

Thylakoid membrane responses to moderately high leaf temperature in Pima cotton

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ABSTRACT

Photosynthesis is inhibited by high temperatures that plants are likely to experience under natural conditions. Both increased thylakoid membrane ionic conductance and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) deactivation have been suggested as the primary cause. The moderately heat-tolerant crop Pima S-6 cotton (*Gossypium barbadense*) was used to examine heat stress-induced inhibition of photosynthesis. Previous field-work indicated that moderate heat stress ($T = 35\text{--}45\text{ }^{\circ}\text{C}$) is associated with very rapid leaf temperature changes. Therefore, a system was devised for rapidly heating intact, attached leaves to mimic natural field heat-stress conditions and monitored Rubisco activation, carbon-cycle metabolites, thylakoid ionic conductance, and photosystem I activity. As a proxy for NADPH and stromal redox status the activation state of NADP-malate dehydrogenase (NADP-MDH) was measured. In dark-adapted cotton leaves, heating caused an increase in thylakoid permeability at temperatures as low as 36 °C. The increased permeability did not cause a decline in adenosine 5'-triphosphate (ATP) levels during steady-state or transient heating. Rapid heating caused a transient decline in ribulose 1,5-bisphosphate without a decrease in Rubisco activation. Sustained heating caused a decline in Rubisco activation and also oxidized the stroma as judged by NADP-MDH activation and this is hypothesized to result from increased cyclic photophosphorylation, explaining the maintenance of ATP content in the face of increased thylakoid membrane ion leakiness.

Key-words: adenosine 5'-triphosphate; heat stress; NADP-malate dehydrogenase; photosynthesis; Rubisco activation.

INTRODUCTION

High temperatures often inhibit plant growth, with photosynthesis considered among the plant functions most sensitive to high temperature (Berry & Björkman 1980; Quinn & Williams 1985). Most temperate C_3 plants exhibit a broad photosynthetic temperature optimum between 20° and 35 °C with peak CO_2 assimilation often occurring near

30 °C. Increasing leaf temperatures beyond this range inhibit photosynthesis. High temperature reduces photosynthetic efficiency by stimulating photorespiration (Ogren 1984; Brooks & Farquhar 1985) as well as by damaging the photosynthetic apparatus.

Photosystem II (PSII) has long been recognized as a prominent heat-labile component of photosynthesis (Santerius 1975; Berry & Björkman 1980) although high-temperature effects on PSII do not often occur below 45 °C (Thompson *et al.* 1989; Gombos *et al.* 1994). Pastenes & Horton (1996) and Bukhov *et al.* (1999) proposed that moderate heat (35–45 °C) caused thylakoid membranes to become leaky and induced cyclic electron transport. Havaux *et al.* (1996) found increased leakiness that could be counteracted by zeaxanthin. Increased cyclic electron transport is believed to involve the introduction of electrons from NADPH at or near cytochrome b_6/f (Thomas *et al.* 1986; Boucher, Harnois, & Carpentier 1989; Bukhov, Samson, & Carpentier 2000). As temperature increases, the capacity for cyclic phosphorylation may no longer be sufficient to compensate for the leakiness of the thylakoid membranes, resulting in reduced adenosine 5'-triphosphate (ATP) or even NADPH availability if too much energy is consumed in cyclic phosphorylation.

On the other hand, deactivation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) at moderate leaf temperature has been known for some time (Kobza & Edwards 1987) confirming the findings of Weis (1980) that Rubisco activity is reduced by heat. Rubisco activase has been shown to be heat labile (Salvucci *et al.* 2001), which could lead to deactivation of Rubisco. Deactivation of Rubisco correlates with the decline in photosynthesis at moderately high temperature (Law & Crafts-Brandner 1999). These authors propose that the heat-induced deactivation of Rubisco is the primary constraint on photosynthesis at moderately high temperature and show that chlorophyll fluorescence signals from PSII are not affected by temperatures that cause significant deactivation of Rubisco (Crafts-Brandner & Salvucci 2000).

Wise *et al.* (2004) measured the functional limitations of photosynthesis of cotton in the field and concluded that at moderately high leaf temperatures ribulose-1,5-bisphosphate (RuBP) regeneration/electron transport contributes significantly to the heat-stress-induced loss of photosyn-

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thetic performance. In this study we investigated mechanisms of heat-induced reductions of photosynthesis of the same cotton cultivars measured by Wise *et al.* (2004). As the field measurements of cotton leaf temperature indicated that moderate heat stress is associated with very rapid temperature changes we devised a system for rapid heating of intact, attached cotton leaves. We measured Rubisco activation, carbon-cycle metabolites, thylakoid leakiness and photosystem I (PSI) activity. As a proxy for NADPH and stromal redox status, we measured the NADP-malate dehydrogenase (MDH) activation state (Scheibe & Stitt 1988). We used this data and the kinetics of heat effects on photosynthetic processes to try to determine which processes are the primary targets of heat effects.

METHODS AND MATERIALS

Plant material

Pima S-6 cotton (*Gossypium barbadense* L.) was planted and grown in a greenhouse in Metro-Mix 366p growth medium (Grace-Sierra, Marysville, OH, USA) until the third or fourth leaf was fully expanded and then transferred to growth chambers. Plants were watered daily with a half-strength Hoagland's solution in the greenhouse and with a solution of Miracle Gro's Excel 15-5-15 Cal-Mag (Miracle Gro, Marysville, OH, USA) in the growth chamber. Plant growth conditions were 32°/26° day/night temperatures with a 16-h photoperiod. Natural daylight was supplemented in the greenhouse by high-pressure sodium vapour lamps that provided 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$. In the growth chamber light was provided by a 1 : 1 mix of high-pressure sodium vapour lamps and high-intensity discharge lamps providing a photosynthetic photon flux of 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Thylakoid 518 nm decay kinetics

Thylakoid membrane energy status was monitored by the flash-induced 518 nm electrochromic shift (ΔA_{518}) as described in Wise & Ort (1989). In brief, an attached leaf was clamped in a thermostatted cuvette of a laboratory-built, single-beam spectrophotometer. Leaves were dark-adapted for 30 min prior to measurements. A dim (i.e. non-actinic), shuttered measuring beam of 518 nm light was passed through the leaf and guided via fibre optics to a photomultiplier tube (PMT). Actinic illumination was provided by a saturating flash from a xenon lamp (6 μs duration) at half peak height, sufficient to turnover 98% of the photosystems only once).

