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The roles of photorespiration and alternative electron acceptors in the responses of photosynthesis to elevated temperatures in cowpea

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Abstract

We explored the effects, on photosynthesis in cowpea (Vigna unguiculata), of high temperature and light — environmental stresses that often co-occur under field conditions. We observed contrasting responses in the light and carbon assimilatory reactions, whereby in high temperature, the light reactions were stimulated while CO2 assimilation was substantially reduced. There were two striking observations. First, the primary quinone acceptor (QA), a measure of the regulatory balance of the light reactions, became more oxidized with increasing temperature, suggesting increased electron sink capacity, despite the reduced CO2 fixation. Second, a strong, O2-dependent inactivation of assimilation capacity, consistent with down-regulation of rubisco under these conditions, a phenomenon that has not been previously reported. The dependence of these effects on CO2, O2 and light led us to conclude that both photorespiration and an alternative electron acceptor, supported increased electron flow, and thus provided photoprotection, under these conditions. Further experiments showed that the increased electron flow was maintained by rapid rates of PSII repair, particularly at combined high light and temperature. Overall, the results suggest that photodamage to the light reactions can be avoided under high light and temperatures by increasing electron sink strength, even when assimilation is strongly suppressed.

Keywords

Photorespiration, high temperature, high-light, alternative electron acceptors, cowpea, , heat stress, net CO_2 assimilation, PSII efficiency

INTRODUCTION

Heat stress is a major environmental challenge for plants, and it is well established that, in a wide range of species, high temperature (HT) stress can lead to declines in photosynthesis (Cui, Li, Fan, Xu, & Zhang, 2006; Panigrahi, Pradhan, Panda, Panda, & Joshi, 2016; Tan, Meng, Brestic, Olsovska, & Yang, 2011; Wise, Olson, Schrader, & Sharkey, 2004). The impact of HT stress on photosynthesis is dependent on factors such as leaf developmental age, duration and previous history of exposure (Hasanuzzaman, Nahar, Alam, Roychowdhury, & Fujita, 2013), as well as other environmental factors, especially high-light (HL) stress, which often co-occur with and can be compounded by HT (Weis, 1982).

While light energy is essential for photosynthesis, excessive light can cause detrimental effects on photosynthesis, a phenomenon known as photoinhibition or photodamage (Aro, Virgin, & Andersson, 1993; Long, Humphries, & Falkowski, 1994; Nishiyama & Murata, 2014). HL stress causes reductions in photosynthesis, especially under rapidly fluctuating environmental conditions, which ultimately results in loss of crop productivity (Slattery, Walker, Weber, & Ort, 2018). Plants have evolved various mechanisms to deal with excessive light and prevent damage, including changes in leaf orientation (Gamon & Pearcy, 1989; Pastenes,

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Pimentel, & Lillo, 2005), chloroplast movement (Suetsugu, Higa, Gotoh, & Wada, 2016; Wada, 2013) and non-photochemical quenching (NPQ) processes that dissipate excitation energy (Ruban, 2016; Stefanov & Terashima, 2008). There are several forms of NPQ, including the rapidly reversible "energy dependent" quenching (q_{E}), which is activated by acidification of the thylakoid lumen (Avenson, Cruz, & Kramer, 2004; Li et al., 2004), and more slowly-reversible forms including q_{I}, which is caused by accumulation of inactive (photodamaged) photosystem II (PSII) centers (Krause, Somersalo, Zumbusch, Weyers, & Laasch, 1990) and state transitions (q_{T}) that can divert PSII-associated light harvesting complexes (LHCs) to PSI. Depending on its redox state, PSI can either use light energy from these LHCs for PSI photochemistry, or dissipate it as heat (Butler, 1978; Joly & Carpentier, 2007).

The importance of light for thermotolerance of photosynthesis has been noted in previous work (Buchner, Stoll, Karadar, Kranner, & Neuner, 2015; Havaux, Greppin, & Strasser, 1991; Weis, 1982). Although, there have been several studies that show that HL combined with HT accentuates photoinhibition (e.g. Chen et al., 2017; Havaux, 1992; Lu et al., 2017), there are also reports, for instance in tomatoes, where combined moderate HT and HL improved tolerance of photosynthesis compared to either treatment alone (Gerganova, Popova, Stanoeva, & Velitchkova, 2016). However, since different species have different light intensity requirements (e.g shade vs. sun adapted plants, (Hemming, 2011; C. H. Lin, McGraw, George, & Garret, 1999)), it is very likely that responses to combinations of HT and HL stress are dependent on genotype, species or acclimation during development. Nevertheless, several components of the responses may be conserved among species.

During photosynthesis, light energy captured by the light harvesting complex is used to drive electrons by linear electron flow (LEF) to store reducing power in NADPH/O_2 and phosphorylation potential in ATP/ADP+Pi, which together drive the carbon assimilation reactions in the Calvin-Benson-Bassham (CBB) cycle and other cellular processes. In principle, both the light reactions and CBB cycle may be affected by HT. For example, it is well-established that HT decreases the specificity of rubisco and solubility of CO_2relative to O_2 (Brooks & Farquhar, 1985; Galmés, Hermida-Carrera, Laanisto, & Niinemets, 2016), thus increasing "photorespiratory pressure" or rates of photorespiration faster than the rate of rubisco carboxylation (Schuster & Monson, 1990).

In addition to this shift from assimilation to photorespiration, moderately high temperatures (35–40 °C) decrease rubisco activity, which can be attributed to the loss of rubisco activase activity, leading to progressive deactivation of rubisco by accumulation of inhibitory products such as sugar phosphates (Crafts-Brandner & Salvucci, 2000; Salvucci & Crafts-Brandner, 2004). While these studies suggest that reduction in rubisco activity is a primary deleterious effect of HT stress, it has also been proposed (Sharkey, 2005) that this effect constitutes an adaptive mechanism possibly to avoid situations where the damage by toxic products of photorespiration outweighs the advantage of higher carbon fixation rates.

