

The temperature response of CO₂ assimilation, photochemical activities and Rubisco activation in *Camelina sativa*, a potential bioenergy crop with limited capacity for acclimation to heat stress

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Abstract The temperature optimum of photosynthesis coincides with the average daytime temperature in a species' native environment. Moderate heat stress occurs when temperatures exceed the optimum, inhibiting photosynthesis and decreasing productivity. In the present study, the temperature response of photosynthesis and the potential for heat acclimation was evaluated for *Camelina sativa*, a bioenergy crop. The temperature optimum of net CO₂ assimilation rate (A) under atmospheric conditions was 30–32 °C and was only slightly higher under non-photorespiratory conditions. The activation state of Rubisco was closely correlated with A at supra-optimal temperatures, exhibiting a parallel decrease with increasing leaf temperature. At both control and elevated temperatures, the modeled response of A to intercellular CO₂ concentration was consistent with Rubisco limiting A at ambient CO₂. Rubisco activation and photochemical activities were affected by moderate heat stress at lower temperatures in camelina than in the warm-adapted species cotton and tobacco. Growth under conditions that imposed a daily interval of moderate heat stress caused a 63 % reduction in camelina seed yield. Levels of cpn60 protein were elevated under the higher growth temperature, but acclimation of photosynthesis was minimal. Inactivation of Rubisco in camelina at temperatures above 35 °C was consistent with the temperature response of Rubisco activase activity and indicated that Rubisco activase was a prime target of inhibition by moderate heat stress in camelina. That photosynthesis exhibited no acclimation to moderate heat stress will likely impact the development of camelina and

other cool season Brassicaceae as sources of bioenergy in a warmer world.

Keywords Biofuel crop · Climate change · Photosynthetic efficiency · Rubisco activase · Temperature optimum

Abbreviations

A	Rate of net CO ₂ assimilation
A_c	Rubisco-limited rate of CO ₂ assimilation
A_j	Electron-transport-limited rate of CO ₂ assimilation
C_i	Intercellular CO ₂ concentration
C_{trans}	Intercellular CO ₂ concentration at which A_c and A_j are co-limiting
F_o	Minimal chlorophyll fluorescence of a dark-adapted leaf
F_v/F_m	Maximum quantum efficiency of photosystem II
ϕ_{PSII}	Operating efficiency of photosystem II
k_{cat}	Turnover number
NPQ	Non-photochemical quenching
J	Maximum rate of electron transport
PPFD	Photosynthetic photon flux density
PSII	Photosystem II
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
V_{cmax}	Maximum velocity of RuBP carboxylation

Introduction

Plant species are adapted to a narrow range of temperatures that restrict their geographic distribution (Berry and Björkman 1980; Atkin et al. 2006; Sage et al. 2008; Keller and Seehausen 2012). Over the last few decades, global air

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temperatures have been increasing steadily, driving a noticeable reduction in plant yield in many locations worldwide (Lobell and Asner 2003; Hatfield et al. 2011). Climate change scenarios predict that global temperatures will increase by 2–5 °C by the end of the century (IPCC 2007), if not sooner (Rowlands et al. 2012), and will be accompanied by a greater frequency of periods of extreme high temperature. These analyses also predict marked changes in water availability in some areas (IPCC 2007) that could exacerbate the general warming trend by reducing the hydration status of the plants and thereby limiting the capacity for leaf cooling via transpiration (Radin et al. 1994; Haldimann et al. 2008; Carmo-Silva et al. 2012).

Unlike respiration (Atkin et al. 2006; Way and Sage 2008; Hüve et al. 2011), photosynthesis is inhibited by temperatures that are only a few degrees above the average ambient temperature (Schlenker and Robert 2009). The temperature optimum of the CO₂ assimilation rate (*A*) differs among species, with the optimum generally matching the daytime temperatures that are prevalent during the growing season in the respective native environments (Berry and Björkman 1980; Bunce 2000; Atkin et al. 2006; Ishikawa et al. 2007; Nagai and Makino 2009; Scafaro et al. 2010). Inhibition of *A* under heat stress has long been linked to the thermal instability of the oxygen-evolving complex of photosystem II (Havaux et al. 1991; Allakhverdiev et al. 2008). However, this idea has been challenged by evidence showing an acute sensitivity of Rubisco activation to inhibition by moderate heat stress (Weis 1981; Kobza and Edwards 1987; Feller et al. 1998; Crafts-Brandner and Salvucci 2000; Haldimann and Feller 2004; Yamori et al. 2006; Scafaro et al. 2012).

The heat-sensitive target in the activation process is Rubisco activase, a molecular motor protein required to reactivate Rubisco sites that become inoperative from dead-end product formation or improper substrate binding (Spreitzer and Salvucci 2002; Portis 2003). Inactivation of Rubisco by moderate heat stress is a phenomenon that has been observed in numerous studies, although there is some disagreement as to whether it is the sole factor inhibiting *A* under moderate heat stress (Crafts-Brandner and Salvucci 2000; Salvucci and Crafts-Brandner 2004a) or one of a number of factors, depending upon the species and conditions (Wise et al. 2004; Cen and Sage 2005; Sage and Kubien 2007; Sage et al. 2008). Also, inhibition of Rubisco activase by moderate heat stress has been attributed to either the inherent instability of Rubisco activase (Salvucci and Crafts-Brandner 2004b; Barta et al. 2010; Carmo-Silva and Salvucci 2011), or considered a secondary consequence of other heat-induced changes in the chloroplast that subsequently impact Rubisco activase's stability in vivo (Sage et al. 2008). Once damaged, Rubisco activase appears to associate with cpn60 (Salvucci 2008), a chaperonin

involved in the folding of nascent polypeptide chains (Hartl and Hayer-Hartl 2009). Association with cpn60 may facilitate refolding of thermally denatured or newly synthesized Rubisco activase, or simply prevent further denaturation.

The sensitivity of *A* to inhibition by moderate heat stress has important consequences for food security and the production of fuel from biomass (Parry et al. 2011). Most scenarios for agricultural production highlight the need for increased yield per unit land area (Godfray et al. 2010). These scenarios often recognize that increased productivity for food, fuel and fiber must occur in the face of a changing global climate that will increase the average temperature in many of the historic growing regions and subject these regions to more frequent episodes of extreme heat stress. Since many of the proposed non-food oilseed plants are cool season species in the Brassicaceae, the impact of a warmer world, punctuated with more frequent episodes of moderate heat, will influence the development of plants as alternatives to petroleum-based fuels.

In the study described herein, the response of photosynthesis to temperature and its ability to acclimate to moderate heat stress was investigated in *Camelina sativa*, an oilseed species that is being developed as a source of biofuel (Fröhlich and Rice 2005; Carlsson 2009). Surprisingly, little is known about the thermostability of photosynthesis and the potential for acclimation to moderate heat stress in this and related Brassica species, even though these properties affect the range of environments that can be used for production. Since these species are generally regarded as cool season plants, knowledge of the early target(s) of photosynthetic inhibition by moderate heat stress is important for developing strategies to improve the tolerance of these plants to heat stress.

