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Temperature Dependence of Photosynthesis in *Agropyron smithii* Rydb.[1]

III. RESPONSES OF PROTOPLASTS AND INTACT CHLOROPLASTS

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JOHN KOBZA, ERNEST G. URIBE*, AND GEORGE J. WILLIAMS III
Department of Botany, Washington State University, Pullman, Washington 99164-4230

ABSTRACT

Protoplasts and intact chloroplasts isolated from *Agropyron smithii* Rybd. were utilized in an effort to determine the limiting factor(s) for photosynthesis at supraoptimal temperatures. Saturated CO_2-dependent O_2 evolution had a temperature optimum of 35°C for both protoplasts and intact chloroplasts. A sharp decline in activity was observed as assay temperature was increased above 35°C, and at 45°C only 20% of the maximal rate remained. The temperature optimum for 3-phosphoglycerate reduction by intact chloroplasts was 35°C. Above this temperature, 3-phosphoglycerate reduction was more stable than CO_2-dependent O_2 evolution. Reduction of nitrite in coupled intact chloroplasts had a temperature optimum of 40°C with only slight variation in activity between 35°C and 45°C. Reduction of nitrite in uncoupled chloroplasts had a temperature optimum of 40°C, but increasing the assay temperature to 45°C resulted in a complete loss of activity. Reduction of *p*-benzoquinone by protoplasts and intact chloroplasts had a temperature optimum of 32°C when measured in the presence of dibromothymoquinone. This photosystem II activity exhibited a strong inhibition of O_2 evolution as assay temperature increased above the optimum. It is concluded that, below the temperature optimum, ATP and reductant were not limiting photosynthesis in these systems or intact leaves. Above the temperature optimum, photosynthesis in these systems is limited in part by the phosphorylation potential of the stromal compartment and not by the available reductant.

It is necessary to identify and characterize the specific chloroplast activities that are sensitive to high temperature inhibition in order to understand the basis of the reduction of leaf photosynthesis at supraoptimal temperature. Studies by Santarius (13–15) have shown that heating of isolated spinach chloroplasts in the range of 35°C to 45°C causes uncoupling of photophosphorylation from electron transport and 55°C was sufficient to totally inhibit electron transport. The decline in coupling has been attributed to thermally induced membrane disorganization. This hypothesis is supported by the studies of Mukohata *et al.* (9, 10) which reveal thermally-induced increases in thylakoid proton permeability and the study by Volger and Santarius (18) which demonstrated a release of membrane protein from thylakoids exposed to high temperatures which inhibited photochemical reactions. Thermolability of O_2 evolution by PSII at temperatures which inhibit electron transport has also been reported for *Atriplex sabulosa* (2).

The response of photosynthesis in the C_3 grass *Agropyron smithii* to supraoptimal temperatures has been investigated at the whole leaf (8) and granal levels of organization (16). Previously reported studies from this laboratory indicated that below 35°C, the temperature optimum for leaf net photosynthesis under analysis conditions of 2% O_2 and 340 $\mu l\ l^{-1}$ CO_2, net photosynthesis of intact leaves of this species is largely limited by photorespiration. It was suggested that a temperature dependent change in the flux of carbon through RuBP[2] carboxylase may limit photosynthesis as the temperature increases up to 35°C since the *in vivo* Q_{10} values for the maximum velocity of the RuBP carboxylase reaction were lower than the *in vitro* Q_{10} values (8). A reduction of quantum yield above 35°C, indicative of an inhibition of the photochemical reactions, was also noted. Our studies on the effect of supraoptimal temperatures on the photochemical reactions of isolated grana of *A. smithii* showed that exposure of coupled grana to temperatures above 35°C led to slight increases in electron transport rates and inhibition of cyclic and noncyclic photophosphorylation (16). This uncoupling was associated with a decreased capacity for net uptake of protons by the thylakoids, again suggesting a thermally induced increase in proton permeability.

The purpose of this study was to determine if the effects of supraoptimal temperature on the thylakoid system may be causally related to the high temperature decline of net photosynthesis of intact leaves. If such a relationship exists, the photochemistry of systems of increasing complexity should exhibit similar responses to high temperature. To this end, intact chloroplasts and isolated protoplasts of *A. smithii* have been used to determine the effects of supraoptimal temperature on electron transport and photophosphorylation in an organized system which can carry out net photosynthesis.

