant proof-of-concept, Kurek et al. (2007) recently demonstrated that improvements in the thermostability of Rubisco activase using gene shuffling increased photosynthesis, growth rates, and yield in transformed Arabidopsis under moderate heat stress.

Studies with a variety of plant species have shown that Rubisco activase often repartitions from the soluble to the insoluble fraction when heat stress is imposed on leaves, protoplasts, and chloroplasts (Feller et al., 1998; Rokka et al., 2001; Salvucci et al., 2001; Haldimann and Feller, 2004; Yang et al., 2005; Feng et al., 2007). Rokka et al. (2001) suggested that repartitioning of Rubisco activase involves binding of the protein to the thylakoid membrane, specifically the ribosomal fraction, and speculated that this binding could reflect a dual function of Rubisco activase in regulating expression of chloroplast proteins. However, treatment of ‘thylakoid-bound’ Rubisco activase with detergent, salt, and chaotropic agents failed to solubilize the Rubisco activase (Rokka et al., 2001; Salvucci et al., 2001). Also, experiments with isolated Rubisco activase in the absence of membranes showed that Rubisco activase loses structural integrity and precipitates from solution when exposed to temperatures only slightly above the temperature optimum for activity (Salvucci et al., 2001). Together these results indicated that Rubisco activase was not redistributing to the insoluble fraction by tightly associating with the membrane, but rather was simply denaturing and co-precipitating with the heaviest fraction from the thylakoid membrane (i.e. polysomes).

It is well known that photosynthesis acclimates to moderate heat stress in both the short and long term when plants receive either a prior or gradual exposure to elevated temperature (Berry and Björkman, 1980). The extent of acclimation is variable and the range of temperatures to which a particular plant species can acclimate is generally related to the temperature range found in the natural environment of that species (Berry and Björkman, 1980). The involvement of Rubisco activase in the thermal sensitivity of photosynthesis suggests that stabilization of Rubisco activase could represent one mechanism for photosynthetic acclimation to heat stress. In fact, several studies have shown that acclimation of Rubisco activation to moderate heat stress can occur in response to both long- and short-term pre-exposure (Crafts-Brandner and Law, 2000; Hikosaka et al., 2006; Yamori et al., 2006). Since this acclimation can take place rather quickly, protective machinery either must already be in place or is rapidly induced to prevent thermal denaturation of Rubisco activase during a heat shock episode. In the present study, an affinity capture technique was used with transgenic Arabidopsis plants containing a modified form of Rubisco activase to identify a chloroplast component that associates with and possibly protects Rubisco activase during moderate heat stress.

**Materials and methods**

**Plant material**

Transgenic Arabidopsis thaliana plants expressing a tagged version of the Arabidopsis β-isofrom (i.e. Δ43 plants) were produced by Agrobacterium-mediated transformation of the Rubisco
activate-deficient rca mutant as described previously (Salvucci et al., 2006). The Arabidopsis cDNA was engineered to include nucleotides that encoded Trp-Ser-His-Pro-Gln-Phe-Glu-Lys, an eight amino acid peptide with binding affinity for the biotin pocket of streptavidin (Skerra and Schmidt, 2000). This Strep- or S-tag was incorporated as a C-terminal extension to the β-form of Rubisco activase via a Ser–Ala linker. Wild-type and transgenic plants were grown in air at 23 °C under an irradiance of 175–250 μmol photons m⁻² s⁻¹ with a 10 h light/14 h dark photoperiod (Salvucci et al., 2006). Plants were grown in soil and watered as necessary with the nutrient solution described previously (Crafts-Brandner and Law, 2000), but at half-strength. Heat stress was imposed on intact plants by increasing the temperature of the growth chamber to the temperatures and for the durations indicated in the text. The relative humidity in the growth chamber was maintained at 80–90% during the treatment. Measurements of leaf temperature with a needle thermocouple verified that leaf temperatures were within 1 °C of air temperatures under these conditions. Immediately following each treatment, plant material was frozen in liquid N₂ and stored at −80 °C until extraction.