Materials and methods

Materials¹

Biochemical reagents of the highest purity available were purchased from Sigma-Aldrich (St. Louis, MO). Radioactive NaH[¹⁴C]CO₃ was purchased from PerkinElmer (Waltham, MA). Ribulose-1,5-bisphosphate was synthesized by isomerization and phosphorylation of ribose-5-phosphate (Jordan and Ogren 1984). Antibodies directed against Rubisco activase and hsp60 have been described previously (Salvucci 2008).

¹ Mention of a trademark, proprietary product or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Plant material and growth conditions

Camelina [*Camelina sativa* (L.) Crantz cv Robinson] plants were grown from seed in controlled-environment chambers (PGR15, Conviron, Winnipeg, Canada) with a 12-h photoperiod and a maximum photosynthetic photon flux density (PPFD) of $600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 1). Seeds were germinated in 3-l pots containing soil mixture (Sunshine mix #1, Sun Gro Horticulture, Canada). Plants were kept well watered and supplemented twice a week with a nutrient solution containing 2 g l^{-1} of 20-20-20 Peters professional water-soluble fertilizer (Scotts-Sierra Horticultural Products Co., USA) and 0.5 ml l^{-1} of a micronutrient solution composed of 2 mM MnCl_2 , $10 \text{ mM H}_3\text{BO}_3$, 0.4 mM ZnSO_4 , 0.2 mM CuSO_4 , $0.4 \text{ mM Na}_2\text{MoO}_4$ and 0.1 mM NiCl_2 , used at half-strength. All plants were initially grown under control conditions of temperature, at $25/18^\circ\text{C}$ day/night, and 70 % relative humidity. After 15 days, plants were thinned to two plants per pot. Measurements of gas exchange, chlorophyll fluorescence, Rubisco activation and protein levels were conducted 5–6 weeks after germination.

For experiments involving acclimation to growth under moderate heat stress, plants at the 22-day growth stage were divided between two identical growth chambers. One chamber was programmed with the control conditions described above and the other with a higher daytime maximum temperature: the temperature increased from 25 to 35°C during 3 h at the middle of the day and this interval was preceded and followed by a 1-h ramp period (Fig. 1). Measurements of gas exchange, chlorophyll fluorescence and protein levels were conducted on fully

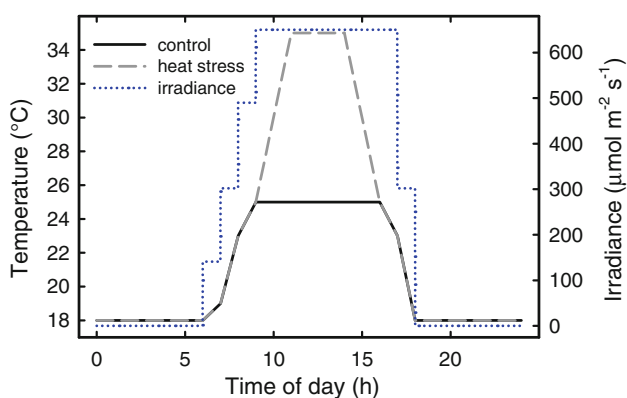


Fig. 1 Temperatures used for growth of *Camelina sativa* under control (dashed line) and heat stress (solid line) conditions. A moderate heat stress of 10°C above the 25°C control was imposed for 3 h each day, at the indicated times, by increasing the chamber temperature to 35°C . The interval of heat stress was preceded and followed by a 1-h ramp period. The same stepped irradiance profile (dotted line) was used for both control and moderate heat stress conditions

expanded leaves 2 weeks after transfer of the plants to the different conditions. At this stage of development, plants were rapidly growing and approaching the flowering stage with no visible signs of leaf senescence. For yield determinations, plants were thinned to one plant per pot and growth was continued through seed set. Seeds were harvested 3 months after germination.

Tobacco (*Nicotiana tabacum* L. cv Petit Havana SR-1) and cotton (*Gossypium hirsutum* L. cv Coker 100A, glandless) plants were grown from seed using the same soil, pots and nutrient solution as described above, except that, for tobacco, the nutrient solution was applied at full strength. Tobacco plants were grown in a controlled-environment chamber at $28/23^\circ\text{C}$ with a 16-h photoperiod and an irradiance of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$. Cotton plants were grown in an air-conditioned greenhouse under natural light. The temperature of the greenhouse was adjusted to maintain daytime temperatures between 25 and 30°C and nighttime temperatures between 25 and 16°C . Measurements of gas exchange, chlorophyll fluorescence and Rubisco activation were conducted 5–6 weeks after germination.

Gas exchange and fluorescence measurements

Gas exchange and chlorophyll fluorescence measurements were conducted using a LiCor (Lincoln, NE, USA) Li-6400 Portable Photosynthesis System equipped with a Li-6400-40 leaf chamber fluorometer. All measurements were conducted at $1,800 \mu\text{mol m}^{-2} \text{s}^{-2}$ PPFD with 10 % blue light on attached leaves of at least four separate plants. Leaf temperatures were increased progressively by adjusting the temperatures of both the growth chamber and the LiCor leaf chamber. To achieve the desired leaf temperatures, the relative humidity of the growth chamber was maintained at $\geq 80\%$ to reduce the vapor pressure deficit. Leaf temperature was measured using an in-chamber type-E thermocouple and was controlled to a precise temperature using the peltier cooling block of the chamber. A reference CO_2 concentration of $380 \mu\text{mol mol}^{-1}$ was used as ambient. For some experiments, gas exchange measurements were conducted at a reduced level of oxygen by supplying the photosynthesis system with gas from a compressed gas cylinder containing 2 % O_2 . Measurements of operating efficiency of photosystem II (ϕ_{PSII}) and maximum quantum efficiency of photosystem II (F_v'/F_m') were conducted with light-adapted plants. Fluorescence parameters requiring dark adaptation (i.e., minimal chlorophyll fluorescence, F_o , maximum quantum yield of photosystem II, F_v/F_m , and non-photochemical quenching, NPQ) were measured with a PAM-2000 fluorometer (Heinz-Walz, Effeltrich, Germany) as described previously (Salvucci and Crafts-Brandner 2004b).

The response of net CO₂ assimilation (*A*) to the intercellular CO₂ concentration (i.e., *A*–*C*_i curve) was measured at 1,800 μmol m^{−2} s^{−2} PPFD by reducing and then increasing the concentration of the reference CO₂, starting from 380 μl l^{−1}, as described previously (Bernacchi et al. 2001; Long and Bernacchi 2003). The data were analyzed using the *A/C*_i Curve Fitting 10.0 utility available at <http://landflux.org/Tools.php>. This utility uses the curve-fitting equations described by Ethier and Livingston (2004), based on the kinetic parameters of Farquhar et al. (1980), Jordan and Ogren (1984) and Bernacchi et al. (2001).