A rubisco activase feedback mechanism of this type could allow for "safe" downregulation of assimilation. However, this mode of regulation should decrease the availability of sinks for the products of the light reactions. Without coordinated downregulation of the light reactions, this mode of regulation could result in the accumulation of reactive intermediates and subsequent photodamage. Indeed, it has been proposed that high rates of photorespiration under environmental stresses can serve a photoprotective role by maintaining electron acceptor sinks (Huang, Hu, & Zhang, 2015; Voss, Sunil, Scheibe, & Raghavendra, 2013). However, if rubisco is completely deactivated, rates of photorespiration would also be decreased. Thus, many times HT results in decreases in assimilation that are not fully accounted for by increased photorespiration (Sharkey, 2005), suggesting that feedback systems must operate to coordinate the capture of light in response to these changes in assimilation, as suggested by observations that HT induced decreases in assimilation in wheat were not accompanied by increased PSII photodamage (Kalituho, Pshybytko, Kabashnikova, & Jahns, 2003).

The light and assimilatory reactions are tightly co-regulated to balance the needs for efficient energy capture, while balancing energy storage into ATP and NADPH to meet the needs of downstream metabolic reactions and prevent the accumulation of reactive intermediates that can lead to photodamage (Kramer & Evans, 2011; (Walker, Kramer, Fisher, & Fu, 2020). The thylakoid proton motive force (*pmf*) plays a central role

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in this co-regulatory network (Avenson et al., 2005; Kanazawa & Kramer, 2002). The ΔpH component of *pmf* causes acidification of the lumen, which activates q_E (Li, Muller-Moule, Gilmore, & Niyogi, 2002; Ruban, Johnson, & Duffy, 2012) and controls electron flow at the level of the cytochrome b_6f complex, in a process known as "photosynthetic control" (Tikhonov, 2014). The *pmf* and its effects on downregulation are sensitive to processes that alter the influx of protons such as LEF, cyclic electron flow (CEF) and the conductivity of the thylakoid membrane to proton efflux (g_{H^+}). The latter is governed mainly by the chloroplast ATP synthase, and the partitioning of *pmf* into its two components, Δψ and ΔpH. For example, decreasing CO_2 level slows assimilation and thus decreases LEF and the flux of protons into the lumen. However, ATP synthase also slows, so despite the fact that proton influx is slowed, the thylakoid builds up a larger *pmf* , leading to activation of q_E and increased photosynthetic control (Kanazawa & Kramer, 2002). Moderate HT has been shown to modulate this co-regulatory network in tobacco by altering g_{H^+}, the rates of violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZE) and the partitioning of *pmf* into Δψ and ΔpH (Zhang, Kramer, Cruz, Struck, & Sharkey, 2011), but it is unclear how HL interacts with these regulatory responses.

In this study, we focused on the effect of light on responses of photosynthesis to HT in cowpea (*Vigna unguiculata*) seedlings having leaves of different maturity, with the aim of determining the relative effects on assimilation and light reactions and their co-regulation. We focused on cowpea because it is an agriculturally important crop that serves as an essential source of livelihood and nutrition for many in developing countries, many of which have hot climates. Characterized by quick growth and broad leaves, it is ideal for studying the interaction between HL and HT since it exhibits considerable genetic variation in tolerance to these traits (Ehlers & Hall, 1996, 1998).

Our data shows that in low light (LL), HT caused substantial decreases in CO_2 assimilation and PSII activity, while HT in combination with HL led to a stimulation of PSII activity, but decreases in CO_2 assimilation compared to growth temperature (GT). Further experiments revealed differences in the rates of PSII photodamage between GT+HL and HT+HL, whereas PSII activity was not enhanced in HT+HL under non-photorespiratory conditions, suggesting photorespiration and other alternative electron acceptors to be essential for tolerance of PSII to HT+HL stress.

MATERIALS AND METHODS

Plant materials and growth conditions

Two cowpea genotypes, Yacine and 58-77, generously provided by Drs Philip Roberts and Bao-Huhyn Lam, from University of California, Riverside were used for all experiments. These lines exhibited apparent tolerance and sensitivity respectively to HL stress. One seed was sown in 1 L pot filled with Suremix potting medium (Michigan Grower Products Inc, USA) and watered with half-strength Hoagland's nutrient solution. Plants were staged in a reach-in growth chamber with the following conditions: RH, 50%; temperature, 30/20 °C day/night; light intensity, up to 400 μmol m^{-2} s^{-1} and photoperiod of 10/14 h light/dark. Seedlings were moved to a Dynamic Environment Photosynthesis Imager (DEPI) chamber (Cruz et al., 2016) for treatments 3–4 d or 12–14 d after germination for the young leaves or mature leaves respectively for treatments and assessment of photosynthetic parameters. Where necessary, older plants were used but leaves of similar maturity were used for all measurements. The relative humidity (RH) varied from 20–40% for the HT treatments whereas it was kept at 50% for the Control treatment.

High temperature and high-light treatments

Various HT and HL treatments are described in detail within the corresponding figure descriptions. Briefly, HT treatments were imposed by heating the air temperature in the chamber using the native environmental control embedded in the growth chamber to 45 °C. For the temperature response curves, the temperature was changed by 5 °C every 2 h (starting from GT of 30 °C) but measurements were made after exposure for at least 1 h. Illumination in the DEPI chamber was provided by LED lights with wavelength in photosynthetically active radiation (PAR) range. The light intensity treatments consisted of low light (LL — 300 μmol $m^{-2}s^{-1}$) and high-light (HL — either 1000 or 1500 μmol m^{-2} s^{-1}).

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Chlorophyll fluorescence and absorbance change measurements

the maximal PSII efficiency (F_V/F_M), plants were dark adapted for 20 min before measurements were made. Most chlorophyll fluorescence measurements were made using either the MultispeQ V1 linked to the PhotosynQ platform [*www.photosynq.org* , (Kuhlgert et al., 2016)] or the image-based DEPI chamber (Cruz et al., 2016). Some fluorescence measurements were also performed simultaneously with gas exchange measurements using the LI-COR 6800 (Lincoln, Nebraska, USA).

