HEAT-STRESS-INDUCED INHIBITION OF THE ACTIVATION STATE OF COTTON RIBULOSE-1,5- BISPHOSPHATE CARBOXYLASE/OXYGENASE (RUBISCO) IS MEDIATED BY RUBISCO ACTIVASE

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Abstract

Glandless upland cotton (*Gossypium hirsutum* L. cv. Coker 100A) plants were subjected to heat stress in a growth cabinet. Carbon exchange rate (CER), ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (rubisco) activation state, and RuBP and 3-phosphoglycerate (3-PGA) content were monitored. Air temperature increases from control (28°C) values were in 2.5°- to 5°-steps to 45°C, with a 1-h equilibration at each step with high ambient humidity (~75%) to allow leaf temperature to approach that of the air. Samples taken from young, fully-expanded leaves indicated that rubisco activation state (mediated by the presence of active, non-heat-denatured rubisco activase) began decreasing when leaf temperature was raised above 35°C and decreased to 25% of control values at 45°C. This was mirrored by decreases in net photosynthetic rate (CER) to 61% of control values at 42.5°C, and by a 50% decrease in the rubisco product 3-PGA, starting at 40°C and reaching a maximum at 45°C. Additionally, these changes in rubisco activity, activation state and 3-PGA content were reversible by subsequently lowering the temperature of the heat-stressed leaves. Fluorescence measurements indicated that increasing leaf temperature adversely affected flux through the photosynthetic electron transport chain. Cumulatively, these results complement a previous study (Feller et al. (1998) Plant Physiol **116**, in press) demonstrating the importance of rubisco activase in the maintenance of maximal rubisco activity at relatively high temperatures.

Introduction

Rubisco is the enzyme that catalyzes the assimilation of atmospheric CO_2 and its activity is considered the rate-limiting step in the photoassimilatory Calvin cycle. Despite the presence of large amounts of rubisco in leaves (often constituting up to 40% of the soluble protein), it is largely unable to catalyze carbon fixation without the presence of the stromal enzyme rubisco activase. Activase facilitates the release of inhibitory sugar phosphates from the catalytic site of noncarbamylated (non-CO_2-bound) rubisco in the light, allowing it to become catalytically competent (for review, see Andrews et al., 1995).

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While rubisco itself is relatively heat-stable, activase is not, and this is thought to limit the assimilation of carbon at higher temperatures. There exist significant differences between species with respect to their ability to withstand high temperatures while maintaining CO_2 assimilation via rubisco. There is currently some evidence suggesting that this variability arises from the molecular form of activase present (Crafts-Brandner et al., 1997). We decided to take a physiological approach in the determination of how increasing temperature affects rubisco activity and its activation state (modulated by rubisco activase). Rubisco activity may also be determined indirectly, via changes in nonphotochemical fluorescence quenching (qN) or by determining the levels of substrate (RuBP) and product (3-PGA) of the rubisco reaction. We hypothesized that photosynthetic response to high temperature stress in cotton leaves is modulated in the short term by reversible disaggregation of active activase multimers, as should be demonstrated by the reversible nature of heat stress on rubisco activity.

Materials and Methods

Glandless cotton plants were germinated in trays for 1 week in a greenhouse under ambient light and a 29°C day/22°C night/~10-h photoperiod regime. The seedlings were transplanted into 20-cm-diameter pots and placed into growth cabinets programmed for 15-h days at 28°C and 350 $\mu E \cdot m^{-2} \cdot s^{-1}$, and 9-h nights at 25°C. Sampling occurred after 3 weeks. All measurements were taken from fully expanded young leaves. Leaf temperatures were measured using a thermocouple (Omega) that rested against the lower surface of a leaf.

1. Photosynthetic Rate and Chlorophyll Fluorescence

Prior to taking measurements, 2 leaves were fully light activated using a cool light source at >1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 30 min. Carbon dioxide exchange rate (CER) was monitored with an ADC infrared gas analyzer (Heddesdon, UK) equipped with a Parkinson leaf chamber. Chlorophyll fluorescence quenching was measured by subsequently dark adapting the leaf for 15 min and was accomplished using a PAM-2000 Walz fluorometer as in Feller et al. (1998), except that determination of qN occurred at the appropriate leaf temperature. Temperature increases from control (28°C) values were in 2.5°- to 5°-steps to 45°C, then back down, with a 1-h equilibration at each temperature.

2. Rubisco Activity and Activation State

Rubisco activity was assayed by monitoring the incorporation of ^{14}C-bicarbonate into acid-stable organic acids as in Salvucci and Anderson (1987). This allowed determination of both total and initial activity, with the intial:total ratio indicating the ability of rubisco activase to maintain rubisco activity under the given temperature treatment conditions.