In dark-adapted leaves (in which the chloroplast ATP synthase is fully oxidized and deactivated), a single-turnover actinic flash will cause an immediate increase in absorbance at 518 nm and the decay of that flash-induced electrochromic shift should be a monophasic process (Oxborough & Ort 1995). However, any increase in thylakoid proton permeability not associated with chloroplast ATP synthase activity increases the rate of decay of the flash-induced electrochromic shift. Therefore, traces were

analysed from 50 to 100 ms after the actinic flash, a time frame during which the fast decay dominates. The slope of the line of $\ln(A - A_{\min})/(A_{\max} - A_{\min})$ versus time was used as the decay constant.

Gas-exchange and PSI redox status

Gas exchange parameters were recorded using a modified fast-kill leaf cuvette equipped with a laboratory-made infrared heating element (Fig. 1). The heating element was constructed of 22 AWG nickel-chromium wire (60% nickel, 26% chrome, and 14% iron; Arcor, Northbrook, IL, USA) woven into a Teflon insulator. The heating element was connected to a variable, step-down transformer (Powerstat, Bristol, CT, USA). Leaf temperature could be elevated by more than 12 °C in less than 10 s, which was sufficient to mimic naturally occurring rapid increases in leaf temperature. Once the desired temperature was reached it could be maintained within ± 0.5 °C to simulate sustained heating episodes. The heating element was mounted on an air cylinder that was used to rapidly pull the heating element away from the leaf cuvette when the fast-kill blocks were clamped. All gas exchange parameters were recorded every 1 s by computer.

The first or second fully expanded leaf from the top of a cotton plant was clamped in the fast-kill cuvette with circular

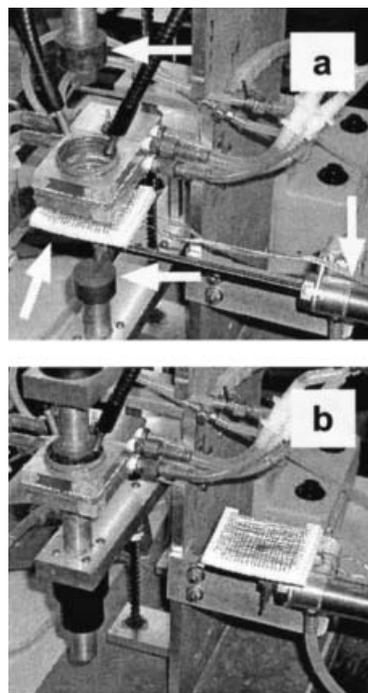


Figure 1. Fast-kill gas exchange cuvette with heating element before (a) and after (b) clamping. The angled arrow points to the heating element with the leaf cuvette directly above it. The horizontal arrows point to the fast-killing metal blocks, which would be stored in liquid nitrogen until needed. The vertical arrow points to the gas cylinder that is used to pull the heating element away from the cuvette.

disposable windows on top (Saran Wrap) and bottom (clear Teflon), exposing 11.46 cm² of leaf tissue for gas exchange. Leaf temperature was monitored with a thermocouple pressed to the abaxial leaf surface. Gas entering the cuvette was controlled with Datametrics (Edwards High Vacuum, Wilmington, MA, USA) type 825 mass-flow controllers. The entering gas was humidified by bubbling a mixture of N₂ and O₂ gas through water, condensing the excess water in an ice-cooled copper coil, and then mixing in 5% CO₂ in air to achieve the desired CO₂ partial pressure. Water and CO₂ entering and exiting the cuvette were analysed with a LiCor 6262 infrared gas analyser (LiCor Inc., Lincoln, NE, USA). Light was provided by a KL1500 (Schott Glas, Mainz, Germany) at 1500 μmol m⁻² s⁻¹ photosynthetic photon flux density, as determined by a LiCor quantum sensor.

The redox status of the reaction centre of PSI was measured *in vivo* using an ED-P700DW-E emitter/detector unit (Heinz Walz, Effeltrich, Germany) connected to a PAM-101 system (Heinz Walz), which measures the absorbance change at 810 nm (ΔA_{810}) relative to that at 860 nm. PSI was transiently reduced by a 15-s episode of darkness and transiently oxidized by a 3-s pulse of saturating white light (8000 μmol m⁻² s⁻¹) provided by a Schott KL2500. A saturating pulse of white light was found to oxidize PSI more completely than a pulse of far-red light in actively photosynthesizing, light-adapted leaves.

Metabolite determination

Leaf metabolism was rapidly stopped during gas-exchange measurements by clamping the leaf between two 8.2 cm² copper blocks that had been cooled in liquid nitrogen (Fig. 1). Frozen leaf discs were immediately cut in half. One half of the sample was used for metabolite analysis and the other half for enzyme analysis. After the first half was ground to a powder, 3.5% HClO₄ (v/v) was added and ground into powder. After thawing and centrifugation, the supernatant was neutralized with 2 N KOH, 0.15 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and 0.01 M KCl, and the precipitate was discarded after further centrifugation. The final solution was snap frozen and stored at -80 °C until analysis. Solutions were analysed by enzyme-linked photometric assays (Lowry & Passonneau 1972) using a dual-wavelength filter photometer (Sigma ZFP22; Sigma Instrumente, Berlin, Germany) measuring the absorbance difference between 334 and 405 nm.