The electrochromic shift was assessed simultaneously with chlorophyll fluorescence measurements using the MultispeQ V1, thus enabling us to connect PSII function with downstream reactions such as the ATP synthase activity. The thylakoid conductivity to protons (g_{H^+}) was determined by the dark interval relaxation kinetics (DIRK) of the electrochromic shift (ECS) at 520 nm (Thomas J Avenson, Cruz, Kanazawa, & Kramer, 2005; Takizawa, Cruz, Kanazawa, & Kramer, 2007) and was utilized as a measure for the chloroplast ATP synthase activity. The proton motive force (*pmf*) was assessed as the amplitude of the first-order decay kinetics of the ECS trace in the first 300 ms [ECSt, (Kanazawa & Kramer, 2002; Kanazawa et al., 2017)].

Photoprotection (the rapidly reversible form of NPQ,q_E) and photo-inhibitory quenching (the slowly relaxing form of NPQ, q_I) were assessed as described in (Tietz, Hall, Cruz, & Kramer, 2017), using the MultispeQ V1 or DEPI platform. Briefly, following initial pulses to determine F_O' and F_M', leaves were dark adapted for 2 min after which q_E would have relaxed, leavingq_I, enabling us to determine F_M''.

Gas exchange measurements

All measurements were assessed with the LI-COR 6800 photosynthesis system with light provided by the light emitting diodes (LEDs) in the fluorescence head. CO_2 concentration in the air (C_a) was set to 400 ppm, whereas the light intensity was set to the ambient light intensity in the chamber, which also corresponded to the light intensity treatment. Air temperature in the leaf chamber was also set to the ambient air temperature in the chamber. RH was set to 35 or 50% for the HT and control treatments respectively. The vapor pressure deficit ranged from 1.5 to 1.9 kPa for the control and 2.5 to 5 kPa for temperatures 35 °C to 45 °C.

TheA/C_i measurements were performed using the auto program settings in the LI-COR 6800 instrument with the following sequence of CO_2 concentration in the chamber: 400, 200, 50, 100, 150, 250, 300, 350, 500, 600, 800 and 1000 ppm. CO_2 levels were changed at 2 min intervals to prevent complete deactivation of rubisco at certain low CO_2concentration, especially under HT conditions. The 2 min was also sufficient to reach the new CO_2 setpoints and establish steady photosynthetic rates. A/C_i analyses were performed according to Sharkey *et al.* (Sharkey, Bernacchi, Farquhar, & Singsaas, 2007), using an improved version of the Excel Sheet from (Sharkey, 2016). Representative individual curves were fitted separately and the extracted parameters were averaged across replicates for each treatment.

Lincomycin treatment

Lincomycin treatments have generally involved infiltration of detached leaves with the lincomycin solution (e.g. Davis et al., 2016; Yang et al., 2007). For the HT treatment in cowpea, however, we found that detached leaves resulted in strong declines in PSII efficiency contrary to what we observed in attached leaves. Therefore, our treatments were either through watering of soil with lincomycin solution or syringe infiltration of attached leaves with lincomycin solution. Both approaches gave similar results, although watering the soil had a slower response (in the order of several hours to days) since uptake was generally slow. For the syringe infiltration, effects were seen within a few hours. Briefly, the abaxial side of the leaf was infiltrated with 0.45 mM lincomycin hydrochloride using a syringe by the solution. Control plants were mock infiltrated with deionized water. Following infiltration, plants were kept under low light (50 µmol m^{-2} s^{-1}) for 30 min to keep the stomata open and then 1 h in the dark before the HL treatment was imposed (GT+HL). For the HT+HL treatment, the temperature was increased to 40 °C 1 h after initiation of HL and to 45 °C after another 1 h.

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The temperature was kept at 45 °C for the next 5 h.

Determination of photorespiration

The velocity of rubisco for oxygenation (v_o — used as a proxy for photorespiration) and for carboxylation (v_c) were calculated from rubisco kinetics using the following equations as described in (Busch, 2013):

$v_c = \frac{A+R_l}{1-\frac{\Gamma^*}{C_c}}$ *(1)*

$v_o = 2 \bullet (v_c - A - R_l)$ *(2)*

where A is net assimilation as measured from gas exchange, C_c is CO_2 concentration in the chloroplast, and R_l is the day (light) respiration, which is also referred to by R_d.

The temperature response function of Bernacchi and others (Bernacchi, Singsaas, Pimentel, Portis Jr, & Long, 2001; Bernacchi, Portis, Nakano, von Caemmerer, & Long, 2002) were used g_m(needed C_c), Γ^* and R_l at different temperatures. v_c calculated using other parameters from the gas exchange measurements or estimated based on data for soybean (Walker et al., 2017). A sample excel sheet for the calculation is attached as supplementary material.

The electron transport rate from gas exchange (ETR_{GE}) was calculated using the following equation:

$ETR_{GE} = 4 \bullet (v_c + v_o)$ *(3)*

To enable direct comparison with the electron transport rate (ETR) obtained by chlorophyll fluorescence, the ETR assessed with LI-COR 6800 simultaneously with gas exchange parameters were used.

Data analysis

Depending on the experiment, we used 2-way (repeated measures) or 3-way ANOVA to determine differences and interactions between treatments (light intensity, temperature and/ or leaf maturity). Means separation was by Tukey's honestly significant difference (HSD) test. Two-sample t-test was also used on specific datasets. Data analysis was performed in Genstat 18th Edition (VSNi, UK), Origin Pro 2019b (OriginLab Corp, Northampton, MA, USA) or in R version 3.6.1 (R Core Team, 2019).

RESULTS AND DISCUSSION

Effects of short-term exposure to elevated temperature on the partitioning of energy to photoprotection and photochemistry

To determine the effect of light intensity on the temperature response of cowpea, 4-or 11- d-old-seedlings of two genotypes, Yacine (HL-tolerant) and 58-77 (HL-sensitive), were exposed to 300, 1000 or 1500 µmol photons m^{-2} s^{-1} PAR while the temperature was increased by 5 °C every 2 h starting from GT (30 °C) to HT (45 °C). Following these treatments, the plants were returned to GT for another 1 h to assess the ability to recover from the treatments.