3. Metabolite Determination

Metabolite content of fully-illuminated (>1000 $\mu E \cdot m^{-2} \cdot s^{-1}$) control and heat-treated cotton leaves was determined by freeze-clamping one 2.1-cm-diameter disc from one side of a young, fully-expanded cotton leaf, then increasing the temperature to the desired level and removing an identical disc from the other side of the leaf after equilibration at the higher temperature. The samples were powdered under liquid N_2, extracted in 500 μL 5% (v/v) hydrofluoric acid with a polytron and centrifuged for 10 min in a microfuge. The freeze-dried supernatants were resuspended in 100 μL dH_2O, ~1 mg activated charcoal added and the mixture incubated on ice for 30 min. After centrifugation the supernatants were sequentially assayed spectrophotometrically for 3-PGA and RuBP as in He et al. (1998).

Results

Leaf temperature proved to be difficult to elevate to air temperature in the chamber without ensuring that humidity levels remained >75%. This was accomplished by maintaining a layer of water on the bottom of the growth chamber by shutting off the chamber drain.

1. Photosynthetic Rate and Chlorophyll Fluorescence

Measurements taken with a hand-held infrared gas analyzer indicated that CER began to be perturbed when leaf temperature was raised from 28 to 32.5°C, and was reduced maximally to 40% of the control at 45°C (table 1). Additionally, the effect on photosynthesis was reversible, with recovery back to 86% of control values when leaf temperature was returned to 30°C (table 1).

Increasing leaf temperatures progressively increased nonphotochemical fluorescence quenching (qN), indicating a significant negative effect on Calvin cycle activity (fig. 1). At higher temperatures, qN was increased ~2-fold compared to the control temperature. Decreasing the leaf temperature led to full recovery of qN to the control level (data not shown).

High leaf temperatures (table 1) decreased maximum fluorescence of a dark-adapted leaf (Fm) and the ratio of variable fluorescence (Fv) to Fm, but the effect was much less pronounced than the effect of high temperature on qN.

2. Rubisco Activity and Activation State

The activity of rubisco activase is difficult to measure directly due to the large amounts of endogenous rubisco present in crude leaf extracts. Thus, the activation state of rubisco (initial activity) is taken as a measure of activase activity, a valid assumption given that the activation state of rubisco in the light is a direct consequence of the activity of activase (Andrews et al., 1995; Eckardt et al., 1997).

Both initial rubisco activity and the activation state of the enzyme declined with increasing leaf temperature, to a minimum of 25% of control values at 45°C (table 1). These decreases were fully reversible, with full recovery to control values by lowering leaf temperature back to 28°C.

3. Metabolite Determination

The product of rubisco, 3-PGA, was found to decrease upon heat treatment of cotton plants. This decrease was maximal (48% of control values) at the highest temperature tested, 45°C (fig. 2). The decrease in 3-PGA content was reversible by subsequently decreasing the temperature of the heat-treated plants back to the control value (28°C) (fig. 2). In contrast to wheat (Kobza and Edwards, 1987) and *Arabidopsis* (Eckardt et al. 1997), RuBP content (fig. 2) was not negatively correlated to rubisco activation state (table 1), and appeared to gradually decline over the time course of heat stress and recovery.

Discussion

Short-term heat stress is an excellent method of demonstrating the heat-lability of photosynthetic carbon metabolism via rubisco. By keeping each temperature increase moderate and the total time course over a matter of hours, it can be demonstrated that heat-related decreases in rubisco activity are reversible and not due to the denaturation of rubisco itself. This is because total rubisco activity in cotton leaf was not affected by heat treatment (Feller et al., 1998), and the enzyme activity is known to be stable at temperatures above 50°C (Ekardt and Portis, 1997).

This work has taken a physiological approach to the examination of the previously-observed deleterious effect of heat stress on the light activation of rubisco (Crafts-Brandner et al., 1997; Eckardt and Portis, 1997; Feller et al., 1998). While studies have been conducted on excised leaf sections floated on water equilibrated to the desired temperature, the present study demonstrates that these observations also hold true when intact plants are used.

Increases in qN with elevated temperature (fig. 1) demonstrated that a decrease in the utilization of ATP and NADPH due to inhibition of Calvin cycle activity (Schreiber et al., 1986) accompany the decrease in rubisco activation state. This is likely due, in part, to the inhibition of activase and consequent decrease in rubisco activity (Feller et al., 1998). The effect of elevated temperature on F_m and F_v/F_m were much less than the effect on qN, thus indicating that electron transport was not as sensitive to high temperature as Calvin cycle activity.