For analysis of RuBP and 3-phosphoglycerate (PGA), a 20 μL extract was buffered to pH 8.0 with 800 μL 50 mM *N,N*-bis(2-hydroxyethyl)glycine (bicine), 15 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM NaCl, 15 mM NaHCO₃, 5 mM phosphocreatine, 10 mM dithiothreitol (DTT), 1 mM ATP and 7 μM NADH. Reactions were started by adding 5 μL creatine kinase (1 U μL⁻¹) and 5 μL glyceraldehyde-3-phosphate dehydrogenase (0.2 U μL⁻¹). Upon completion of these reactions, 5 μL phosphoglycerate kinase (0.2 U μL⁻¹) was added to determine PGA levels. Upon completion of this reaction, 5 μL of activated Rubisco was added to determine RuBP levels.

RuBP results were divided by two to account for the stoichiometry of the Rubisco-catalysed reaction.

For analysis of glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), and ATP, 20 μL extract was buffered to pH 8.0 with 800 μL 50 mM bicine, 5 mM MgCl₂, 1 mM EDTA, 500 μM NADP and 0.25 mM glucose. Reactions were started by adding 5 μL G6P dehydrogenase to determine G6P. Upon completion of this reaction, 5 μL hexose isomerase was added to determine F6P. Upon completion of this reaction, 5 μL hexokinase was added to determine ATP.

For analysis of adenosine 5'-diphosphate (ADP), 50 μL extract was buffered to pH 7.0 with 800 μL 50 mM Hepes, 5 mM MgCl₂, 90 mM KCl, 7 μM NADH and 0.3 mM phosphoenolpyruvate (PEP). Reactions were started by adding lactic acid dehydrogenase. Upon completion of this reaction, 5 μL pyruvate kinase was added to determine ADP.

Rubisco activation and stromal redox status

Leaf samples stored at -80 °C in 1.5 mL tubes were briefly ground, then 800 μL extraction buffer was added. The extraction buffer was 50 mM bicine pH 8.0, 30 mM NaCl, 5 mM MgCl₂, 2 mM EDTA, 0.5% Triton X-100, 10 mM mannitol, 0.5% polyvinyl poly pyrrolidone (PVPP), 1% bovine serum albumin (BSA), 5 mM DTT, and 1 mM phenylmethylsulphonyl fluoride. The extract was used for Rubisco activation and redox analysis. Reactions to determine initial Rubisco activity were started immediately after centrifuging the sample for 10 s by adding 15 μL extract to a scintillation vial containing 200 μL 50 mM bicine buffered at pH 8.0, 20 mM MgCl₂, 0.2 mM EDTA, 15 mM NaH¹⁴CO₃ (1 mCi mmol⁻¹), and 0.5 mM RuBP, and stopped after 30 s with 200 μL formic acid. Reactions to determine total Rubisco activity were conducted equivalently, with the exception that 177 μL extract was incubated for 5 min with 23 μL 20 mM MgCl₂, 15 mM NaHCO₃, and 0.05 mM 6-phosphogluconate. Reactions to determine background radioactivity were conducted equivalent to initial Rubisco reactions except that formic acid was added before the leaf extract. All samples were evaporated in a sand bath overnight, rehydrated with 100 μL H₂O and 3 mL Biosafe II liquid scintillation cocktail (Research Products International Corp., Mt. Prospect, IL, USA), and radioactivity was determined by scintillation counting for 2 min.

After analysing for initial Rubisco activity, analysis for initial NADP-MDH activity was initiated with 10 μL extract buffered to pH 8.0 with 50 mM bicine, 1 mM EDTA, 0.05 mM NADPH, 1 mM DTT and 0.01% BSA. Reactions were started by adding 5 μL 200 mM oxaloacetic acid. Analysis for total NADP-MDH activity was conducted equivalently except that 100 μL extract was incubated for 5 min with 25 μL of 2 M bicine buffered to pH 9.0 and 200 mM DTT. Analysis for NAD-MDH activity was initiated with 10 μL extract that had been diluted 50-fold and buffered to pH 8.0 with 50 mM bicine, 1 mM EDTA, 5 mM MgCl₂, and 0.05 mM NADH. Reactions were started by adding 5 μL 200 mM oxaloacetic acid. Solutions were analysed using a

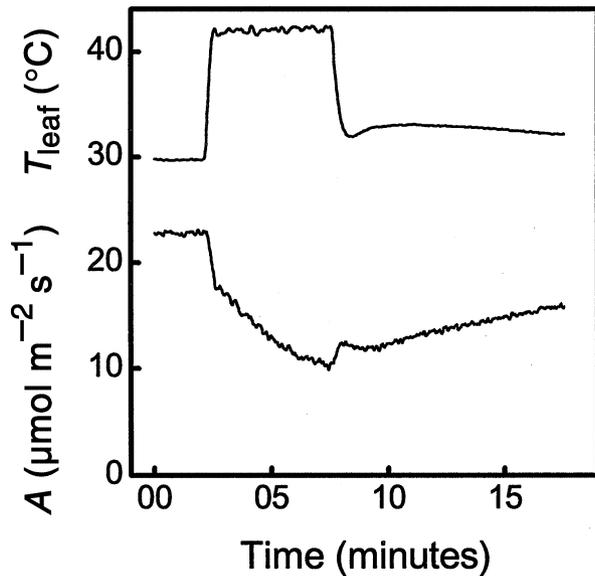


Figure 2. Leaf temperature (T_{leaf} ; top trace) and CO_2 assimilation rate (A ; bottom trace) of cotton heated by infrared radiation to 42°C for 5 min.

dual-wavelength filter photometer (Sigma ZFP22; Sigma Instrumente) measuring the absorbance difference between 334 and 405 nm.

77 K fluorescence

The chlorophyll emission spectra at liquid nitrogen temperature (77 K) were obtained using a Photon Technologies Incorporated (PTI; Severna Park, MD, USA) Alphascan-1 emission spectrometer equipped with a xenon arc lamp excitation source and fitted with an Oxford Instruments DN-1704 liquid nitrogen cryostat thermostated by an ITC-4 digital temperature controller (Oxford Instruments, Chicago, IL, USA). Samples were collected in the field, immediately frozen between two blocks of dry ice, and stored at -80°C until analysis, approximately 2 weeks later. Control experiments revealed that fluorescence at 77 K did not change as a consequence of the time that the tissues were stored at -80°C (data not shown). Whole leaf samples (approximately 1 cm^2) were held in the cryostat using mounts that positioned the samples at a 45° angle relative to the 440 nm-excitation beam and were temperature-equilibrated for no less than 75 min. Emitted light was collected from the samples at 90° to the excitation source by passing the fluorescence through a series of Schott colour glass filters (470, 550 and 580 nm), a monochromator, and finally detecting the signal with a Hamamatsu R928 photomultiplier (Hamamatsu City, Japan).