Gas exchange and fluorescence measurements

The gas exchange of attached leaves was measured at air levels of CO₂ (internal CO₂ ~280 μbar) and 202 mbar O₂ with an irradiance of 1000 μmol photons m⁻² s⁻¹ on 4–5-week-old plants using a Li-Cor 6400 as described previously (Salvucci et al., 2006). Control plants were measured after at least 1 h at 23 °C. Leaf temperatures were measured using a thermocouple placed directly on the underside of the leaf. Heat stress was imposed by increasing the temperature of the growth chamber and the leaf cuvette rapidly from 23 °C to 37.5 °C over a 5–10 min period. After 30 min at 37.5 °C, the temperature was decreased to 23 °C for 1 h. Gas exchange was measured continuously, before, during, and after the 30 min of heat stress. In parallel experiments, chlorophyll fluorescence was measured at 250 μmol photons m⁻² s⁻¹ as described previously (Salvucci et al., 2006). The results of the gas exchange and fluorescence measurements are the means ± SEM of individual measurements of 4–8 separate plants.

Rubisco activation

Rubisco activation was determined at 25 °C as described previously (Salvucci et al., 2006). Leaf discs (0.5 cm²) were excised in the light from intact plants during a time-course of heat stress and recovery as described above, and immediately frozen in liquid N₂ and stored at −80 °C. Duplicate assays were conducted on each sample, each sample consisting of a disc from a separate plant. The results presented are the means ± SEM of 3–5 samples.

Rubisco activase protein determination

The relative amounts of Rubisco activase protein in the soluble and insoluble fractions from leaf extracts of transgenic and wild-type Arabidopsis plants were determined by image analysis of immunoblots. Serial dilutions of extracts prepared from discs sampled under control conditions were used as standards. Leaf discs (0.5 cm²) were sampled as described above and the frozen discs were extracted in the same buffer used for Rubisco assays, but supplemented with 0.1% (v/v) Triton X-100. Extracts were centrifuged for 10 min at 10 000 g to separate soluble and insoluble proteins. Insoluble (i.e. pellet) material was suspended in the same volume of buffer used for extraction and the solution was supplemented with SDS and dithiothreitol to solubilize the proteins. Polypeptides in both the soluble and insoluble fractions were separated and analysed by SDS–PAGE and immunoblotting (Salvucci et al., 2001).

Affinity purification of Rubisco activase from leaf extracts

All procedures except electrophoresis and immunoblotting were performed at 4 °C. Frozen leaf tissue (~0.9 g fresh weight) from heat-stressed and control plants was extracted in 8 ml of 0.1 M TRIS-HCl, pH 8, and 75 mM NaCl (S-75 buffer) in a Ten Broeck glass homogenizer. For some experiments, the buffer was supplemented with 85 μg of purified, recombinant S-tagged Rubisco activase prior to extraction. Extracts were centrifuged for 5 min at 23 500 g and the supernatant loaded on a 0.15–0.8 ml column of Strep-Tactin-Sepharose (IBA GmbH, Göttingen, Germany). After three passes of the extract through the column, the column was rinsed with 8 ml of S-75 buffer. Rubisco activase was eluted by incubation of the column for 30 min with one column volume of 5 mM desthiobiotin in S-75 buffer followed by two additional column volumes of this buffer. The eluted material was concentrated by ultrafiltration on a Centrifcon YM-30 membrane and the polypeptides were separated and analysed by SDS–PAGE and immunoblotting (Salvucci et al., 2001). For some experiments, blots were stained with Coomassie Brilliant Blue, and the stained proteins were submitted to the Protein Analysis and Synthesis Laboratory at Arizona State University for N-terminal sequencing by Edman degradation analysis.

Miscellaneous

The recombinant β-isofrom of Arabidopsis Rubisco activase containing a C-terminal S-tag, but without a chloroplast transit peptide was expressed in Escherichia coli cells from a cDNA as described previously (Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004a). Monoclonal antibodies directed against human hsps60 were purchased from StressGen Bioreagents (Victoria, British Columbia, Canada). Electrophoresis under non-denaturing conditions was performed at 4 °C as described by Barraclough and Ellis (1980) using 5% (w/v) polyacrylamide gels. Rubisco was purified from spinach leaves as described previously (Salvucci, 1992).