Figure 1 shows the responses of photosynthetic parameters in Yacine to the temperature regime. Similar results were obtained in the HL-sensitive line, 58-77 (Supplementary Figure 1A). At LL (300 µmol m^{-2} s^{-1}), F_{II}in young leaves remained nearly constant as the temperature was increased, consistent with electron flow predominantly light-limited. Mature leaves showed F_{II} values that decreased significantly ($p<0.001$) with increasing temperature, down by ~10% of initial values at 45 °C, similar to earlier reports in tomato (Meng et al., 2017), At 1000 and 1500 µmol m^{-2} s^{-1}, mature leaves showed only small changes in F_{II} with temperature, but young leaves showed F_{II} that increased significantly (by about 30% $p<0.001$) as temperature was increased to 45 °C, likely indicating an increase in the capacity for photochemistry, possibly reflecting an increase in heat tolerance with illumination as reported earlier (Havaux et al., 1991; Weis, 1985).

The relatively small effect of temperature on $F_{II}$is somewhat surprising given the expected strong temperature dependence of photosynthetic reactions and suggests that photosynthesis is subject to compensatory

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regulation or control by multiple processes. All of these effects were reversed , suggesting that they were not caused by irreversible changes.

In all cases, short-term exposure to increasing temperature led to reversible decreases in light-induced thylakoid *pmf* , as estimated by the amplitude of the dark-interval relaxation kinetics (DIRK) of the electrochromic shift [ECSt, Figure 1B (Takizawa et al., 2007)]. The decreases in *pmf* can be attributed to increased thylakoid proton conductivity, g_{H^+}, estimated from ECS decay kinetics (Baker, Harbinson, & Kramer, 2007; Kanazawa & Kramer, 2002; Kramer, Avenson, & Edwards, 2004) (see Figure 1C), and thus likely reflects increased ATP synthase activity (Kanazawa & Kramer, 2002) as was seen previously with moderate temperature increases in other species (Zhang & Sharkey, 2009; Zhang et al., 2009), although other factors such as proton slippage or membrane leakage cannot be ruled out. The *pmf* , acting through its pH component, is suggested to be responsible for activating q_E (Li et al., 2002). The exception was mature leaves at low light, for which*pmf* decreased, but g_{H^+}remained relatively constant with temperature. In this case, the decreases in *pmf* at higher temperature could be attributed to decreased F_{II} , which should translate into lower proton influx by LEF.

Figure 1D shows the responses of NPQ to temperature. Note that these values were estimated using the $NPQ_{(T)}$ method (Tietz et al., 2017), which allows for more rapid and less invasive measurements, but results in NPQ values that are typically higher than those obtained with the classical method. In the case of older leaves, $NPQ_{(T)}$ (the sum of $q_{I(T)}$ and$q_{E(T)}$) increased with temperature, but the opposite behavior was observed in younger leaves (Figure 1D), though in all cases the changes were relatively modest. In general, changes in total $NPQ_{(T)}$ were inversely related to changes inF_{II} . $NPQ_{(T)}$ was reversed during the recovery except under HL (1000 and 1500 µmol $m^{-2}s^{-1}$) where it remained elevated or increased, after return to GT. The q_L fluorescence parameter, which estimates the fraction of PSII centers with oxidized Q_A (Kramer, Johnson, Kiirats, & Edwards, 2004) increased by ˜25% at the highest temperature in HL, regardless of leaf age (Figure 1E). This result implies that electrons did not accumulate on the intersystem electron transfer chain, suggesting that changes in the capacity for LEF were compensated for by complementary adjustments in $NPQ_{(T)}$ so that the quantum yield of unregulated energy dissipation (F_{NO}) remained relatively constant over the course of the experiment (Supplementary Figure 1B).

CEF has been reported to increase under HT conditions in some species (Essemine, Qu, Mi, & Zhu, 2016; Sun, Geng, Du, Yang, & Zhai, 2017), possibly to provide extra ATP needed to maintain metabolic processes, or to maintain *pmf* and initiate *pmf* -related regulation of light capture and electron flow. Figure 1F plots the relative flux of protons through the ATP synthase (v_{H^+}), estimated by the decay of the DIRK or the ECS signal, against LEF. Increases in the ratio (v_{H^+}/LEF) would indicate elevated CEF relative to LEF (Livingston, Kanazawa, Cruz, & Kramer, 2010; Strand et al., 2015; (Walker, Strand, Kramer, & Cousins, 2014)). The slope of v_{H^+}/LEF is also dependent on the content of chloroplasts and ECS-active pigments. Thus, without calibration, we cannot directly compare slopes between leaves of different developmental stages. However, we can assess changes over short time periods, and thus we normalize the ratios to those obtained at 30 °C and note that in the mature leaves, generally the ratio increased with temperature (Figure 1G) at HL (both 1000 and 1500 µmol m^{-2} s^{-1}), but declined in LL. Since we know that F_{II} and thus LEF increased slightly or remained nearly constant in mature leaves with increasing temperature, the only way that the ratio would increase in HL is for CEF to increase. Thus, this data suggests that CEF increased with temperature in mature leaves under HL. On the other hand, the ratio declined with increasing temperature in young leaves, regardless of light intensity, indicating that CEF decreased with temperature. The observation in mature leaves is consistent, whereas that in the young leaves is contrary to earlier reports (Sun et al., 2017), suggesting different responses in leaves of different ages.

Photoinhibition of PSII is controlled by altering the rates of damage and repair processes (Aro et al., 1993; Murata, Takahashi, Nishiyama, & Allakhverdiev, 2007; Nishiyama & Murata, 2014), and it is possible that HT impacts one or more of these processes. We thus compared the effects of lincomycin, an inhibitor of chloroplast protein translation that abolishes PSII repair (Tyystjärvi & Aro, 1996), on changes in maximal PSII quantum efficiency (F_V /F_M), measured 20 min after exposure to light, to dissipate q_E (Figure 2).

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Exposure to 1500 µmol m^{-2} s^{-1} at GT (GT+HL) over a period of six hours resulted in a loss ofF_V /F_M of about 50% in the presence of lincomycin, but only ˜20% in the absence of the inhibitor, suggesting that the HL treatments induced substantial photodamage, which was partly reversed by repair. On the other hand, the loss of F_V /F_M in the presence of lincomycin was only ˜25% when the temperature was ramped from 30 to 45 °C during exposure to HL (HT+HL), implying that HT decreased the rate of PSII photodamage. The fact thatF_V /F_M deceased only slightly under HT in the absence of lincomycin, suggests that the lower rate of PSII photodamage and repair were able to maintain nearly full PSII efficiency.