RESULTS

Rapid leaf heating

When leaf temperature was rapidly increased from 30 to 42°C (temperature transition completed in less than 30 s),

net photosynthesis declined instantaneously with the temperature increase by about 17%, followed by a progressive decay of photosynthesis at 42°C of approximately $8\% \text{ min}^{-1}$ (Fig. 2). Lowering the temperature to 32°C was accompanied by a nearly instantaneous recovery of photosynthesis by 8–10% followed by a slower recovery at approximately $3\% \text{ min}^{-1}$. The slow decline was temperature-dependent, being progressively worse from 39 to 45°C (data not shown). The extent of the rapid decline is consistent with the increased rate of photorespiration that occurs at high temperature (Ogren 1984; Brooks & Farquhar 1985) but increased mitochondrial respiration (Bednarz & Van Isreal 2001) may also have contributed.

Leaf metabolites

Rubisco activation did not decline during very short heating episodes (Fig. 3). On the other hand, the amount of RuBP in the leaves declined by 28% in response to just 7 s of heating, which may reflect the 144% increase in the rate

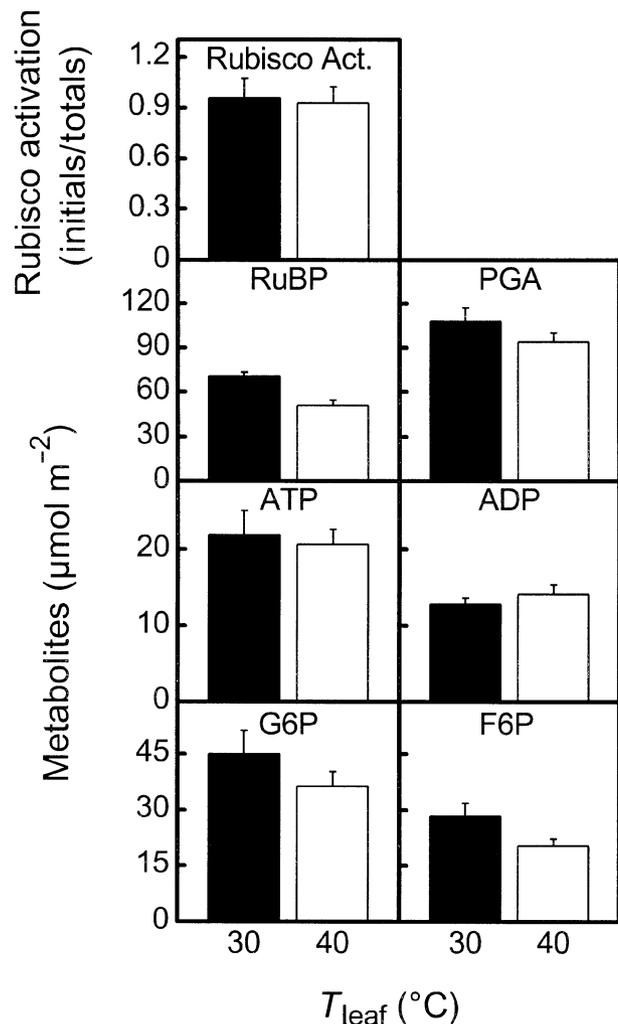


Figure 3. Rubisco activation and leaf metabolites at 30°C before heating (grey bars) and in leaves heated to 40°C in 7 s (black bars), ($n = 4$, mean + SE).

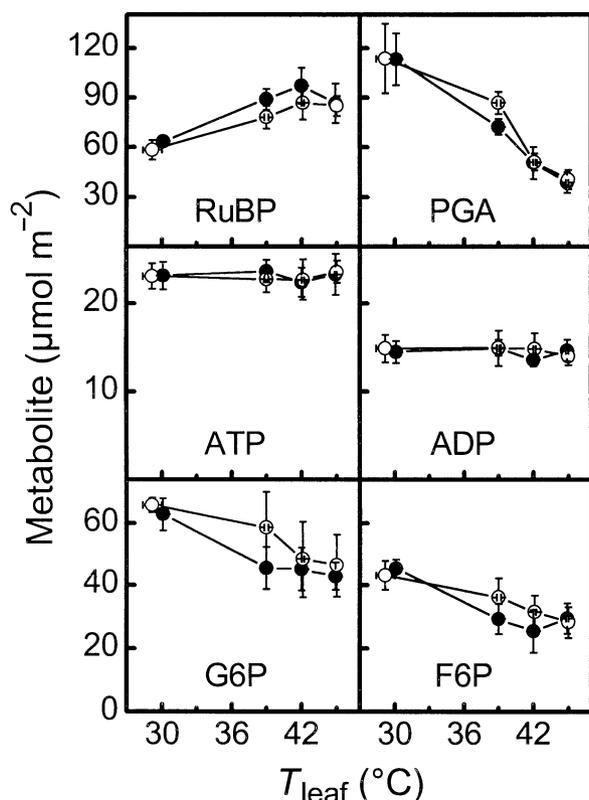


Figure 4. Leaf metabolites in leaves heated for 3 min (closed) and following 1-min recovery from a 3-min heating event (open), ($n = 4$, mean \pm SE).

of photorespiration (estimated using the photosynthesis parameters of Bernacchi *et al.* 2001). Leaf content of ATP and ADP was little affected and PGA, G6P and F6P declined only marginally following rapid heating.