Results

Photosynthesis, chlorophyll fluorescence, Rubisco activation, and Rubisco activase distribution during heat stress and recovery

The inhibition and recovery of net photosynthesis after moderate heat stress were compared for wild-type and transgenic Δ43 Arabidopsis plants. Compared with the wild type, Δ43 plants contained ~12% of the amount of Rubisco activase (Salvucci et al., 2006). At the control temperature and a saturating irradiance of 1000 μmol m⁻² s⁻¹, the Δ43 plants exhibited photosynthetic rates that were 44% of wild-type rates (Fig. 1A). These rates of net photosynthesis in air were similar to rates measured previously by us (Salvucci et al., 2006) and others (Eckardt et al., 1997; Zhang et al., 2002) for Arabidopsis plants grown under relatively high irradiance and with supplemental nutrients. When plants were exposed for 30 min to a moderate heat stress of 37.5 °C, net photosynthesis decreased for both wild-type and Δ43 plants. Net photosynthesis in the Δ43 plants was completely inhibited by the moderate heat stress, whereas net photosynthesis in wild-type plants was only inhibited by ~20%. For wild-type plants, recovery of net photosynthesis was complete within
30 min of transfer back to the control temperature of 23°C, whereas marked inhibition of net photosynthesis persisted in the Δ43 plants, even after 60 min at 23°C. Differences between wild-type and Δ43 plants were also apparent in the response of the effective quantum yield of PSII (ΦPSII) to heat stress (Fig. 1B). When measured at 250 μmol m⁻² s⁻¹, the same irradiance used for growth, ΦPSII increased slightly in wild-type plants in response to a 30 min heat stress, but then decreased over the 60 min recovery period. In contrast, ΦPSII in the Δ43 plants decreased in response to heat stress and continued to decrease markedly over the 60 min recovery period.

The activation state of Rubisco was also measured before and after heat stress and during recovery at the growth irradiance of 250 μmol m⁻² s⁻¹ (Fig. 1C). Compared with the wild type, Rubisco activation was lower in the Δ43 plants under control temperatures and decreased to a greater extent in response to heat stress, that is 36% for Δ43 plants compared with 18% for wild-type plants (see also Salvucci et al., 2006). After 60 min of recovery at the control temperature, the activation state of Rubisco in wild-type plants fully recovered to the level at the control temperature prior to imposition of the heat stress, whereas Rubisco activation in the Δ43 plants was 18% lower than the level at the control temperature.

The amounts and distribution of Rubisco activase protein were examined in wild-type and Δ43 plants at three stages of the time-course in Fig. 1; while at the control temperature of 23°C prior to heat stress, after 30 min at 37.5°C, and after 60 min of recovery at the control temperature. Immunoblots of Rubisco activase in soluble and insoluble fractions from extracts of wild-type plants showed that neither the total amount of Rubisco activase nor its distribution changed in response to moderate heat stress (Fig. 2). The leaves of wild-type plants contained both isoforms of Rubisco activase, and both remained soluble after heat stress, with <8% of the total Rubisco activase associating with the insoluble fraction. In contrast, leaves of the Δ43 plants contained only the β-isoform of Rubisco activase, and the amount and, particularly, the distribution of this Rubisco activase changed markedly in response to moderate heat stress. At the control temperature prior to imposition of heat stress, >93% of the Rubisco activase was soluble, similar to the distribution in wild-type leaves. However, after 30 min of moderate heat stress, most of the Rubisco activase (i.e. 65%) was recovered in the pellet fraction and a large percentage (i.e. 35%) of the total Rubisco activase from the Δ43 plants was still associated with insoluble material after 60 min of recovery at the control temperature. The total amount of Rubisco activase in the Δ43 plants decreased by ~10% after 30 min of heat stress and by ~15% after an additional 60 min of recovery.