Overall, the results in Figure 2 imply that photoinhibition was lower at HT+HL, both because of decreased rates of PSII damage and relatively unhindered rates of PSII repair. This view is consistent with the increased q_L and decreased *pmf* at HT (discussed above), which could ameliorate PSII photodamage caused by recombination reactions that would otherwise lead to$^1O_2$ production (Davis et al., 2016). An increase in PSII repair with temperature was proposed to contribute to the tolerance to HT observed in tobacco with enhanced synthesis of the osmoprotectant, glycine betaine (Yang et al., 2007).

Effects of high temperature on CO_2 assimilation and photorespiration

Figure 3 shows temperature response curves for net CO_2assimilation (A) under conditions similar to those used in Figure 1. For simplicity, we present data on young leaves in the main text, but found similar results with mature leaves, as presented in Supplementary Figure 2. At LL (300 µmol m^{-2} s^{-1}),A declined steadily as temperature was increased from 30 to 45 °C (Figure 3A), but recovered fully (even increasing slightly) after returning to growth temperature. Under 1000 or 1500 µmol m^{-2} s^{-1}, A remained relatively constant from 30-40 °C, but decreased significantly (p<0.01) by ˜20% from 40-45 °C, consistent with previous work on other species (Crafts-Brandner & Salvucci, 2002; Lu et al., 2017). Similar to what was found in Loblolly pine (*Pinus taeda*) and eastern cottonwood (*Populus deltoides*) (Urban, Ingwers, McGuire, & Teskey, 2017a, 2017b), temperature-dependent decreases in A were not related tog_s, which increased with temperature (p<0.01) under all light intensities but recovered when returned to growth temperature (Figure 3B). Indeed,C_i increased with temperature (Figure 3C and Supplementary Figure 2C), due to increased g_s and decreased A . These results imply that the drop in A was likely related to decreased carboxylation or increased oxygenation. It is possible that rubisco was deactivated (Salvucci & Crafts-Brandner, 2004) and/or, as is well established, the specificity for CO_2 over O_2 declined with increasing temperature (Brooks & Farquhar, 1985; Galmés et al., 2016; Prins et al., 2016).

velocities of rubisco carboxylation (v_c) and oxygenation (v_o — used as a proxy for photorespiration) (Figures 4 A and B) from the gas exchange data shown in Figure 3. Results from experiments taken at 1500 µmol m^{-2} s^{-1} were omitted because they were very similar to those observed at 1000 µmol m^{-2}s^{-1}. At LL (300 µmol m^{-2}s^{-1}, Figure 4A), v_c andv_o remained relatively constant over temperature with v_o /v_c ratio of about 0.3. However, at HL (1000 µmol m^{-2}s^{-1}, Figure 4B), v_o more temperature-dependent and increased by more than 36% at 45 °C compared to v_c, which only increased by about 16%. These effects were independent of leaf age.

This increase in photorespiration over CO_2 assimilation can account for some of the reduction in net CO_2assimilation at HT. It is also possible that some LEF was diverted into "alternative" electron sinks (not related to assimilation or photorespiration) at HT. These sinks include the Mehler-peroxidase reaction (MPR, water-water cycle) (Asada, 2000; Miyake, 2010), the plastid terminal oxidase (PTOX) (Rumeau, Peltier, & Cournac, 2007), nitrogen (N) reduction, and lipid biosynthesis.

To assess this possibility, we compared the electron transport rate (ETR) measured by chlorophyll fluorescence with that calculated from gas exchange (ETR_{GE}). The difference between the two parameters indicates flow of electrons that cannot be accounted for by carbon fixation and photorespiration. Figure 4C shows that under LL, in mature leaves, there was no significant (p>0.05) difference between ETR and ETR_{GE} at 30 °C, but the difference increased significantly (p=0.03) at 45 °C, due to a decrease in ETR_{GE} which likely stems from the decrease inv_c observed in Figure 4A. A decrease inv_c can affect ETR_{GEGE} throughout the entire temperature range, except during recovery when ETR_{GE} in both leaves increased to the level of ETR. This

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suggests the persistence of alternative electron acceptors, whose activity likely reduced following recovery.

In HL however, upon increasing the temperature to 45 °C, there was no significant difference (p=0.9) between ETR and ETR_{GE} in mature leaves, although in young leaves, differences were significant (Figure 4D). This strongly suggests that, at HT+HL and especially in mature leaves, the utilization of electrons by CO_2 assimilation and photorespiration could account for most of the electrons coming from the light reactions. Nevertheless, in young leaves, there is evidence for the existence of a large pool of alternate electron acceptors which were active regardless of temperature. These results are generally consistent with the v_{H^+}/LEF data in Figure 1F–G, which shows elevated CEF in mature leaves but a decline in young leaves under HT+HL. Thus, the case in the mature leaves is generally consistent with an increase in processes with higher ATP demand, such as photorespiration. The low CEF in young leaves is consistent with more active alternative electron acceptors, although photorespiration was also increased as shown in Figure 4B.

The larger alternative electron flow in young leaves could be explained by more electron consuming processes. Such processes include nitrogen metabolism and lipid synthesis, electrons from the light reactions, although these were not directly assayed in this study. Differential nitrogen metabolism between young and mature leaves has been reported in other species and stresses, e.g. in rice (Wang, Wu, Han, Zheng, & Yang, 2012). There have also been reports that nitrate (NO_3^-) reduction can use up to 25% of electrons from LEF (Bloom, Caldwell, Finazzo, Warner, & Weissbart, 1989). Considering that the Hoagland's nutrient solution used to fertilize the cowpea plants in our experiments provide nitrogen mostly as NO_3^-, this explanation may account for at least some of the alternative electron sinks.

, photons going to alternative electron acceptors also increased. These observations suggest that the combined activity of photorespiration and other alternate electron acceptors could account for a significant portion of electrons from LEF under HT, especially in HL, although, photorespiration was the dominant electron acceptor when considering the relative change in photon or electron utilization.