MATERIALS AND METHODS

Plant Material. *Agropyron smithii* Rybd. was grown from seed obtained from the Plant Resources Center, Bridger, MT. Growing conditions were those described by Monson *et al.* (8).

Isolation of Protoplasts. The enzymic digestion procedure of Edwards *et al.* (3) was used to prepare protoplasts from 14- to 21-d old leaves. The digestion medium contained 0.5 M sorbitol, 1 mM $CaCl_2$, 3.0% cellulase (Onozuka-4S), 0.3% macerozyme, 0.1% BSA, and 5 mM Mes (pH 5.5, KOH). Protoplasts were released by washing the digested leaf segments in a solution containing 0.5 M sorbitol, 1.0 mM $CaCl_2$, and 5.0 mM Mes (pH 5.5, KOH) and then recovered by centrifugation at 100*g* for 3 min. The crude protoplast pellet was suspended in a solution

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[2] Abbreviations: RuBP, ribulose bisphosphate; DBMIB, dibromothymoquinone; PGA, 3-phosphoglyceric acid.

containing 0.5 M sucrose, 1.0 mM $CaCl_2$, and 5.0 mM Mes (pH 5.5, KOH). A solution containing 0.4 M sucrose, 0.1 M sorbitol, 1.0 mM $CaCl_2$, and 5.0 mM Mes (pH 5.5, KOH) was layered over this crude protoplast suspension. A third solution containing 0.5 M sorbitol, 1.0 mM $CaCl_2$, and 5.0 mM Mes (pH 5.5, KOH) was then layered over this. This discontinuous gradient was centrifuged at 250*g* for 5 min. The purified protoplasts were found at the interface between the top two solutions. These were collected with a Pasteur pipette and used directly or processed for preparation of intact chloroplasts. Evan's blue exclusion by the protoplasts revealed that the preparations exceeded 80% intactness.

Isolation of Intact Chloroplasts. Intact chloroplasts were obtained by passing an aliquot of the purified protoplast suspension through a 20-μm net. BSA was added during breakage to maintain a concentration of 0.1%. The broken protoplast suspension was centrifuged at 250*g* for 4 min. The supernatant was discarded and the intact chloroplast pellet was resuspended in a solution containing 0.33 M sorbitol, 10 mM Na_2 EDTA, 10 mM $NaHCO_3$, and 50 mM Hepes (pH 7.6, KOH). Chloroplasts were used within 30 min of isolation. Intactness was greater than 80% in all preparations as determined by the ferricyanide method (6). Chl was determined by the method of Arnon (1).

Measurement of Oxygen Evolution. Chloroplasts or protoplasts equivalent to 25 to 35 μg/ml of Chl were preincubated at the assay temperature for 30 s prior to initiation of the assay. Upon illumination, the reaction was followed polarographically in a system described previously (16). CO_2-dependent O_2 evolution by protoplasts was measured in a solution containing 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM Na_2EDTA, 10 mM $NaHCO_3$, and 50 mM Hepes (pH 7.6, KOH). Benzoquinone-dependent O_2 evolution by protoplasts was measured using the same solution substituting 4.0 μM DBMIB and 0.5 mM *p*-benzoquinone for $NaHCO_3$. CO_2-dependent O_2 evolution by intact chloroplasts was measured in an assay solution containing 0.33 M sorbitol, 10 mM Na_2EDTA, 10 mM $NaHCO_3$, 25 μM KH_2PO_4, and 50 mM Tricine (pH 8.1, KOH). When measuring PGA reduction, 1 mM PGA was substituted for $NaHCO_3$ in the assay solution and the phosphate concentration increased to 5 mM to inhibit CO_2-dependent O_2 evolution. Nitrite-dependent O_2 evolution was measured in an identical assay solution to that for PGA reduction except 1 mM NO^-_2 was substituted for PGA. The assay solution was altered in content for *p*-benzoquinone-dependent O_2 evolution such that there was a final concentration of 0.5 mM benzoquinone and 4.0 μM DBMIB.