Identification of a protein that potentially interacts with Rubisco activase during heat stress
A C-terminal S-tag was engineered into the sequence of Rubisco activase to facilitate affinity purification of
Rubisco activase and any proteins bound to it from leaf extracts of the Δ43 plants. Separation of polypeptides by SDS–PAGE after elution under gentle conditions with desthiobiotin showed that a 43 kDa polypeptide was the major protein retained on the Strep-Tactin resin from leaf extracts of the Δ43 (Fig. 3), but not wild-type plants (data not shown, but see below). The identity of this protein as Rubisco activase was confirmed immunologically (see below), as well as by N-terminal sequencing (data not shown). In addition to the 43 kDa Rubisco activase polypeptide, two other polypeptides of ~52 kDa and 33 kDa bound to the affinity resin and co-eluted with Rubisco activase. These polypeptides were also present in the bound fraction from columns treated with extracts of wild-type plants (data not shown), indicating that their presence in this fraction was not related to the presence of Rubisco activase.

A 60 kDa polypeptide co-eluted with Rubisco activase in extracts prepared from heat-stressed Δ43 plants (Fig. 3). Interestingly, the amount of the 60 kDa polypeptide eluting in the bound fraction increased with increasing leaf temperature, although the amount of Rubisco activase decreased at the highest temperatures and the amounts of the 33 kDa and 52 kDa polypeptides were relatively unaffected. The 60 kDa polypeptide was not present in the bound fraction from affinity chromatography of wild-type extracts, suggesting that its presence in this fraction was related to the presence of Rubisco activase (see below). The N-terminal sequence of this polypeptide, NH₂-AAKELHFNKDGTTI, corresponded to the sequence of the β-subunit of chaperonin 60 (cpn60β), the chloroplast homologue of GroEL (Hendrick and Hartl, 1993; Hill and Hemmingsen, 2001).
abundance during the 30 min exposure to a moderate heat stress of 37.5 °C (data not shown).

**Evidence for association of Rubisco activase and cpn60β in a high molecular mass complex**

Electrophoresis on non-denaturing gels was used to determine if the Rubisco activase and cpn60β that eluted from the affinity column were associated in a complex (Fig. 5). Staining of the native gels with Coomassie Brilliant Blue showed that the desthiobiotin fraction from affinity chromatography of extracts from heat-stressed Δ43 plants contained a very high molecular mass protein band that was extremely faint in this same fraction from non-heat-stressed Δ43 plants. Antibodies to hsp60 recognized this band on immunoblots of replicate lanes of the gel. Replicate lanes probed with antibody to tobacco Rubisco activase (left panel) or human hsp60 (right panel), and visualized using alkaline phosphatase conjugated to a secondary antibody. The arrow indicates the position of cpn60β on the blot.

**Fig. 4.** Dependence on Step-Tactin-bound Rubisco activase from heat-stressed plants for affinity capture of cpn60β. Wild-type (wt) and transgenic Δ43 (Δ43) Arabidopsis plants were exposed for 1 h to control (C) or heat stress (HS) temperatures of 23 °C or 40 °C, respectively, before freezing in liquid N2. Rubisco activase and associated proteins were affinity purified from leaf extracts as described in Fig. 3. Polypeptides were separated by SDS–PAGE and transferred to a PVDF membrane. Blots were probed with antibody to tobacco Rubisco activase (left panel) or human hsp60 (right panel), and visualized using alkaline phosphatase conjugated to a secondary antibody. The arrow indicates the position of cpn60β on the blot.

**Fig. 5.** Evidence for an association of Rubisco activase with cpn60β in a high molecular mass complex from heat-stressed plants. (A) Transgenic Δ43 Arabidopsis plants were exposed for 1 h to control (C) or heat stress (HS) temperatures of 23 °C or 37.5 °C, respectively, before freezing in liquid N2. Rubisco activase and associated proteins were affinity purified from leaf extracts as described in Fig. 3. Proteins were separated by electrophoresis under non-denaturing conditions and visualized directly either by staining with Coomassie Brilliant Blue (left panel) or by immunoblotting with anti-hsp60 (middle panel) or anti-Rubisco activase (right panel) antibodies after transfer to a PVDF membrane as described in Fig. 4. The band labelled R on the stained gel marks the position of the 520 kDa spinach Rubisco holenzyme. (B) Immunoblots of affinity-purified proteins from transgenic Δ43 Arabidopsis plants that were exposed for 1 h to 40 °C. Proteins were separated by non-denaturing electrophoresis and visualized by immunoblotting with anti-hsp60 (left panel) or anti-Rubisco activase (right panel) antibodies. The arrows in the panels indicate the position of Rubisco activase accumulation on the blots, which coincided with the position of cpn60β.