Rubisco activation

The activation state of Rubisco was determined under the same conditions of light and temperature used for the gas exchange measurements. Leaf discs of 0.5 cm² were punched from identical leaves and floated on a solution of 25 mM MES-NaOH, pH 5.5, contained within a water-jacketed beaker. The solution was flushed with 380 μmol mol^{−1} CO₂/21 % O₂ or 380 μmol mol^{−1} CO₂/2 % O₂ prior to the addition of the leaf discs. Leaf discs were illuminated for 1 h with 1,800 μmol m^{−2} s^{−1} PPFD and maintained in the respective atmosphere by continuous sparging during illumination. The solution temperature was maintained by circulating water through the beaker jacket and monitored using a thermocouple placed just below the solution surface. After 1 h in the light at the temperatures indicated in the text, leaf discs were plunged into liquid N₂ and maintained at −80 °C until extraction. Discs were homogenized and the extracts were assayed for Rubisco initial and total activity, i.e., assay immediately upon extraction or after incubation for 3 min in assays without RuBP to fully carbamylate the enzyme (Carmo-Silva et al. 2012). Previous studies have shown that response of Rubisco activation to temperature is similar in leaf discs and in intact tissue, indicating that diffusion of CO₂ into leaf discs floating on liquid was adequate to prevent decarbamylation (Salvucci and Crafts-Brandner 2004a). Also, visual inspection showed that camelina leaves were amphistomatous, i.e., with stomata on both surfaces of the leaf.

Immunoblot analysis

Leaf discs (0.5 cm²) were excised from intact leaves, immediately frozen in liquid N₂ and then stored at −80 °C. Two discs (1 cm²) were extracted at 4 °C in 0.5 ml of 50 mM Tricine-NaOH, pH 8.0, 10 mM EDTA, 1 % PVP-40, 10 mM 2-mercaptoethanol, 1 mM PMSF and 10 μM leupeptin. The extract was centrifuged for 3 min at 12,000g and the supernatant was added to a solution containing 5 % SDS at 95 °C (Salvucci 2008). Polypeptides were separated by SDS-PAGE on 12 % gels, transferred to

polyvinylidene difluoride (PVDF) membranes, probed overnight with Rubisco activase or hsp60 antibodies and then visualized using alkaline phosphatase-conjugated secondary antibody (Salvucci 2008). The commercially available hsp60 antibody recognizes cpn60 in plant extracts (Salvucci 2008). A separate immunoblot was prepared for each of three replicate samples to establish that the patterns shown in the text were representative.

Statistical analyses and reproducibility

All measurements were conducted in at least triplicate for each treatment and the results presented are the mean ± standard error of the mean (SEM) for each treatment. Where indicated in the text, statistical differences between treatments were evaluated using Student's *t* tests. Immunoblot analyses were performed in at least three independent biological samples and produced identical results.

Results

Temperature response of gas exchange, Rubisco activation and chlorophyll fluorescence

The temperature responses of net CO₂ assimilation rate (*A*) and Rubisco activation in camelina were measured at ambient CO₂ (380 μmol mol^{−1}) under photorespiratory (21 % O₂ or 195 mbar O₂) and non-photorespiratory (2 % O₂ or 19.4 mbar O₂) conditions (Fig. 2). Although *A* was higher when photorespiration was eliminated, the temperature optima of *A* were similar under photorespiratory and non-photorespiratory conditions, i.e., about 30 and 32 °C, respectively. The rate of net CO₂ assimilation decreased with increasing leaf temperatures above 30 °C under photorespiratory conditions and above about 35 °C under non-photorespiratory conditions.

Both under photorespiratory and non-photorespiratory conditions, the temperature response of Rubisco activation mirrored the response of *A* at temperatures ≥30 °C. In the lower temperature range (i.e., 14–30 °C), *A* increased with increasing leaf temperature, particularly under non-photorespiratory conditions, whereas Rubisco was nearly fully activated over this entire temperature range under both conditions. In tobacco, the temperature optimum of Rubisco activation under photorespiratory conditions was about 5–10 °C higher than for camelina (Fig. 2 inset), and was similar to the optimum temperature of *A* reported by others for this and other warm-adapted species (Crafts-Brandner and Salvucci 2000; Kubien and Sage 2008; Yamori and von Caemmerer 2009).

Under both photorespiratory and non-photorespiratory conditions, the decrease in *A* observed at supra-optimal

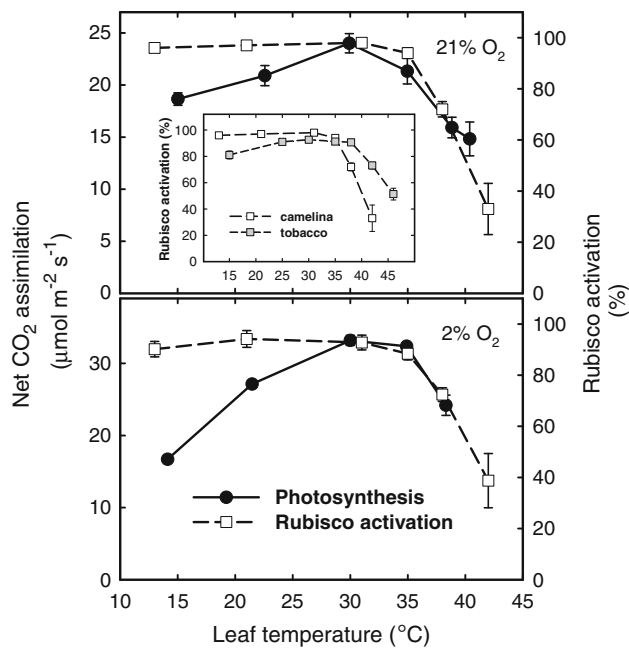


Fig. 2 Temperature response of net CO₂ assimilation rate (filled circle) and Rubisco activation (open squares) in camelina plants under photorespiratory and non-photorespiratory conditions. Gas exchange was measured in attached leaves at the indicated leaf temperatures using a reference CO₂ concentration of 380 μmol mol⁻¹, a PPFD of 1,800 μmol m⁻² s⁻¹ and either 195 mbar O₂ (21 %, top panel) or 19.4 mbar O₂ (2 %, bottom panel). Rubisco activation was determined by measuring Rubisco activities in extracts prepared from leaf discs that were exposed to the same conditions of temperature, light, CO₂ and O₂ used for the gas exchange measurements. *Inset* the temperature response of Rubisco activation in tobacco (filled squares) under photorespiratory conditions. The response for camelina under photorespiratory conditions is duplicated in the inset for reference

temperatures was not accompanied by a decrease in the intercellular CO₂ concentration (C_i), or by reduced stomatal conductance and transpiration rates (Fig. 3). In fact, stomatal conductance and transpiration rates increased with increasing leaf temperature, and C_i was relatively unaffected, causing no diffusional limitations to photosynthesis.

The temperature response of the operating efficiency of PSII (ϕ_{PSII}) resembled the response of A (Fig. 4). The efficiency was greater at ambient than at 2 % O₂ because of the alternative electron sink provided by the photorespiratory pathway. In contrast, the maximum quantum efficiency of PSII in the light (F_v'/F_m') was similar under photorespiratory and non-photorespiratory conditions, except at the lowest temperature, and decreased with increasing leaf temperature.