Figure 5 shows A/C_i curves with concurrent chlorophyll fluorescence measurements for young leaves under both GT (30 °C) and HT (45 °C) at 300 and 1000 µmol photons $m^{-2}s^{-1}$ and under ambient (21%) and low (1%) O_2 atmospheres. Similar results were obtained for mature leaves (Supplementary Figure 5). A summary of the fitted parameters based on Sharkey et al. (Sharkey et al., 2007; Sharkey, 2016) is shown in Supplementary Figure 4.

As described in detail in the Materials and methods, the measurements were performed sequentially, starting at ambient CO_2(400 ppm) followed by lowering CO_2 to 50 ppm through 200 ppm and followed by a gradual rise to high CO_2 levels (1000 ppm). Under 300 µmol photons $m^{-2}s^{-1}$ and 30 °C (Figure 5A), A increased with CO_2 over the entire range measured. In low oxygen, A appeared to become saturated with CO_2. In Figure 5B, ETR/4 was mostly independent of CO_2 indicating the RuBP regeneration was the rate determining process. In 1 and 21% O_2 ETR/4 was about the same except that in 1% O_2 ETR/4 declined at low CO_2, the behavior that indicates RuBP-saturated rubisco kinetics was rate determining. The initial measurement at 400 ppm (circled data) fell on the line observed during the CO_2 response curve measurement.

Under 300 µmol photons m^{-2} s^{-1} and 45 °C, the data were similar (Figure 5 C and D) except that the decline in ETR/4 at low CO_2 was more pronounced, indicating the RuBP-saturated rubisco kinetics were more limiting than at 30 °C. Initial measurements (circled) were the same as measurements made during the response curve analysis. Because the maximum ETR/4 was the same at 1 and 21% O_2 at both temperatures there was no indication of triose phosphate use limitation, as would be expected at this moderate light intensity.

The responses were different at 1000 µmol photons $m^{-2}s^{-1}$. In 21% O_2 and 30 °C, the initial response of A to C_i was much steeper and the CO_2 dependence of ETR/4 indicated a significant control by rubisco up to ~200 ppm (compare Figure 5 B with 5 F). In Figure 5E, A at 1 and 21% O_2 reach the same rate and then are independent of CO_2. This has been labeled the triose phosphate use limitation (Sharkey 1985a), which can often be observed at saturating light and slightly higher C_i than the leaves likely experienced during growth. Because of photorespiration, when A in 1 and 21% O_2 is the same at the same C_i, ETR/4 must be

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higher at 21% O_2 as was observed (Figure 5F). Because A was the same at 1 and 21% O_2 and no reverse sensitivity was observed there is no evidence for photorespiration increasing A by providing amino acids to the plant as is sometimes observed (Busch et al. 2018). Initial measurements (circled) fell on the same line as determined during the measurements from low to high CO_2 (Figure 5 E and F). The behaviors reported in Figure 5 A through F show the expected relationships among A , ETR/4, light, and C_i (Sharkey 1985b)

In 1000 µmol photons m^{-2} s^{-1} and 45 °C, novel behavior was observed. Especially at high C_i , A was approximately the same in 1 and 21% O_2 (Figure 5G). This would require higher ETR/4 in 21% O_2, which was observed (Figure 5H). However, the O_2 insensitivity of A was not correlated with a C_i insensitivity (Figure 5G). This phenomenon was seen in both young and mature leaves (supplemental Figure 6G) and in both 1000 and 1500 µmol photons $m^{-2}s^{-1}$ (supplemental Figure 7 C and G). This behavior is not predicted by current gas exchange theory (Sharkey 2016). Also observed only in saturating light and high temperature is a decline in A and ETR/4 between the first measurement (circled) and the same C_i measured during the response curve measurements. We suspect this was a change in metabolic state with time but could also have been induced by the low C_i experienced during measurements made to construct the curve.

The strong response of both A and ETR/4 to C_i in 1000 µmol photons $m^{-2}s^{-1}$ and 45 °C indicates that rubisco activity was determining the behaviors. Rubisco activation is known to decline at high temperature (Salvucci and Crafts-Bradner 2004). The unusual behavior could reflect a variable rubisco activation state at saturating light and high temperature. Study of this unusual behavior including determining how widespread it is, the activation states of rubisco, metabolomics, and more detailed measurements of electron and proton flows may provide insight into how cowpea tolerates high temperature.

Longer-term effects of elevated temperature and fluctuating light on photosynthesis

We have shown that short term HT combined with HL caused a shift toward photochemistry with the increased electron flux attributed to decreased PSII photodamage, enhanced PSII repair and increased photorespiration as well as activity of other alternative electron acceptors. However, it was not clear whether the effects on PSII function were only for a short period or whether it persisted over a relatively longer time scale and under dynamic conditions as occurs in the field. We, therefore, tested the hypothesis that prolonged exposure to HT+HL especially under dynamic conditions could lead to more severe loss in efficiency of PSII for photochemistry (F_{II}) under the combined stress relative to HL stress alone. Dynamic (fluctuating) light conditions have been shown to produce phenotypes not detected under constant light conditions normally used in growth chambers (Cruz et al., 2016; J. Li et al., 2019). Figure 6 shows results from a four-day experiment, with altered temperature and light intensity patterns, as indicated above Figure 6A, to expose the plants to a range of light and temperature conditions. On Day 1, the light intensity was fluctuated between 300 and 1500 µmol photons m^{-2} s^{-1} in two steps. On Days 2 and 3, the light was held constant at 1500 and 300 µmol photons m^{-2} s^{-1} respectively, and on the final day the fluctuating pattern used on Day 1 was repeated. Plants were also exposed to either a constant GT (30 °C), a constant HT (45 °C), or a daily fluctuation in temperature between GT in the morning, HT at midday and returning to GT by end of the day (HTR — HT with recovery). The night temperature was kept constant (20 °C) for all treatments, except the constant HT, where it was kept at 35 °C.