Decreased CER at elevated temperatures was correlated with decreased rubisco activation state (table 1). Lowering of initial rubisco activity and rubisco activation state (table 1) were likely due to lessening of the ability of activase to activate rubisco at elevated temperatures. The reversible nature of this inhibition and the knowledge that it is the aggregation of activase subunits which allows the enzyme

to safeguard rubisco against temperature-induced inhibition (Salvucci and Ogren, 1996) suggests that this interaction is perturbed in cotton leaves at high temperature, but quickly restored when temperatures are subsequently lowered. Metabolite measurements indicate that rubisco is indeed inhibited at these temperatures, due to a decrease in the level of 3-PGA detected (fig. 2). However, unlike previous studies in wheat (Kobza and Edwards, 1987) and *Arabidopsis* activase mutants (Eckardt et al., 1997), RuBP content did not rise in tandem with the 3-PGA decrease, and in fact appeared to decrease slightly during heat stress and recovery (fig. 2). These results suggest that cotton activase may not be as heat labile as, for example the wheat enzyme (Kobza and Edwards, 1987), and that other Calvin cycle enzymes are adversely affected by high temperature.

In conclusion, these results indicate that activase is reversibly inhibited by elevated leaf temperature in cotton, contributing to decreased CER. However, the results also suggest that other Calvin cycle enzymes may be equally sensitive to the effects of high temperature.

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Table 1. Photosynthetic constants affected by leaf temperature in cotton leaves. CER (carbon exchange rate, or net photosynthetic rate), initial rubisco activity, rubisco activation state (initial/total activity × 100%), maximum fluorescence of a dark-adapted leaf (F_m), and the ratio of variable fluorescence (F_v) to F_m. Measurements of each parameter were taken on the same leaf after a 1-h adaptation at the indicated temperature. --, not determined.

Leaf temp.	CER	Initial rubisco activity	Rubisco activation state	F_m	F_v/F_m
°C	*μmol CO_2·m^{-2}·s^{-1}*		*% maximum*		
30	17.1±0.6	56	100	0.750	0.790
35	14.8±0.5	56	90	0.720	0.758
37.5	14.4±0.4	28±2	59±3	0.679	0.710
40	12.6±0.3	27±1	65±9	0.649	0.688
42.5	9.6±0.5	19±1	66±6	0.538	0.652
45	6.6±0.9	15	25	0.591	0.633
40	9.8±0.4	41	85	0.506	0.614
35	--	22	73	0.559	0.711
30	14.7±0.4	89	100	--	--

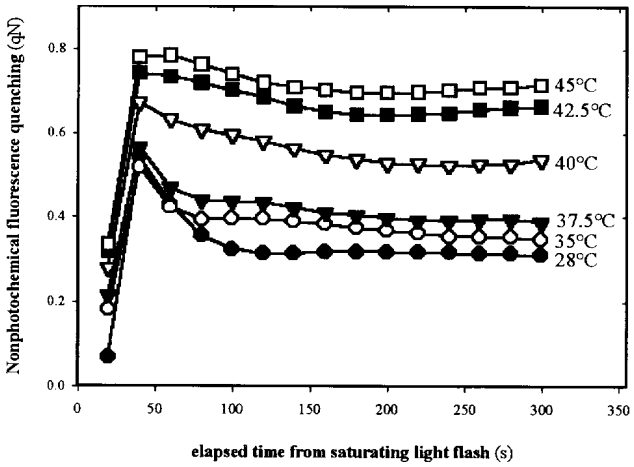

Figure 1. Effect of leaf temperature on the time course of nonphotochemical fluorescence decay (qN) in cotton leaves. Cotton plants were heated to the indicated leaf temperature for 1 h under ambient light (350 $\mu E \cdot m^{-2} \cdot s^{-1}$) and leaves dark-adapted for 15 min prior to qN determination using continuous actinic light (125 $\mu E \cdot m^{-2} \cdot s^{-1}$ emitted at 665 nm) and saturating (>4000 $\mu E \cdot m^{-2} \cdot s^{-1}$) pulses of 0.8 s every 20 s.

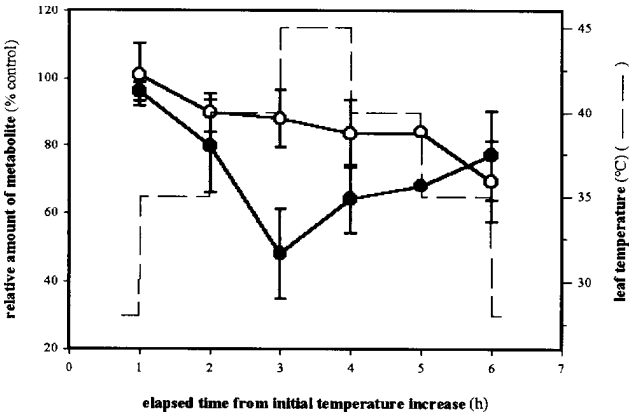

Figure 2. RuBP and 3-PGA content of cotton leaves relative to leaf temperature. Leaf temperature was sequentially increased to a maximum of 45°C, then decreased back to control (28°C) levels as indicated. Metabolites were sampled from freeze-clamped leaf discs removed from pre-illuminated young leaves. Error bars represent means ± S.E.M. of 3 determinations from 3 separate leaves. •, 3-PGA; o, RuBP.