The dynamics of the association between Rubisco activase and cpn60β

Thus far, the data suggest that cpn60β was associated with Rubisco activase in extracts from heat-stressed plants.
However, to eliminate the possibility that a heat-activated form of cpn60β was associating with undamaged Rubisco activase after Rubisco activase had already bound to the column, Strep-Tactin columns were pre-loaded with saturating amounts of purified recombinant S-tagged Rubisco activase prior to chromatography of leaf extracts from control and heat-stressed Δ43 plants (Fig. 6A). Immunoblots of the desthiobiotin elutions showed that cpn60β did not bind to the purified Rubisco activase that was pre-loaded on the column, but was bound to Rubisco activase when leaf extracts alone were chromatographed through untreated columns (see Fig. 4) or when extracts were supplemented with purified Rubisco activase prior to passage through untreated columns (Fig. 6B). For extracts supplemented with purified Rubisco activase, the amount of cpn60β recovered with Rubisco activase was greater in extracts from heat-stressed leaves, suggesting that cpn60β was associated with the endogenous and possibly heat-damaged Rubisco activase from the plant rather than binding to the exogenously added protein.

Recovery experiments were conducted to characterize the dynamics of the apparent association of cpn60β with Rubisco activase during heat stress (Fig. 7). Immunoblots showed that the amount of cpn60β that was recovered with Rubisco activase from heat-stressed plants decreased progressively with the duration of recovery. Imposition of a second 1 h episode of heat stress after 2 h of recovery from the initial heat stress increased the amount of cpn60β that was associated with Rubisco activase compared with just 1 h of heat stress followed by 1–4 h of recovery.

**Discussion**

**Heat stress and recovery experiments with the Δ43 plants provide new insights into the involvement of Rubisco activase in photosynthetic thermotolerance**

The activation state of Rubisco in leaves reflects the balance between the rates of deactivation, caused by catalytic misfire and/or decarbamylation, and reactivation of inactive Rubisco sites by Rubisco activase (Andrews et al., 1995; Spreitzer and Salvucci, 2002; Portis, 2003). The relatively low temperature optimum of Rubisco activase activity coupled with the high temperature optimum for deactivation drive the equilibrium towards...
Deactivation as temperatures approach and then exceed the thermal optimum of Rubisco activase (Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004a, b). Deactivation reduces the $k_{cat}$ of Rubisco by decreasing the number of sites that are competent for carboxylation. Thus, when photosynthesis is limited by Rubisco, a decrease in the activation state of Rubisco should reduce the rate of net photosynthesis when other conditions are equivalent. Transgenic plants with lower amounts of Rubisco activase, including the Δ43 plants (Salvucci et al., 2006), support this conclusion since their reduced rates of net photosynthesis correlate with a lower activation state of Rubisco (cf. Mate et al., 1993; Eckardt et al., 1997).

Under moderate heat stress, Rubisco activation was inhibited to a greater extent in transgenic plants with lower amounts of Rubisco activase than in wild-type plants (Fig. 1; see also Salvucci et al., 2006). In the present study, immunoblots of leaf extracts revealed that a large portion of the Rubisco activase in the Δ43 plants became insoluble during a brief exposure to moderate heat stress, whereas almost all of the Rubisco activase remained soluble in wild-type plants under these same conditions. These results provide additional evidence for a cause and effect relationship between Rubisco activation and Rubisco activase thermal stability (Salvucci et al., 2001; Salvucci and Crafts-Brandner, 2004a, b).