After 3 min at moderately high temperature, leaf RuBP content increased (Fig. 4) and PGA declined, consistent with deactivation of Rubisco. The whole-leaf ATP and ADP content did not change whereas G6P and F6P declined. Unlike the data at 7 s after the beginning of the heat spike, after 3 min at moderately high temperature Rubisco activation declined (Fig. 5). However, NADP-MDH activation state also declined under these conditions (Fig. 5). Photosynthesis and Rubisco activation were well correlated with NADP-MDH activation (Fig. 6).

Electrochromic shift at 518 nm

The thylakoid membrane permeability increased with temperature as indicated by relaxation rates of ΔA_{518} during and after heating. Relaxation rates were approximately 50% faster at 42 °C (Figs 7 & 8). More rapid relaxation rates were evident in leaves heated to 36 °C compared with leaves at 30 °C whereas peak absorbance amplitude was not affected until 45 °C. Relaxation rates did not differ during and after heating except at 33 and 36 °C, where decay rates were faster after heating than during heating. Peak absorbance amplitude did not differ during and after heating at any temperature.

The electrochromic shift experiments were done with dark-adapted leaves. To examine the effect of light during the heat spike we equilibrated leaves at 30 °C, turned off the light, and heated the leaf in the dark to 42 °C for 1 min. After the leaf temperature returned to 30 °C, the leaf was re-illuminated (total time in darkness was less than 2 min). This heat spike in the dark caused a 42% decline in net photosynthesis. When the light was left on, the heat spike caused a 25% decline (Fig. 9).

PSI response to high temperature

Responses of PSI were compared between leaves heated to 39 and 42 °C (Fig. 10). These changes were also quantified and compared with fully reduced PSI (darkness) and fully oxidized PSI (saturating pulse of white light, Fig. 11). Immediately upon heating, the PSI became reduced, behaving the same at 39 and 42 °C. However, during heating at 39 °C, PSI remained reduced, whereas at 42 °C PSI oxidized during heating, becoming slightly more oxidized after 5 min than before heating. Upon cooling, PSI oxidized rapidly with the relative change in oxidation equivalent between 39 and 42 °C. Five minutes following this initial

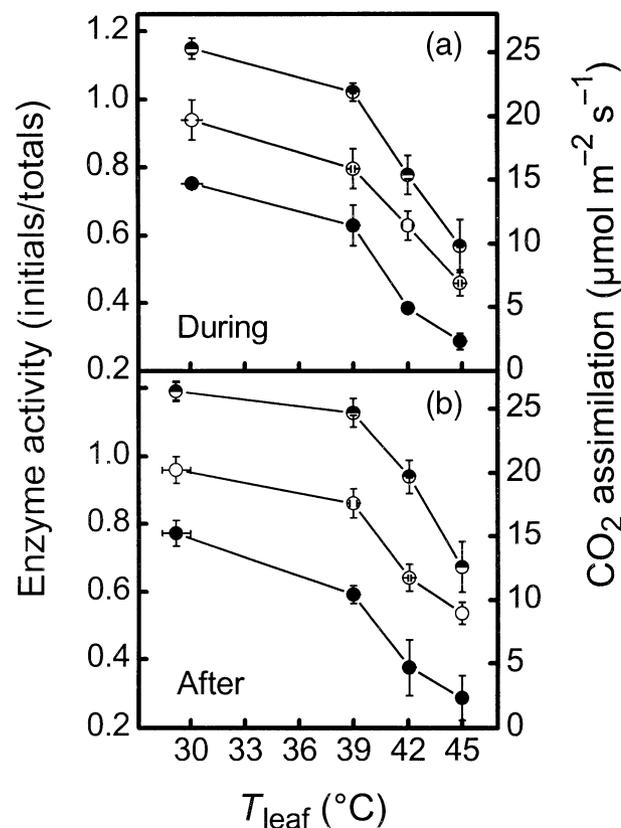


Figure 5. Photosynthetic rates (half closed symbol, right axis), Rubisco activation (open symbols, left axis), and NADP-malate dehydrogenase activity (closed symbols, left axis) for leaves heated for 3 min (a) and for leaves heated for 3 min and cooled for 1 min (b). Note the enzyme activity scale begins at 0.2 for both graphs, ($n = 4$, mean \pm SE).

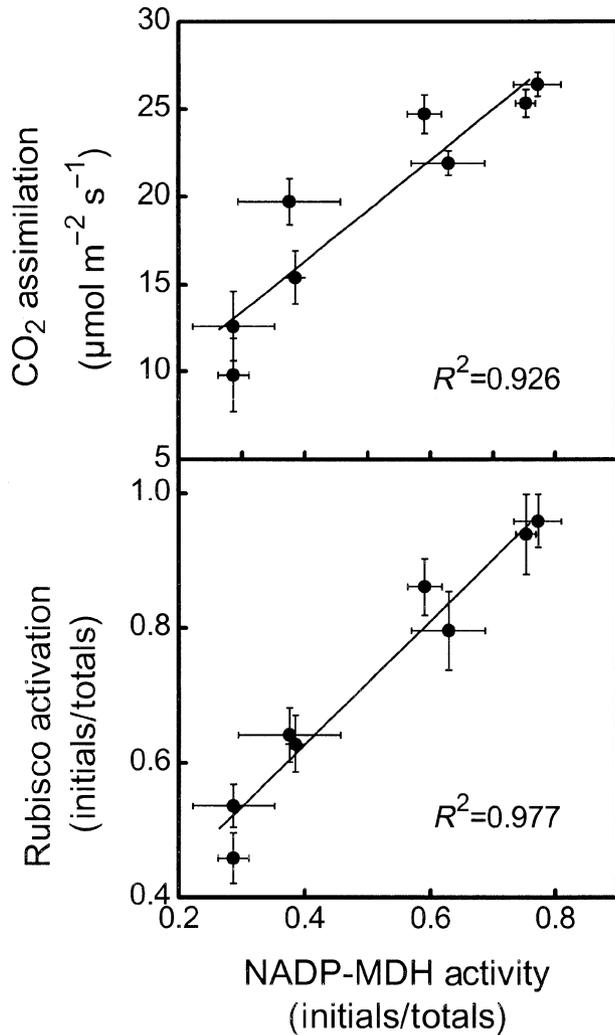


Figure 6. Correlation of NADP-malate dehydrogenase activity with Rubisco activation and CO₂ assimilation rates. Data are from the same experiment reported in Fig. 5, ($n = 4$, mean \pm SE).

oxidation upon cooling, PSI re-reduced, returning to pre-heat redox levels in leaves heated to 39 °C and to slightly oxidized levels in leaves heated to 42 °C. ΔA_{810} was also recorded for dark-adapted leaves that were kept in the dark during the entire course of heating (data not shown). Absorbance in darkness did not change before, during, or after heating, but remained at a constant level.