RESULTS

Chloroplast preparations were found to remain relatively intact over the temperature range of 20°C to 40°C (Table I). At temperatures above 40°C, electron transport to ferricyanide in the presence of NH_3 rapidly declined to zero. As a result, no data for intactness could be obtained using the ferricyanide method. Electron acceptors such as NO^-_2 and PGA that are known to cross the chloroplast envelope were found to support electron transport at temperatures above 40°C (Figs. 1 and 2). Since these acceptors require an intact system in order to function as Hill oxidants, it can be assumed that within the temperature range utilized in these experiments the chloroplasts remained intact. It can therefore be concluded that the observed responses by chloroplasts to increasing assay temperature were not due to loss of chloroplast integrity.

Table I. *The Per Cent Intactness of Chloroplasts Isolated from Protoplasts of Agropyron smithii as a Function of Assay Temperature*

Osmotically shocked and intact chloroplasts were incubated for 30 s at the given temperature in an assay solution containing 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5.0 mM K_2HPO_4, 1.0 mM $K_3Fe(CN)_6$, and 50 mM Hepes (pH 7.6, KOH). After preincubation, ferricyanide reduction was followed polarographically.

Assay Temperature	Intactness[a]
°C	%
20	89.5
25	87.3
30	86.0
35	90.0
40	86.0

[a] Each value is a mean of three replicates. The maximum range of values for any one mean was 12.5%.

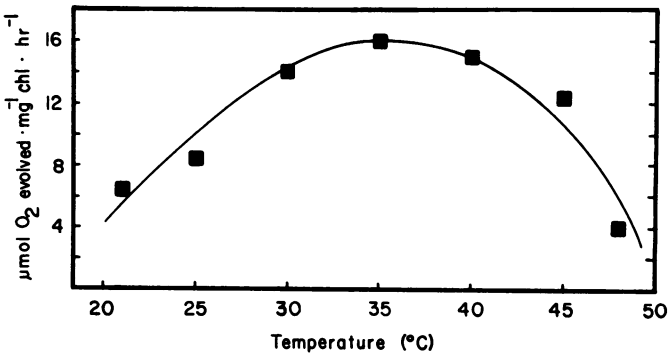

FIG. 1. Phosphoglycerate-dependent O_2 evolution (polarographic) of intact chloroplasts of *A. smithii* as a function of assay temperature. Each point is the mean of at least three replicates. Maximum and minimum standard deviations were 2.1 and 1.1, respectively.

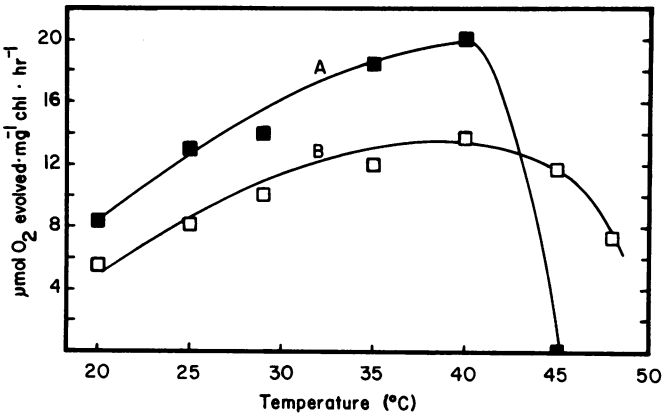

FIG. 2. Nitrite-dependent O_2 evolution (polarographic) of both coupled (A) and uncoupled (with 5.0 mM NH_4Cl, B) intact chloroplasts of *A. smithii* as a function of assay temperature. Each point is the mean of at least three replicates. Maximum and minimum standard deviations were 2.8 and 0.5, respectively.

CO_2-dependent O_2 evolution by both protoplasts and chloroplasts responded similarly to increasing temperature (Fig. 3). Rates of O_2 evolution increased gradually with a Q_{10} of 1.2 to 1.4 in the 20°C to 35°C interval. Both preparations had optimal CO_2-dependent O_2 evolution at 35°C and sharp declines in activity in the 35°C to 45°C interval. At 45°C, only 15% to 20% of the activity present at 35°C remained.