Both lines showed decreased F_{II} when exposed to fluctuating and HL under constant GT, but was particularly evident in the HL-sensitive line (58-77) on days 2 through 4 (Figure 6A). The decreased F_{II} was accompanied by increases in long-lived NPQ ($q_{\text{I(T)}}$), suggesting that the regiment of light treatments induced substantial photoinhibition or photodamage. By contrast, both regimes with elevated temperatures resulted in smaller losses of PSII activity on Day 4 (Figure 6A), when compared to the initial values at the beginning of Day 1, suggesting that the HT prevented the buildup of photoinhibition. Supplementary Figure 7 shows the $\log_2$ fold change of F_{II} which indicates that both HT treatments resulted in relatively higher PSII activity compared GT, especially in HL.

When the temperature was first increased in Day 1, there was an abrupt spike in $NPQ_{(T)}$ (Figure 6B); the rapid recovery of this spike suggests that it is caused by $q_{\text{E(T)}}$ (Figure 6D). This effect was observed in both

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lines, but was larger for Yacine, especially under HL, and was regardless of whether the light was constant or fluctuating. However, with time, NPQ declined, likely due to the decreasing $q_{\mathrm{E(T)}}$, and reached values similar to the GT treatment at the end of the day. By the 4th day, $\mathrm{NPQ}_{\mathrm{(T)}}$ and photoprotection ($q_{\mathrm{E(T)}}$) had declined in Yacine below values of the GT plants. Since F_{II} increased with the decline in NPQ as was seen in Figure 1, it suggests that PSII photodamage was not increased and/or that PSII repair was able to maintain high activity, in line with Figure 2.

By contrast, $q_{\mathrm{I(T)}}$ was persistently low in the HT treatments compared to the GT plants under HL regardless of the light intensity (Figure 6C). Thus, it is possible that the increased $q_{\mathrm{I(T)}}$ in GT resulted in impaired PSII activity as indicated by the low F_{II} in HL. These results suggest that HT prevented loss of PSII activity under HL by reducing the slowly relaxing form of NPQ. It should be noted that compared to values at the beginning of Day 1, the HT treatment slightly increased $q_{\mathrm{I(T)}}$. However, when compared to GT at the same time points, $q_{\mathrm{I(T)}}$ was lower in both HT treatments. This reduced $q_{\mathrm{I(IT)}}$ is consistent with enhanced activity of alternative electron acceptors, and particularly photorespiration.

The data presented imply that, at least in these cowpea lines and in particular younger leaves, the light reactions of photosynthesis were relatively unimpaired under combined HT+ HL as has been reported in tomatoes (Gerganova et al., 2016). Further, these effects apparently persist over a relatively long term and under dynamic light and temperature conditions. Using two genotypes with differing sensitivity to HL revealed that the effects of combined HT and HL were common regardless. In fact, the HL-sensitive genotype, 58-77, appeared to show more tolerance to HL when combined with HT. These observations allude to the fact that cowpea plants are better equipped to deal with the combination of HL and HT stresses since these often occur together in the field. However, it is not known whether the exhibited tolerance of the light reactions would lead to increased productivity since carbon metabolism was inhibited under these conditions.

Conclusions

Several remarkable results lead us to propose that multiple electron acceptors can protect PSII against photodamage under combined high light and elevated temperatures. One remarkable observation from our work was the nearly temperature-independent responses of the light reactions. Most strikingly, the redox state of $\mathrm{Q_A}$, often taken as a measure of the regulatory balance of the light reactions, tended to become more oxidized as temperature increased (Figure 1E). These results imply that, even as assimilation was significantly reduced by temperature, the light reactions were stimulated (rather than inhibited) in a light intensity dependent manner. Under fluctuating light conditions, increasing temperature increased F_{II}, decreased both q_{I} and q_{E} components of NPQ, and resulted in a less reduced $\mathrm{Q_A}$, suggesting that HT decreased the accumulation of states that would normally result in photodamage (Figure 6). These effects were more pronounced following acclimation to HT and fluctuating/high light. Separate experiments suggest that the rates of photodamage are decreased and the rates of PSII repair increased under HT.

The small impact of temperature on the light reactions can in large part be explained by the fact that the sinks for electrons from LEF remained highly active. When A decreased, electrons flowed to other electron acceptors. A large fraction of these acceptors can be accounted for by increased photorespiration, but an additional fraction appears to be sent to a light intensity dependent process, possibly involving $\mathrm{O_2}$ reduction by a process with a relatively low K_{m} for $\mathrm{O_2}$, or other metabolic electron sinks.

Similarly, the regulation (or control) of the light reactions through the *pmf* was less engaged at higher temperatures, with increased ATP synthase activity (g_{H^+}), and decreased *pmf* (Figure 1). This combination of increased LEF and high g_{H^+} are consistent with increases in sinks for both electrons and ATP, i.e. there did appear to be a depletion of sinks or substrates for ATP synthase. These results pose an interesting question: how is the supply of outputs of ATP and NADPH from the light reactions maintained in balance with metabolic demand despite the diversion of energy to alternative processes? In particular, photorespiration requires additional ATP/NADPH compared to the CBB cycle, and one might expect that ATP supplementation would be required under high photorespiratory conditions at HT. However, CEF (Figure 1F–G), which is thought to supplement ATP under such conditions (Strand, Fisher, & Kramer, 2017), actually decreased

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under elevated temperatures in the young leaves, suggesting that other processes allowed for ATP/NADPH balance. One possibility is alternative electron flow to O_2, as suggested by comparisons of ETR calculated from the sum of the velocity for carboxylation and oxygenation (ETR_{GE}, Figure 4) and chlorophyll fluorescence (ETR), which should have increased ATP production without net changes in NADPH (Miyake, 2010). On the other hand, in mature leaves, CEF increased in HL, consistent with the increased photorespiration accounting for a large fraction of electrons under HT (Figure 4). In either case, we show clear evidence that photorespiration is an essential electron sink that appears to help maintain open PSII redox sites under high light and temperature stresses.

A second remarkable observation is the light intensity-and temperature-dependent down-regulation or inhibition of rubisco activity at low O_2 in (Figure 5, Supplementary Figure 5 and 6). This effect can most easily be explained by deactivation of rubisco itself, though our data cannot rule out other indirect effects that result in apparent loss of rubisco activity estimated by gas exchange. Nevertheless, the effect was mostly seen in HL, but was more pronounced at HT and under low CO_2 conditions, and was rapidly reversible upon increasing CO_2, suggesting that it serves a regulatory role.