Response of net CO₂ assimilation to intercellular CO₂ concentration ($A-C_i$) under moderate heat stress

To investigate the factors limiting A in camelina under moderate heat stress, the $A-C_i$ response was measured at

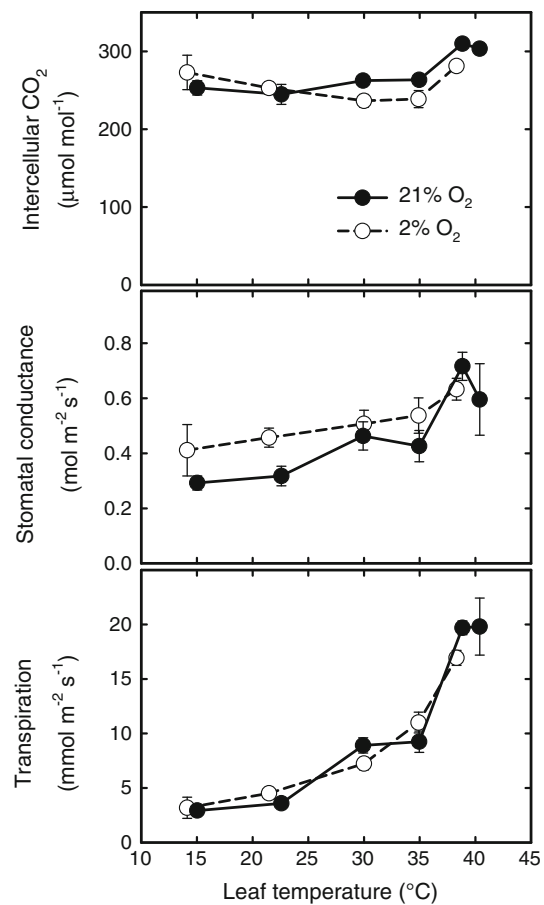


Fig. 3 Temperature response of intercellular CO₂ concentration, stomatal conductance and transpiration in camelina plants under photorespiratory and non-photorespiratory conditions. Gas exchange was measured as described in Fig. 2, at either 21 % (filled circles) or 2 % (open circles) O₂

23 °C, a control temperature, and at 38 °C, a temperature that caused moderate inhibition of A . The experimental data were modeled (Farquhar et al. 1980) to estimate Rubisco- and RuBP-regeneration-limited A (Fig. 5) and compared with the $A-C_i$ response of tobacco (Fig. 6). Since tobacco is adapted to warmer environments, the temperatures used for control and moderate heat stress were 4–5 °C higher than those used for camelina.

Experimental and modeled $A-C_i$ data for camelina and tobacco are summarized in Table 1. At ambient CO₂, A was Rubisco limited under control temperatures in both camelina and tobacco; i.e., the C_i was lower than the transition point between Rubisco- and RuBP-regeneration-limited A (C_{trans}). Interestingly, C_{trans} increased under moderate heat stress, but the C_i at ambient CO₂ concentrations was relatively unaffected because stomatal conductance increased in response to the higher leaf temperatures. Thus, the difference between C_i and C_{trans} increased under moderate heat stress, making the Rubisco limitation even more prominent.

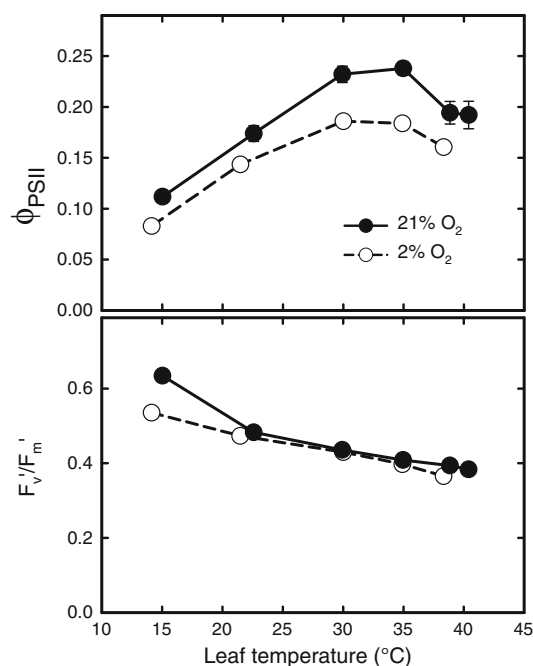


Fig. 4 Temperature response of the operating efficiency of PSII (ϕ_{PSII}) and the maximum quantum efficiency of PSII in the light (F_v/F_m) in camelina plants under photorespiratory and non-photorespiratory conditions. Fluorescence parameters were determined in attached leaves during the gas exchange measurements described in Fig. 2 in an atmosphere containing either 21 % (filled circle) or 2 % (open circle) O_2

The C_i at ambient CO_2 was similar at control and elevated temperatures, but A was inhibited at the higher temperature in both camelina and tobacco. Even though A decreased, as temperature increased above the optimum, a higher rate of RuBP regeneration was required to sustain the measured A , mainly because of an increased rate of RuBP consumption in the oxygenase reaction (Jordan and Ogren 1984). At saturating CO_2 , A was greater at elevated than at control temperatures. When CO_2 is saturating, RuBP consumption by the oxygenase reaction is minimized, but the rate of RuBP carboxylation is faster, driven by the greater availability of CO_2 and a faster turnover number (k_{cat}). Both the maximum velocity of carboxylation (V_{cmax}) and the maximum rate of electron transport (J) increased at high temperatures, but V_{cmax} exhibited a greater increase at the higher temperature, reducing the J/V_{cmax} ratio in camelina from 2.2 at 23 °C to 1.4 at 38 °C.

Temperature dependence of the photochemical activities of camelina and two warm-environment plants, tobacco and cotton

The higher temperature optimum of A in tobacco (see Kubien and Sage 2008; Yamori and von Caemmerer 2009) compared with camelina was paralleled by a higher