State 1 to state 2 transition

Three leaves were collected from field-grown plants at the site in Maricopa, AZ early in the morning when air temperature was 28 °C and three were collected at 1330 h when air temperature was 41 °C. Fluorescence was measured at 77 K. The ratio of fluorescence at 735 to that at 685 nm was significantly higher (at the 5% probability level, by Tukey's test) at mid-day when the air temperature was 41 °C than at 0830 h when air temperature was 28 °C (Fig. 12).

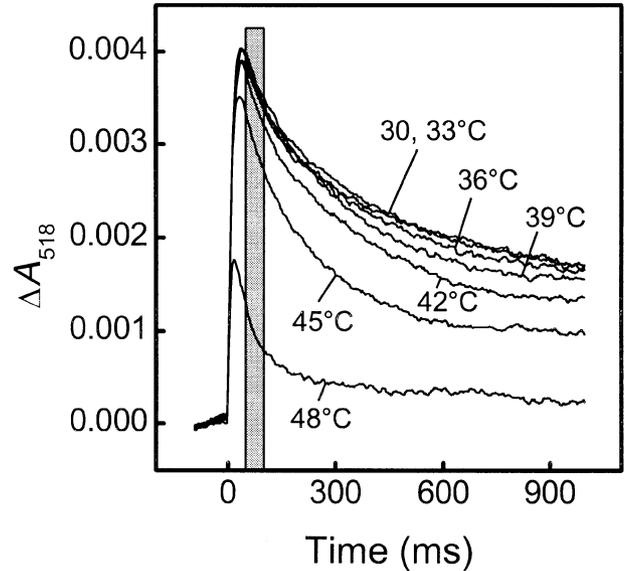


Figure 7. Absorbance of dark-adapted cotton leaves at 518 nm following a saturating flash of light.

DISCUSSION

The instantaneous decline in net photosynthesis at moderately high temperature was consistent with increased photorespiration when the data were modelled using the

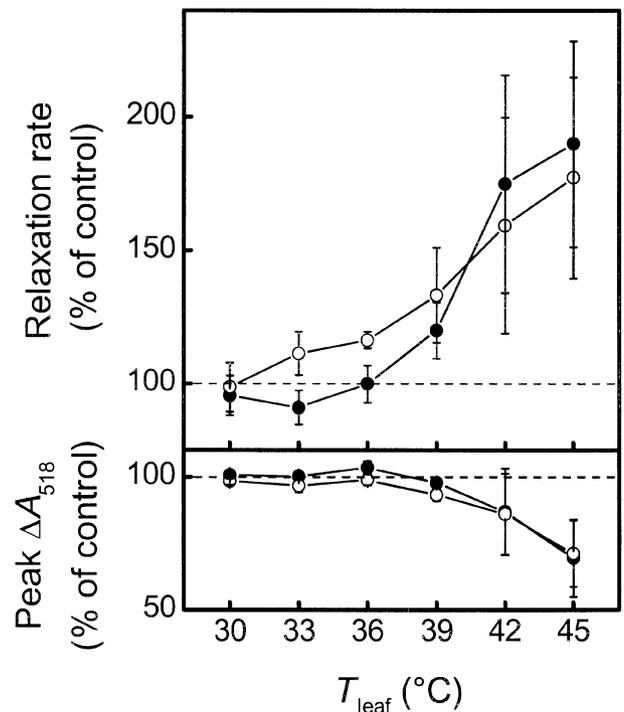


Figure 8. Rate of thylakoid energy dissipation (top panel) and peak energization (bottom panel) for various leaf temperatures. Attached, dark adapted leaves were analysed for the change in absorbance at 518 nm during (closed symbols) and after heating (open symbols), ($n = 5$, mean \pm SE).

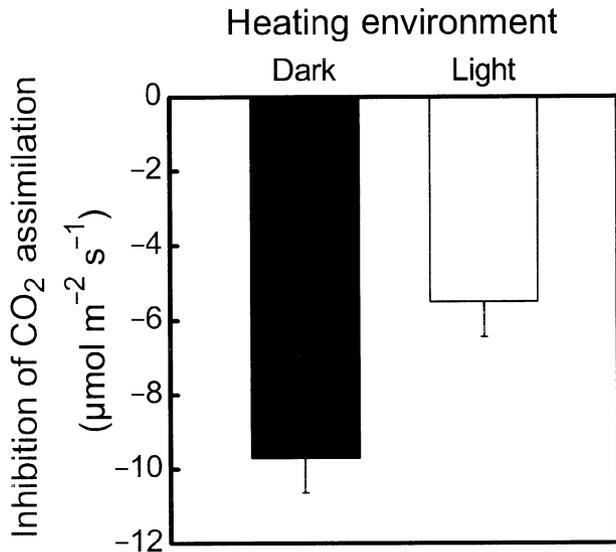


Figure 9. Inhibition of CO₂ assimilation for leaves heated 1 min in darkness or 1 min with the lights remaining at 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Leaves averaged a CO₂ assimilation rate of 22.6 $\mu\text{mol m}^{-2} \text{s}^{-1}$ before heating, ($n = 4$, mean \pm SE).

parameterization of Bernacchi *et al.* (2001, 2002). However, damage to the photosynthetic apparatus was quickly apparent. After heating for 3 min at 39 °C and above, Rubisco deactivated and the decline in activity was well correlated with the decline in photosynthesis, but so was a decline in NADP-MDH activity. The lack of significant change in some PSII chlorophyll fluorescence

parameters has been taken as an indication that membrane reactions are less sensitive to moderately high temperature (Law & Crafts-Brandner 1999). However, we found that moderately high temperature affected other measures related to photosynthetic electron transport, namely PSI reduction state and NADP-MDH activity, supporting the involvement of electron transport components in the heat sensitivity of photosynthesis. A role for thylakoid membranes in moderate heat damage to photosynthesis is also supported by work showing that zeaxanthin can reduce the leakiness of thylakoid membranes induced by 15 min of heat stress thereby reducing the heat damage to photosynthesis (Havaux 1993; Havaux & Gruszecki 1993; Havaux & Tardy 1996; Havaux *et al.* 1996; Havaux 1998).