Overall, cowpea appears to have mechanisms that allow the light reactions to maintain high activity and low propensity for ROS generation, through a combination of highly active alternative energy sinks, including photorespiration and other, yet undefined, electron sinks. We speculate that breeding or engineering crops to increase the capacity of these sinks could lead to more robust crop productivity under field conditions where acute combined high light and temperature stresses occur.

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FIGURE LEGENDS

Figure 1:Temperature response of photosynthesis under different light intensities and in leaves of different maturity. A) photosynthetic efficiency (F_{II}), B) light-induced thylakoid proton motive force (*pmf*) estimated by the ECSt parameter, C) ATP synthase activity (g_{H+}), D) non-photochemical quenching (NPQ), E) redox state of primary quinone acceptor (q_L), F)v_{H+}/LEF and G) Relative change in thev_{H+}/LEF ratio. Data were normalized to values at 30 °C from Figure 1F. Leaf maturity is indicated by the line type: dashed line, mature leaves (ML, unifoliate leaves from 12 -day old seedlings); solid line, young leaves (YL, unifoliate leaves from 4-day old seedlings). The different colored lines represent measurements at low (300 µmol m^{-2} s^{-1}) and high (1000 µmol m^{-2} s^{-1} and 1500 µmol m^{-2} s^{-1}) light intensities as indicated in (A). Grey area represents recovery for at least 1 h. Data are from cowpea genotype 'Yacine'. The temperatures reported are the air temperature in the chamber, which was changed every 2h, although, measurements were made after at least 1 h under each temperature. Data are means of 8-14 replicates from 2-3 independent experiments ±1 S.E.

Φιγυρε 2: Εφφεςτς οφ λινςομψςιν ον μαξιμυμ εφφιςιενςψ οφ ΠΣΙΙ (Φ_v/Φ_M) υνδερ ΗΛ (1500 µµολ μ^{-2} ς^{-1}) ορ ςομβινεδ ΗΛ ανδ ΗΤ στρεσς. Starting from 1 h after lincomycin treatment, the kinetics of relative F_V/F_M are shown for A) GT+HL and B) HT+HL treatments. Values are expressed relative to the initial F_V/F_M values for each day. Leaves were syringe infiltrated with 0.2 g/L lincomycin or deionized water. Dashed lines with hollow markers represent lincomycin treatment and solid lines with filled markers represent mock (water) infiltrated leaves. Line color indicates the temperature and light intensity treatment. Red = HT+HL (high temperature + high-light), black=GT+HL (growth temperature + high-light). For the GT+HL treatment, the temperature was kept constant (30 °C) throughout

the 6 h period whereas the temperature for the HT+HL are indicated in the colored boxes above the data points. Data are from young cowpea leaves and the two genotypes (Yacine and 58-77) are used to show the differences in HL sensitivity which is abolished in HT+HL. The means of 4 replicates ±1 S.E are shown.

Figure 3: Temperature responses of CO_2assimilation-related processes in young leaves of cowpea seedlings under different light intensities. A) Net CO_2 assimilation (A), B) Stomatal conductance (g_s) and C) Intercellular CO_2 concentration (C_i). The different colored lines represent measurements at low (300 µmol m^{-2} s^{-1}) and high (1000 µmol m^{-2} s^{-1} and 1500 µmol m^{-2} s^{-1}) light intensities as indicated in the legend in Figure 3B. Grey area represents recovery for at least 1 h. Data are from young leaves (YL) of cowpea genotype 'Yacine'. The temperatures reported are the air temperature in the chamber, which was changed every 2h, although, measurements were made after at least 1 h under each temperature. Data are means ±1 S.E of at least 8 biological replicates from 2–3 independent experiments.

Figure 4: Effect of temperature on velocity of rubisco for carboxylation (v_c) and oxygenation (v_o) and electron transport rate in cowpea.A–B) Temperature response of v_c andv_o in leaves of different maturity and under light intensities of A) 300 µmol photons $m^{-2}s^{-1}$ (low light — LL) and B) 1000 µmol photons m^{-2} s^{-1} (high-light — HL). C–D). The temperature response and light intensity dependence of electron transport rate measured by chlorophyll fluorescence (ETR) or calculated from gas exchange (ETR_{GE}). The colors represent the different parameters as indicated in the y-axes of the leading (far left) figures. Closed symbols = young leaves (YL), open symbols = mature leaves (ML). LL Data are means of 6–8 replicates with error bars being ± 1 S.E. Assumptions used in calculating v_c andv_o are outlined in the Materials and Methods.

Figure 5: Comparison of responses of net CO_2assimilation (A) and ETR/4 to internal CO_2concentration (C_i) in different light intensities, O_2 levels and temperature in young cowpea leaves. Response of A and ETR/4 against C_i under low light (300 µmol photons m^{-2} s^{-1}) at A–B) 30 °C and C–D) 45 °C respectively. Corresponding measurements at high light (1000 µmol photons $m^{-2}s^{-1}$) are shown in E–F and G–H respectively. The circled points represent the first measurements made under ambient CO_2. Data are means ± 1 S.E of 3–10 biological replicates from 1–3 independent experiments.

Figure 6: HT treated plants exhibit less photo-inhibitory quenching under prolonged dynamic and high light conditions. Changes in A) F_{II}, B) Non- photochemical quenching ($NPQ_{(T)}$), C) Photo-inhibitory quenching ($q_{I(T)}$) and D) the rapidly reversible (photoprotective) quenching ($q_{E(T)}$) during a 4-day experiment with corresponding temperature and light intensity (PAR=photosynthetically active radiation — µmol photons m^{-2} s^{-1}) changes indicated in the top panel of Figure 6A. The color of the lines corresponds to the colors of the temperature. Dashed line = 58-77, solid lines=Yacine. The suffix after the genotype names indicates the temperature treatment. Data are means of 5–8 replicates from 1–2 independent experiments. Measurements were made at 20 min intervals on days 1 and 4 and at 1 h intervals on days 2 and 3 using the CCD (charge coupled device) cameras in the DEPI (Dynamic Environment Photosynthesis Imager) chamber (Cruz et al., 2016).

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