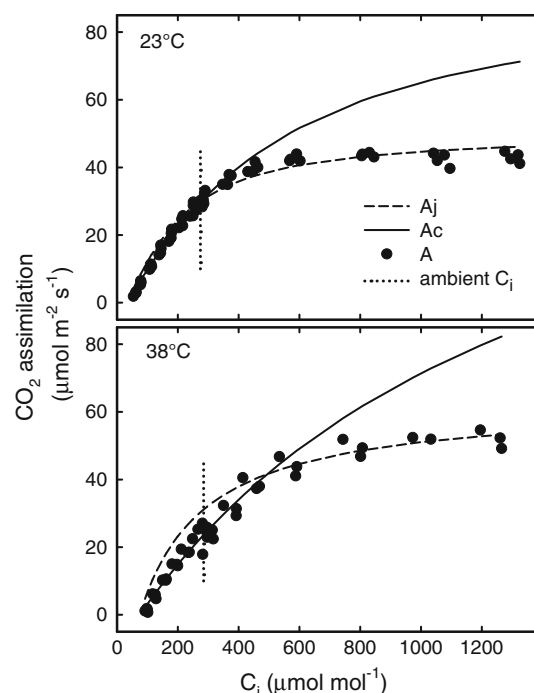


Fig. 5 Effect of temperature on the response of net CO_2 assimilation rate (A) to intercellular CO_2 concentration (C_i) in camelina plants. Gas exchange was measured at a leaf temperature of 23 °C (top panel) or 38 °C (bottom panel) on attached leaves using reference CO_2 concentrations of 75–1,500 $\mu\text{mol mol}^{-1}$ and a saturating PPFD of 1,800 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Each symbol represents one measurement. Three different plants were analyzed under each condition. The modeled average electron-transport-limited A (A_j , dashed line) and RuBP-consumption-limited A (A_c , solid line) are shown. The C_i at atmospheric CO_2 levels is indicated by the vertical dotted line

temperature optimum of Rubisco activation (Fig. 2 inset). To determine if the photochemical activities in warm-environment plants are also adapted to higher temperatures than in camelina, the temperature responses of several chlorophyll fluorescence parameters of camelina were compared with those of tobacco and cotton. Since F_m , F_v and F_o require dark adaptation, it was necessary to heat the plants in the dark prior to measurement. Consequently, the temperature responses of these parameters are not directly comparable to the data in Figs. 2, 3 and 4, determined under the more physiologically relevant condition of heating in the light (see Havaux et al. 1991). Still, these measurements provide an indication of relative species differences in the thermal stability of photochemical activities.

Figure 7 shows the temperature response of minimal fluorescence (F_o) and the maximum quantum efficiency of photosystem II (F_v/F_m). An increase in F_o and a decrease in F_v/F_m occurred in camelina above about 35 °C. In contrast, in both cotton and tobacco, F_o was relatively unaffected by temperature and F_v/F_m was only affected when leaf temperatures exceeded 40 °C. The temperature

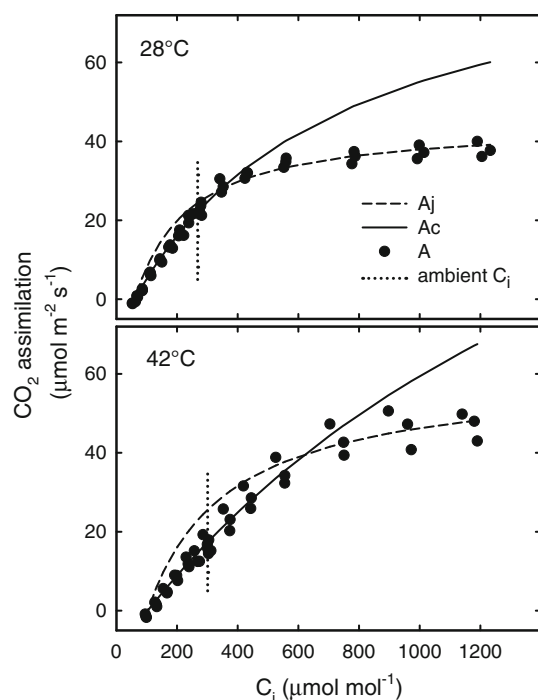


Fig. 6 Effect of temperature on the response of CO_2 assimilation rate (A) to intercellular CO_2 concentration (C_i) in tobacco plants. Gas exchange was measured at a leaf temperature of 28 °C (*top panel*) and 42 °C (*bottom panel*) on attached leaves using reference CO_2 concentrations from 75 to 1,500 $\mu\text{mol mol}^{-1}$ and a saturating PPFD of 1,800 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Each symbol represents one measurement. Three different plants were analyzed under each condition. The modeled average electron-transport-limited A (A_j , *dashed line*) and RuBP-consumption-limited A (A_c , *solid line*) are shown. The C_i at atmospheric CO_2 levels is indicated by the *vertical dotted line*

response of the operating efficiency of photosystem II (ϕ_{PSII}) and non-photochemical quenching (NPQ), measured after illuminating dark-adapted plants, exhibited similar species differences (Fig. 8). In camelina, ϕ_{PSII} decreased and NPQ increased with temperatures exceeding

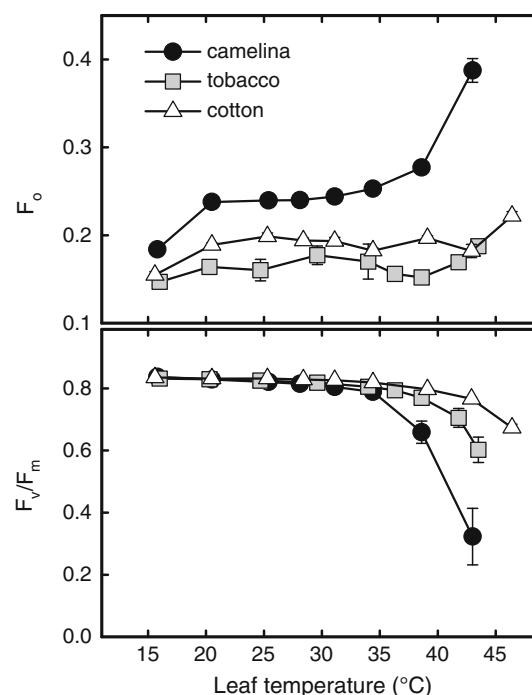


Fig. 7 Temperature response of the minimal chlorophyll fluorescence (F_o) and maximal quantum efficiency of PSII (F_v/F_m) in camelina, tobacco and cotton plants. Camelina (*filled circle*), tobacco (*filled square*) and cotton (*open triangle*) plants were dark adapted at the indicated temperature for at least 1 h prior to measurement

35 °C, whereas higher temperatures were required to affect ϕ_{PSII} and NPQ in cotton and tobacco.

Effect of elevated growth temperatures on acclimation of photosynthesis in camelina

To determine if photosynthesis in camelina could acclimate to moderate heat stress, plants were grown under conditions that imposed a 3-h interval of moderately high temperatures

Table 1 Effect of temperature on the gas exchange properties of camelina and tobacco at ambient and elevated CO_2 concentrations

Plant species	Leaf temp. (°C)	C_i ($\mu\text{mol mol}^{-1}$)	A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Stomatal conductance ($\text{mol m}^{-2} \text{s}^{-1}$)	C_{trans} ($\mu\text{mol mol}^{-1}$)	V_{cmax} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	J (J/V_{cmax}) ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
Camelina	23	274 ± 5	29.0 ± 0.7	0.64 ± 0.06	287 ± 7	93 ± 1	201 ± 2 (2.2)
		822 ± 10	43.7 ± 0.3	0.58 ± 0.04			
		1,240 ± 22	52.0 ± 1.6	0.68 ± 0.07			
Tobacco	28	268 ± 8	21.6 ± 0.2	0.44 ± 0.05	365 ± 11	92 ± 2	175 ± 4 (1.9)
		1,209 ± 12	38.0 ± 5.3	0.29 ± 0.01			
		1,170 ± 15	46.9 ± 2.0	0.46 ± 0.03			
Tobacco	42	301 ± 1	15.7 ± 5.3	0.66 ± 0.02	629 ± 33	194 ± 13	244 ± 13 (1.3)
		1,170 ± 15	46.9 ± 2.0	0.46 ± 0.03			
		1,170 ± 15	46.9 ± 2.0	0.46 ± 0.03			