Deactivation of Rubisco may be advantageous to plants at moderately high temperature. First, the potential for very high rates of photorespiration at high temperature can be held in check by Rubisco deactivation. This would lower the total amount of photorespiratory enzymes that plants would need to invest in as well as limit the production of cytotoxic photorespiratory products (Husic, Husic, & Tolbert 1987). The activation state of Rubisco can be reduced by high rates of photorespiration (Chastain & Ogren 1985) because of high levels of the photorespiratory intermediate glyoxylate (Chastain & Ogren 1989; Campbell & Ogren 1990b). Second, heat damage is more severe in darkness than in the light (Fig. 9; Weis 1982; Havaux, Greppin & Strasser 1991) indicating that a transthylakoid energy gradient helps thylakoid membranes cope with high temperature. Deactivation of Rubisco (together with increased

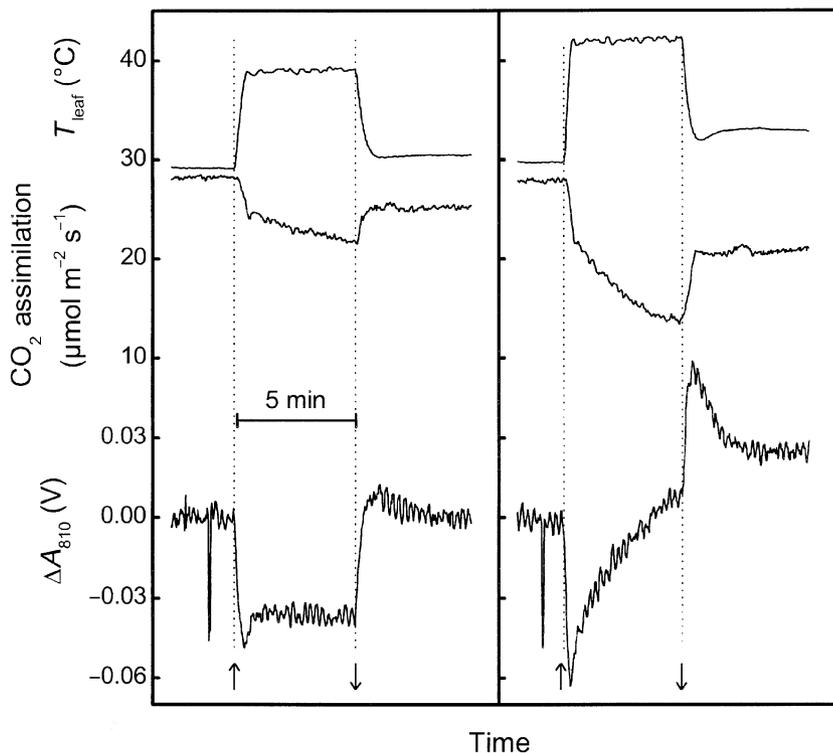


Figure 10. Representative traces of ΔA_{810} (bottom trace), CO₂ assimilation rate (middle trace), and leaf temperature (top trace) for leaves heated to 39 °C (left panel) and 42 °C (right panel) for 5 min. Up arrows indicate the onset of heating using infrared heating and down arrows indicate the removal of infrared heating. The transient decline in the ΔA_{810} signal just prior to heating is a calibration signal and is equal to 0.1% transmittance.

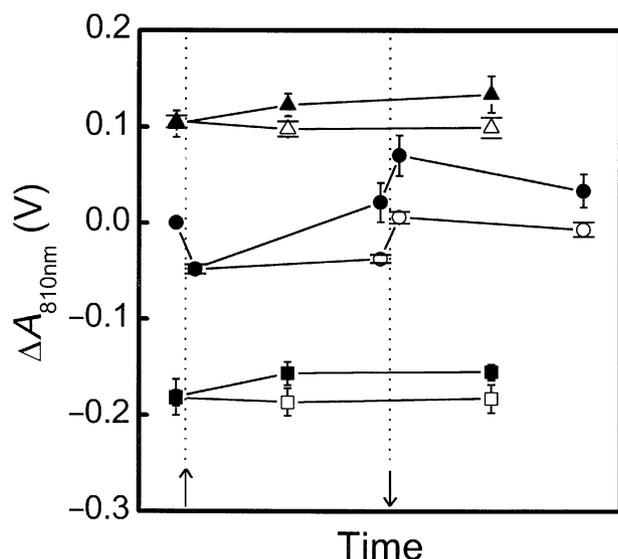


Figure 11. ΔA_{810} before heating, during heating, and after heating for leaves heated to 39 °C (open symbols) and 42 °C (closed symbols) for 5 min. ΔA_{810} were measured at 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light (circles), in darkness (fully reduced, squares), and during a brief saturating flash of white light (fully oxidized, triangles). Up arrows indicate the onset of heating using infrared heating and down arrows indicate the removal of infrared heating, ($n = 4$, mean \pm SE).

cyclic electron transport) would help maintain a transthylakoid energy potential at high temperature in the face of increasing membrane leakiness. At moderately high temperature, maintenance of a high transthylakoid energy gradient may become more important than extra carbon fixation to protect the thylakoid membranes. This is consistent with the state 1 to state 2 transition observed in plants experiencing heat stress in the field (Fig. 12). Pea leaves