That net photosynthesis was completely inhibited in the Δ43 plants during moderate heat stress was consistent with the negative effect of reduced Rubisco activation on photosynthesis (Crafts-Brandner and Salvucci, 2001) combined with the stimulatory effect of higher temperatures on photorespiration and dark respiration (Berry and Björkman, 1980; Sharkey et al., 2001). Less clear is why very low rates of net photosynthesis persisted in the Δ43 plants long after heat stress was removed and Rubisco had partially reactivated. Similar slow recovery responses to heat stress have been reported for other plants that contain little or no Rubisco activase (Sharkey et al., 2001; Kim and Portis, 2005), indicating that slow recovery of photosynthesis from heat stress might be a general characteristic of plants with very low amounts of Rubisco activase. The biochemical basis for persistent inhibition of photosynthesis following a heat stress is unknown, but might involve (i) injury to thylakoid membranes caused by the severe level of photosynthetic inhibition suffered during the heat stress; (ii) changes in the nature of the inhibited form of Rubisco upon recovery, specifically to a form with a looser binding inhibitor (Kim and Portis, 2006); or (iii) direct effects of lower amounts and/or activities of Rubisco activase on Rubisco catalysis, independent of changes in the activation state (He et al., 1997; Sharkey et al., 2001).

Another perplexing response observed with the Δ43 plants was the acute sensitivity of their Rubisco activase to thermal denaturation. While it is possible that modification of an S-tag decreased the thermal stability of Rubisco activase in these plants, this possibility seems unlikely given that the balanced amino acid composition of the short S-tag generally has little effect on protein stability or activity (Skerra and Schmidt, 2000). In addition, the photosynthetic response of the Δ43 plants to heat stress and recovery was similar to the response of rwt46, a transgenic Arabidopsis line that expresses only the α-isof orm of Rubisco activase (Zhang et al., 2002; Kim and Portis, 2005; Salvucci et al., 2006). In both the Δ43 and rwt46 plants, the amount of Rubisco activase is reduced compared with the wild type, indicating that the greater thermal instability of their Rubisco activase might be a consequence of the lower amount of Rubisco activase protein in these plants. Rubisco activase has been shown to be a highly self-associating protein whose specific activity and molecular mass increase with increasing concentrations of protein (Salvucci, 1992; Portis, 2003). Consequently, the low levels of Rubisco activase protein in the Δ43 and rwt46 plants would favour a more dissociated state in vivo, making the enzyme more prone to thermal denaturation. This idea might also be relevant to the results of Yang et al. (2005) who showed that accumulation of glycine betaine, a compatible solute known to promote self-association reactions (Feltsky et al., 2004), prevented denaturation of Rubisco activase in transgenic tobacco plants that were exposed to heat stress.

**Association of Rubisco activase with cpn60β suggests a possible role for this chaperonin as a conventional heat shock protein in chloroplasts**

Using an affinity capture procedure, cpn60β was identified as a protein that potentially interacts with Rubisco activase during heat stress. The acute sensitivity of Rubisco activase in the Δ43 plants probably improved the specificity of the procedure for cpn60β since a lower temperature could be used to induce thermal denaturation of Rubisco activase in these plants. Key to the approach was the use of very gentle elution conditions that preserved the Rubisco activase–cpn60β complex through isolation by affinity chromatography. That these proteins remained associated after extensive washing of the column with buffer and following electrophoresis through non-denaturing gels suggested that cpn60β forms a very tight complex with Rubisco activase that persists through extraction and subsequent chromatography.

Control experiments with wild-type and unstressed Δ43 plants provided considerable evidence that the Rubisco activase–cpn60β complex was formed in vivo during heat stress by the binding of cpn60β to heat-damaged Rubisco activase (Fig. 6). Other experiments indicated that the formation and apparent dissociation of the complex was dynamic, dependent on the duration and intensity of the...
heat stress and recovery applied to the living plant. Taken together, the results seem to rule out, but not completely exclude, the possibility that cpn60β associated with Rubisco activase in solution during extraction rather than in planta. This possibility seems unlikely given that the propensity for protein–protein interaction would be much greater when Rubisco activase and cpn60β are present together at high concentrations and temperatures in the chloroplast, than at much lower concentrations in diluted leaf extracts at 4 °C.