C_{trans} , V_{cmax} and J were estimated from modeling the A – C_i response curves shown in Figs. 5 and 6. When $C_i < C_{\text{trans}}$, A is A_c limited and, when $C_i > C_{\text{trans}}$, A is A_j limited

C_{trans} , intercellular CO_2 concentration at which Rubisco activity (A_c) and electron transport (A_j) co-limit A , V_{cmax} , maximum RuBP carboxylation rate, J maximum electron transport rate

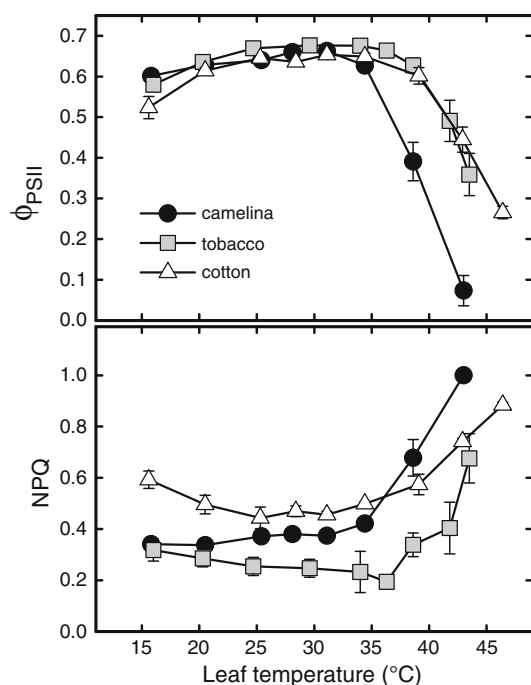


Fig. 8 Temperature response of the operating efficiency of PSII (ϕ_{PSII}) and the non-photochemical quenching (NPQ) in camelina, tobacco and cotton plants. Camelina (filled circles), tobacco (filled squares) and cotton (open triangles) plants were dark adapted at the indicated temperature for at least 1 h prior to illumination at a PPFD of $125 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. ϕ_{PSII} and NPQ were determined after 6 min of illumination

at midday, but with the same nighttime temperatures as those used for the control (Fig. 1). The temperature used for these experiments, 35°C , exceeded the temperature optimum of photosynthesis in 25°C -grown plants by about 5°C (Fig. 2). When plants were taken to maturity under this temperature regime, total seed weight, weight per seed and seed number were reduced by 63, 34 and 50 %, respectively, compared to growth at a constant day temperature of 25°C (Table 2).

For plants grown for 2 weeks under the elevated temperature regime, the temperature response of A was not statistically different from the response observed for plants grown at 25°C (Table 3). For example, A was highest at 30°C for plants grown under both regimes and was inhibited at 35 and 40°C . In addition to A , the temperature responses of F_o , F_v/F_m , ϕ_{PSII} and NPQ in plants that were

Table 3 Effect of growth under moderate heat stress on the gas exchange parameters of camelina measured at optimal and supra-optimal leaf temperatures

Growth temp. ($^\circ\text{C}$)	Leaf temp. ($^\circ\text{C}$)	A^a ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	C_i ($\mu\text{mol mol}^{-1}$)	Stomatal conductance ($\text{mol m}^{-2} \text{s}^{-1}$)
25	25	20.4 ± 0.8	261 ± 8	0.43 ± 0.03
	30	22.7 ± 0.6	270 ± 5	0.50 ± 0.04
	35	20.0 ± 0.9	276 ± 5	0.53 ± 0.05
	40	14.8 ± 1.6	303 ± 7	0.60 ± 0.13
35	25	22.6 ± 0.6	241 ± 5	0.49 ± 0.04
	30	23.8 ± 0.6	282 ± 5	0.60 ± 0.04
	35	22.6 ± 0.6	292 ± 5	0.71 ± 0.06
	40	13.5 ± 0.6	318 ± 3	0.80 ± 0.04

Camelina plants ($n = 14\text{--}16$) were measured after continuous growth under control temperatures of 25°C or after a 2-week exposure to conditions that imposed a regular interval of moderate heat stress of 35°C at midday

^a At each measured leaf temperature, differences in A between plants grown at 25 and 35°C were not statistically significant at the $\alpha = 0.05$ level

grown at control and elevated temperatures were nearly identical (Fig. 9). Taken together, the results demonstrate that photosynthesis exhibited no detectable acclimation to heat stress when camelina plants were given a daily exposure to moderate heat stress for 2 weeks.

Effect of elevated growth temperatures on the abundance of Rubisco activase and cpn60 in camelina

The chloroplast homolog of GroEL, cpn60, has been shown to associate with Rubisco activase in heat-stressed *Arabidopsis* leaves (Salvucci 2008). Consequently, the levels of cpn60 were examined three times during the day in leaf extracts of camelina plants grown under control and moderate heat stress conditions (Fig. 10a). The levels of cpn60 were higher in plants grown at elevated temperature than at the control temperature. Interestingly, cpn60 levels were higher at all sampling times, even though the chamber temperature was not elevated at the first and third sampling times, i.e., 9:15 and 16:00 h (see Fig. 1). When plants grown at elevated temperature were moved to control

Table 2 Effect of moderate heat stress on seed yield in camelina

Growth condition	Total yield (g plant^{-1})	Seed weight (mg seed^{-1})	Seed number ($1,000 \text{ seeds plant}^{-1}$)
Control	8.6 ± 1.2	0.71 ± 0.04	12.6 ± 2.3
Heat stress	3.1 ± 0.8^a	0.47 ± 0.02^a	6.4 ± 1.4^a

Seeds were collected from camelina plants ($n = 5$) grown under control temperatures of 25°C or under conditions that imposed a regular interval of moderate heat stress of 35°C at midday

^a All differences between control and heat stress were statistically significant at the $\alpha = 0.05$ level