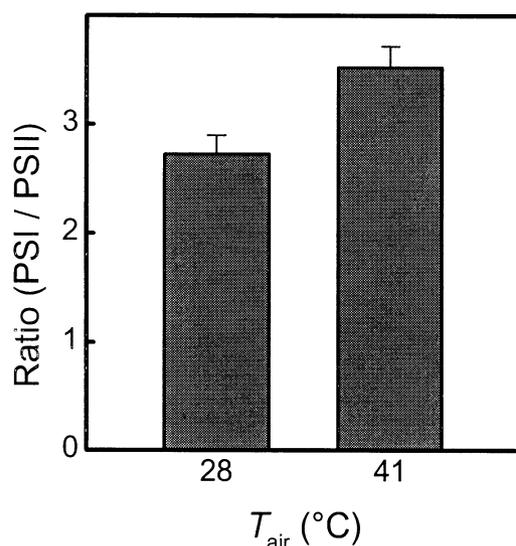


Figure 12. Ratio of fluorescence from PSI to PSII at 77 K. Leaves were taken early in the morning, when air temperature was 28 °C or midday when air temperature was 41 °C.

subject to 42 °C show increased phosphorylation of light-harvesting complex II (LHCII) and migration to non-appressed thylakoids increasing the supply of light energy to PSI (Mohanty, Vani, & Prakash 2002).

Experiments using tobacco plants with antisense genes to Rubisco activase indicated that when plants cannot deactivate Rubisco they are more damaged by moderate heat stress than control plants (Sharkey *et al.* 2001). Heat causes different forms of activase to be made by plants but also leads to a reduction in mRNA for activase (Law & Crafts-Brandner 2001). Therefore the effect of moderately high temperature may be primarily on thylakoid reactions with Rubisco deactivation either a consequence of, or an adaptive response to, the damage to thylakoids or potentially high rates of photorespiration. Parsimony makes it easier to imagine how stromal oxidation status could affect Rubisco activase (Zhang & Portis 1999; Zhang, Schürmann, & Portis 2001) than to imagine how Rubisco deactivation could cause the stroma to become more oxidized. Deactivation of Rubisco should lead to less NADPH consumption and increased redox status, rather than the effects observed here.

The ability to form a transthylakoid gradient was not impaired at temperatures that significantly increased the decay of the gradient (Fig. 8). This indicates that PSII was more heat stable than the integrity of the thylakoid membranes. We were surprised to find that ATP levels were not affected by heat stress in cotton. We hypothesize that the stability of ATP pools during heat stress may be explained by the decreased use of ATP by the deactivation of Rubisco and maintenance of transthylakoid potential by the induction of cyclic electron transport. Plants in which one or the other cyclic electron transport pathway has been knocked out may provide evidence for which pathway is important during heat stress.

Rapid heating caused a transient reduction in RuBP levels without deactivating Rubisco. This transient reduction corresponded with a rapid increase in photorespiration as measured by gas exchange parameters. Although RuBP levels recovered after prolonged heating, this initial decline could facilitate the deactivation of Rubisco. RuBP bound to Rubisco prevents Rubisco from deactivating by holding the carbamate in place (Badger & Lorimer 1981), and under non-stressful conditions in the light, RuBP is saturating for Rubisco active sites. A rapid increase in RuBP use by either photosynthesis or photorespiration could lower RuBP to subsaturating levels for Rubisco active sites, and thus allow Rubisco to deactivate (Portis, Lilley, & Andrews 1995). The change in stromal redox status could reduce the activity of activase (Campbell & Ogren 1990a) and in some cases aggregation of activase (Feller, Crafts-Brandner, & Salvucci 1998) or binding of activase to thylakoid membranes (Rokka, Zhang, & Aro 2001) could result in substantial deactivation of Rubisco allowing RuBP levels to recover. An initial decline in RuBP content followed by a recovery as Rubisco deactivates has been observed in response to reducing light level (Mott *et al.* 1984) or imposing end-product-synthesis feed-

back on plants (Sharkey *et al.* 1986). Thus there are other examples of Rubisco deactivation in response to treatments that reduce the capacity for electron transport. Rubisco deactivation appears to be a mechanism of regulation rather than a limiting process, just as electron transport can be regulated when there is excess light by mechanisms that result in q_E quenching of chlorophyll fluorescence (Niyogi 2000).

Photosystem II damage has long been considered the primary site of heat damage to photosynthetic electron transport (Berry & Björkman 1980). However, many investigators have found that moderate heat stress increases PSI activity, often at the expense of the redox status of the stroma (Boucher *et al.* 1989; Havaux *et al.* 1991; Havaux 1996; Bukhov, Boucher, & Carpentier 1998; Bukhov *et al.* 2000). In addition, there is a substantial shift of light-harvesting complexes from PSII to PSI (a state 1 to state 2 transition) (Fig. 12 and Pastenes & Horton 1996). This may come about because of a stimulation of thylakoid-associated kinase 1 (Snyders & Kohorn 2001) a kinase that phosphorylates LHCI so that it can migrate to unappressed regions of the thylakoid and transfer energy to PSI (Allen 1995). However, it has also been reported that PSII itself is very rapidly dephosphorylated at moderately high temperature, perhaps to facilitate repair (Rokka *et al.* 2000).

The response of PSI to heat is quite different in leaves varying in temperature by only 3 °C, with PSI in leaves at 39 °C remaining reduced and at 42 °C re-oxidizing during heating (Figs 10 & 11). It is unclear what causes this difference but we speculate on a few possibilities here. First, the initial reduction of PSI is very rapid and corresponds closely with the leaf temperature. This reduction could be due to stromal reductants reducing elements in the intersystem electron transport chain. One obvious source is NADPH donation to NDH (Nixon 2000; Peltier & Cournac 2002) but donation of electrons from ferredoxin to FQR (Peltier & Cournac 2002; Munekage *et al.* 2002) probably would also lower the stromal redox potential.

In summary, moderate heat stress affects the permeability of the thylakoid membranes and stimulates PSI activity at the expense of stromal reductants. Among many consequences of these effects is the deactivation of Rubisco, which can be an adaptive response to maintain the thylakoid energy gradient and reduce accumulation of high levels of photorespiratory intermediates.

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