The term molecular chaperone has been used to describe the ability of cpn60 and other GroEL-type proteins to influence the assembly and disassembly of oligomeric proteins (Hendrick and Hartl, 1993; Horwich et al., 2006). While much attention has been paid to the involvement of cpn60β in aiding the folding and assembly of chloroplast proteins such as Rubisco (Viitanen et al., 1995; Lubben et al., 1989), its role as a conventional heat shock protein has received considerably less attention. In plants, cpn60β does not usually exhibit a rapid increase in abundance in response to heat stress (Schmidt et al., 1996; Holland et al., 1998; Ferreira et al., 2006), suggesting that its function in the chloroplast might differ from that of its counterpart, hsp60, in other organisms. However, Prezewski et al. (2000) reported that cpn60β levels in nine tomato genotypes increased from 1–6.5-fold within 12–24 h of a 4 h heat stress and that the increase was highest in genotypes whose photosynthesis was most thermotolerant. Attempts to elucidate the precise role of cpn60β in plants by reducing or eliminating the amount of cpn60β protein using antisense suppression of cpn60β mRNA in tobacco or T-DNA disruption of the cpn60β gene in Arabidopsis have been inconclusive, producing plants with abnormal phenotypes (Zabeleta et al., 1994; Ishikawa et al., 2003). Curiously, the levels of Rubisco in both tobacco and Arabidopsis were unaffected by a severe reduction in the levels of cpn60β.

The Arabidopsis genome contains three paralogues of cpn60β (Hill and Hemmingsen, 2001). The N-terminal sequence of the cpn60β polypeptide that was identified in the present study corresponds to either the cpn60β-2 or -3 gene, which are located on chromosomes III and I, respectively (Hill and Hemmingsen, 2001). Deletion of cpn60β-3 in the len1 T-DNA mutant drastically reduced the amount of cpn60β and produced a severely stunted lesion mimic mutant in plants that were grown under short photoperiods, but had no obvious phenotype under a long photoperiod (Ishikawa et al., 2003). Heat stress accelerated the death of seedlings of the len1 mutant compared with wild-type plants under short days, but experiments with mature plants grown under long photoperiods were not reported. An accumulation of cpn60β in response to NaCl stress was reported in tobacco, but only at the seedling stage, leading to the conclusion that the involvement of cpn60β in stress tolerance in plants only occurs at certain developmental stages (Holland et al., 1998).

The association of cpn60β with Rubisco activase in heat-stressed Δ43 plants suggests that cpn60β could function in plants as a mechanism for stress tolerance, not just in seedlings, as with tobacco (Holland et al., 1998), but in mature plants. It has long been recognized that hsp60 improves the thermal tolerance of a large number of organisms by associating with and stabilizing a wide variety of proteins during episodes of heat shock (Parsell and Lindquist, 1991; Hendrick and Hartl, 1993). To my knowledge, the results with Rubisco activase are the first to document an association of cpn60β with another chloroplast protein during heat stress. It seems likely that the association of Rubisco activase with cpn60β during heat stress could function to protect Rubisco activase from thermal denaturation, thus providing a mechanism to protect and acclimate photosynthesis to heat stress. In this way, the function of cpn60β in chloroplasts during heat shock would be analogous to its function in other organisms, but without the requirement for additional accumulation of cpn60β protein. Perhaps the large amount of cpn60β in the chloroplast that is presumably required for assembly of Rubisco and other abundant chloroplast proteins (Barraclough and Ellis, 1980; Viitanen et al., 1995) is sufficient to protect Rubisco activase and other heat-labile proteins during moderate heat stress. It is interesting to note that Rubisco synthesis in cotton leaves was severely inhibited when plants were subjected to moderate heat stress (Law et al., 2001). Although speculative, reduced synthesis of Rubisco and enhanced association of cpn60β with Rubisco activase during episodes of heat stress could be an indication that the function of cpn60β shifts during heat stress from folding and assembly of newly synthesized oligomeric proteins to protection of thermally labile proteins.

Acknowledgements

The author wishes to acknowledge Nancy Parks for technical assistance and Dr Ben DeRidder, Grinnell College, for producing the Δ43 transgenic plants. The author would like to thank Dr Elizabeth Vierling, University of Arizona, for initially suggesting the use of an S-tagged Rubisco activase, and Drs Archie R Portis, Jr (USDA-ARS) and Scott Holaday (Texas Tech University) for their critical review of the manuscript. The research was supported by the USDA-ARS and a grant from the United States Department of Energy (97ER20268).

References


and under photosynthetic and non-photosynthetic conditions.  