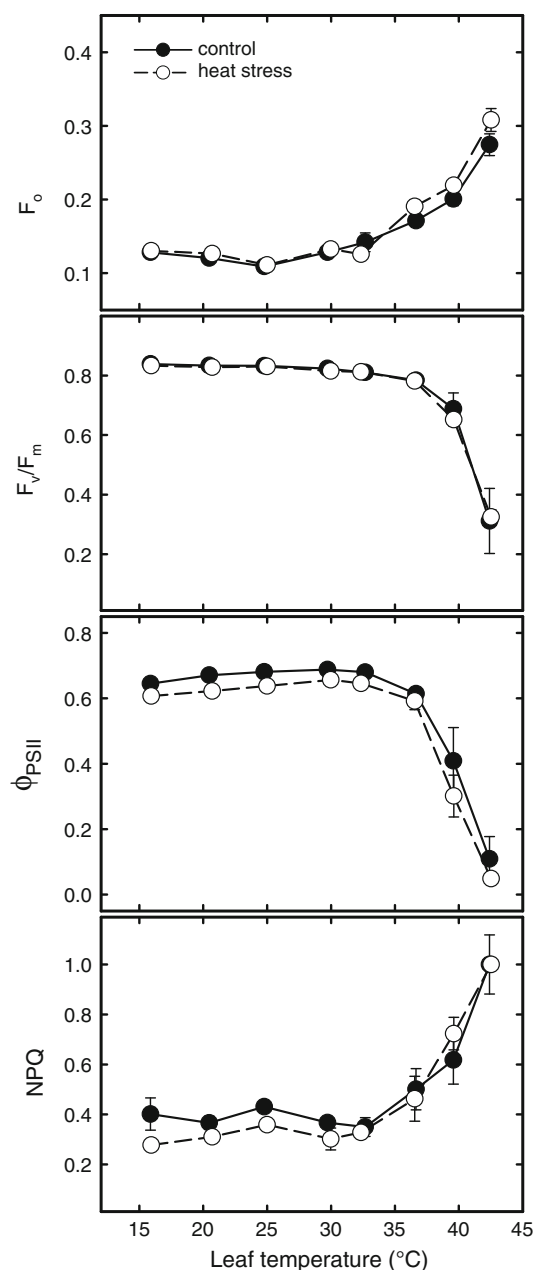


Fig. 9 Temperature response of the minimal chlorophyll fluorescence (F_0), maximum quantum efficiency of PSII (F_v/F_m), operating efficiency of PSII (ϕ_{PSII}) and non-photochemical quenching (NPQ) in camelina plants grown under control and moderate heat stress conditions. Camelina plants were analyzed after continuous growth at control temperatures (filled circles) or after growth for 2 weeks under conditions that imposed a regular interval of moderate heat stress of 35 °C at midday (open circles). Plants were dark adapted at the indicated temperature for at least 1 h prior to illumination at a PPFD of 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$. F_0 and F_v/F_m were determined immediately prior to illumination and ϕ_{PSII} and NPQ were determined after 6 min of illumination

conditions at the beginning of the light period, *cpn60* levels remained high throughout the day even though the heat stress had been removed (Fig. 10b, 35 → 25). When plants

grown under control conditions were moved to elevated temperature at the beginning of the light period, *cpn60* levels increased in leaves harvested at the last sampling time, i.e., after the plants had experienced 3 h of heat stress.

In contrast to *cpn60*, Rubisco activase levels were relatively unaffected by growth at elevated temperature (Fig. 10c). However, plants grown at elevated temperature contained more high molecular weight polypeptides that reacted with Rubisco activase antibodies than plants grown at control temperatures (Fig. 10d). An accumulation of high molecular mass polypeptides that react with Rubisco activase antibodies has been reported previously after short-term exposure of wheat and cotton to heat stress (Feller et al. 1998), and probably represents protein targeted for degradation.

Discussion

Rubisco activation shapes the response of CO_2 assimilation to moderate heat stress

The temperature response of photosynthesis was examined for camelina, a cool season crop with potential for use as a source of biofuel. The temperature optimum of *A* in air was about 30 °C and increased only slightly under non-photorespiratory conditions. Thus, *A* decreased at temperatures that were well below 55–60 °C, the thermal optimum for Rubisco (Crafts-Brandner and Salvucci 2000; Sage 2002; Salvucci and Crafts-Brandner 2004a; Cen and Sage 2005). The decrease in photosynthesis could not be attributed solely to an increase in the oxygenase/carboxylase ratio with increasing temperature, since a similar response occurred under non-photorespiratory conditions (see also Kobza and Edwards 1987). As shown in other studies (Bunce 2000; Haldimann and Feller 2004; Reynolds-Henne et al. 2010; Scafaro et al. 2012), the decrease in *A* with increasing leaf temperature was not accompanied by a decrease in C_i when plants were under high humidity, suggesting the absence of diffusional limitations to *A* under moderate heat stress.

Analysis of *A*– C_i curves indicated that *A* was Rubisco limited at both control and elevated leaf temperatures. The temperature response of Rubisco V_{cmax} , reported by Jordan and Ogren (1984) for the isolated enzyme and estimated by Bernacchi et al. (2001) from *A*– C_i response curves, suggests that an increase in temperature from 23 to 38 °C in camelina should increase the V_{cmax} from 93 to about 271 or 335 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. Both predictions assume that Rubisco remains fully activated at the higher temperature. However, Rubisco activation state decreased in camelina at 38 °C and the magnitude of the decrease, i.e.,

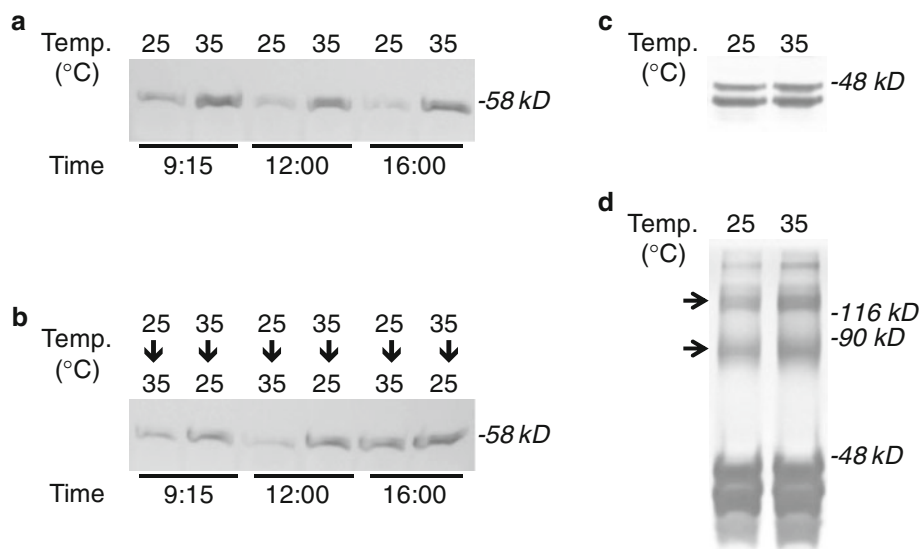


Fig. 10 Abundance of cpn60 (**a** and **b**) and Rubisco activase (**c** and **d**) polypeptides in camelina plants grown under control and moderate heat stress conditions. Camelina plants were grown under control temperatures (daytime temperature = 25 °C) or exposed for 2 weeks to conditions that imposed a regular interval of moderate heat stress of 35 °C at midday before analyses. Polypeptides in leaf extracts were separated by SDS-PAGE before immunoblotting with hsp60 or Rubisco activase antibodies. The positions of apparent molecular weight markers are indicated to the right of each panel. (**a** cpn60) Camelina extracts were prepared from plants grown under control (25 °C) and heat stress (35 °C) conditions that were harvested at the

times indicated below the blot. (**b** cpn60) Camelina extracts were prepared from plants that were transferred from control to heat stress (25 → 35 °C) or from heat stress to control (35 → 25 °C) growth conditions at the beginning of the photoperiod and harvested at the times indicated below the blot. (**c** Rubisco activase) Camelina extracts were prepared from plants grown under control (25 °C) and heat stress (35 °C) conditions that were harvested at 12:00 h. (**d** Rubisco activase) Camelina extracts were the same as in (**c**). Blots were overloaded with protein to reveal high molecular mass species (arrows)

from 97 to 72 %, could largely account for the lower V_{cmax} determined from the $A-C_i$ response at 38 °C (Table 1). Thus, the responses of Rubisco activation state and A to supra-optimal temperatures in camelina were closely correlated both qualitatively and quantitatively. A similar relationship between inhibition of A and deactivation of Rubisco has been reported for several other species (Weis 1981; Kobza and Edwards 1987; Feller et al. 1998; Crafts-Brandner and Salvucci 2000; Haldimann and Feller 2004; Yamori et al. 2006; Carmo-Silva et al. 2012; Scafaro et al. 2012).

Species differences in the thermal stability of Rubisco activase explain the respective temperature responses of Rubisco activation and photosynthesis

In their seminal review, Berry and Björkman (1980) showed that the temperature optimum of A differed among higher plants species, varying from about 12 °C to as high as 40 °C, mostly in accord with the temperatures that are prevalent in the species' native environments. In the present study, the temperature response of Rubisco activation in camelina and tobacco differed by about 4–5 °C, resembling the respective responses of A to elevated temperature (this work; Kubien and Sage 2008; Yamori and

von Caemmerer 2009). The differences in the response of A and Rubisco activation to high temperature were consistent with different temperature responses of Rubisco activase activity reported for the native enzymes in leaf extracts (Carmo-Silva and Salvucci 2011). Since Rubisco activase activity was measured under optimal conditions in Carmo-Silva and Salvucci (2011), inactivation of Rubisco activase appears to be a direct effect of temperature on the thermal stability of the protein (see also Barta et al. 2010) and not a secondary consequence of heat-induced changes in the chloroplast environment that subsequently impact Rubisco activase. However, we cannot rule out the possibility that heat-induced changes in redox state (Schrader et al. 2007; Zhang et al. 2009; Sharkey and Zhang 2010) or the concentrations of chloroplast constituents necessary for Rubisco activase function (Sage et al. 2008) might moderate or exacerbate the effects of high temperature on Rubisco activase and either lessen or worsen the impact.

Species differences in the thermal stability of electron transport activities—the next in a cascade of heat-related metabolic failures?

Like A and Rubisco activation, reactions associated with the thylakoid membranes were also more susceptible to

heat stress in camelina compared with warm-environment plants. Specifically, thylakoid membrane integrity and PSII stability, indicated by F_o and F_v/F_m , respectively, were less stable at high temperature in camelina than in tobacco and cotton. Judging from the clear Rubisco limitation at atmospheric CO_2 levels and the higher A at saturating CO_2 , it is unlikely that reduced reaction center and electron transport activities contributed to the inhibition of A observed for camelina at 38 °C. However, the data indicate that photochemical reactions would become a significant factor limiting A under more severe heat stress.

The impact of reduced carbon assimilation under moderate heat stress on camelina seed yield

Camelina exhibited a large decrease in seed yield when grown under a relatively brief (3 h daily) and moderate (35 °C) heat stress during the day, but with normal night temperatures. Similar and even more profound effects of moderate heat stress on seed yield have been reported for *Arabidopsis* (Kurek et al. 2007; Kumar et al. 2009; Vile et al. 2012). These effects are likely to be associated with a reduced assimilate supply from the inhibition of carbon fixation under moderate heat stress, since improvements in the thermostability of photosynthesis improved yield under moderate heat stress (Kurek et al. 2007; Kumar et al. 2009).

Acclimation of camelina to moderate heat stress—induction of cpn60 but minimal adjustments in carbon assimilation or photochemical activities

The apparent inability of camelina to acclimate to growth under conditions that imposed regular intervals of moderate heat stress was somewhat surprising. The temperature sensitivity of A and the photochemical activities associated with PSII and membrane stability were unchanged in camelina, in contrast to findings with other species that showed enhanced tolerance to heat stress after growth at a higher temperature (Berry and Björkman 1980; Atkin et al. 2006; Yamori et al. 2006). However, Sage et al. (2008) noted that plants from cooler environments had a reduced capacity for acclimation of photosynthesis to heat stress compared with plants from warmer environments (see also Atkin et al. 2006). It appears that seasonal plants have limited biochemical plasticity and cannot acclimate Rubisco activase and/or other components of the photosynthetic apparatus to heat stress.

The levels of cpn60, a GroEL homolog that associates with Rubisco activase in heat-stressed leaves (Salvucci

2008), were elevated after growth under conditions that imposed regular intervals of moderately high temperatures. Higher levels of cpn60 after 2 weeks of growth under these conditions provide a clear indication for a lack of acclimation to heat stress in camelina, since a heat shock response was still being elicited. That the levels of cpn60 were elevated before, during and after the actual heating interval suggested that the response to heat stress in camelina involved continual accumulation of this protein. This result is consistent with the results of proteomic studies that have identified cpn60 among the proteins that increase in abundance in heat-stressed rice plants (Scafaro et al. 2010). Given the vital role of GroEL-type chaperones in protein folding, expression of higher levels of this protein in anticipation of, or upon exposure to, heat stress might protect proteins like Rubisco activase from heat denaturation or facilitate an increased rate of de novo synthesis. The elevated levels of cpn60 did not improve the thermostability of photosynthetic activities, but their presence might have lessened the impact of prolonged and regular exposure to supra-optimal temperatures.

Prospects for improving the thermal stability of photosynthesis in camelina

Camelina sativa is typically grown in cool environments where daytime temperatures rarely exceed 30 °C (Dixon 2007). In Chile, the yields of this crop were highest at the earliest planting dates, i.e., when temperatures were coolest (Berti et al. 2011). In camelina, A was linked to the low temperature optimum of Rubisco activation and exhibited little capacity for acclimation. Consequently, improvements in the thermotolerance of photosynthesis would require increasing the temperature optimum of Rubisco activase by insertion of a more thermostable Rubisco activase transgene. Proof of concept for this approach has already been established for *Arabidopsis*, whose temperature optimum for A (Kumar et al. 2009) and Rubisco activase activity (Carmo-Silva and Salvucci 2011) are similar to camelina. The success achieved with *Arabidopsis* (Kurek et al. 2007; Kumar et al. 2009) indicates that improvement of the thermal stability of camelina Rubisco activase by a few degrees would be sufficient in itself to improve photosynthetic performance and yield under moderate heat stress, the most prevalent type of heat stress.

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