The PGA-dependent O_2 evolution temperature profile of intact chloroplasts (Fig. 1) was generally similar to that seen with CO_2 as the electron acceptor. In this case, the temperature-dependent increase in activity in the range of 20°C to 35°C was considerably larger (Q_{10} of 2.4) than that seen with CO_2-dependent O_2 evolution by intact chloroplasts. The decline in activity in the 35°C to 48°C interval was much less pronounced than that seen for CO_2-dependent O_2 evolution, the activity at 45°C being approximately

75% of the activity measured at 35°C.

Nitrite ion was also effective in supporting O_2 evolution in intact chloroplasts (Fig. 2). Photosynthetic reduction of nitrite in a coupled system (minus ammonium ion) was observed to be more thermostable than either CO_2 or PGA reduction. The system had an optimum of 40°C with only slight changes in activity in the 35°C to 45°C range. The data of Figure 2 also illustrate the effect of supraoptimal temperature on uncoupled electron flow. The uncoupler, ammonium ion, caused a 60% increase in the rate of O_2 evolution in the 20°C to 40°C interval. The temperature optimum for this enhanced electron transport was again 40°C, but in this uncoupled system, an additional 5°C increment in temperature to 45°C was sufficient to completely inhibit O_2 evolution.

Both protoplasts and intact chloroplasts evolved O_2 at appreciable rates when *p*-benzoquinone was provided as the electron acceptor (Fig. 4). The suboptimal temperature response of the PSII-mediated photoreduction was similar to that seen for the PSII- and PSI-mediated reduction of nitrite in intact chloroplasts. There was a 2-fold increase in the rate of *p*-benzoquinone-dependent O_2 evolution in the interval 20°C to 30°C. The temperature optimum was at 32°C, and higher temperatures caused a strong inhibition of O_2 evolution. At 45°C, O_2 evolution was completely inhibited in both protoplasts and intact chloroplasts

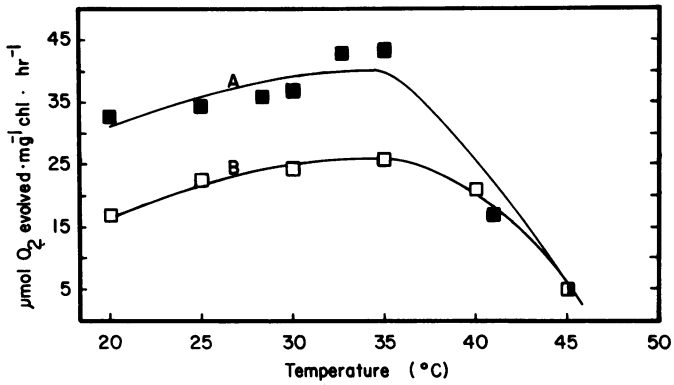

FIG. 3. CO_2-dependent O_2 evolution (polarographic) of protoplasts (A) and intact chloroplasts (B) of *A. smithii* as a function of assay temperature. Each point is the mean of at least three replicates. Maximum and minimum standard deviations were 3.1 and 0.2, respectively.

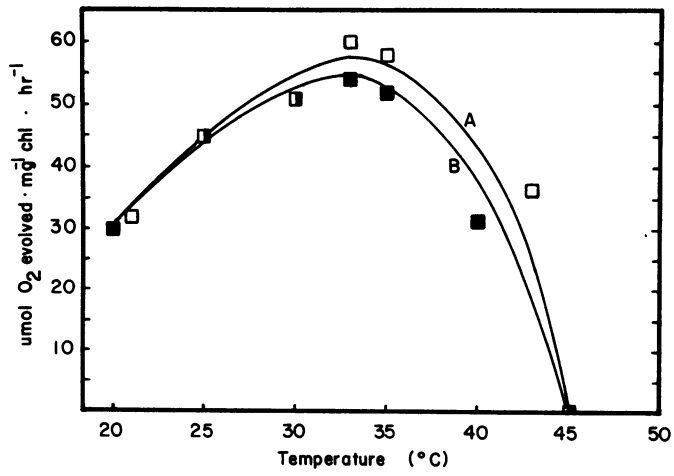

FIG. 4. Benzoquinone-dependent O_2 evolution (polarographic) of protoplasts (A) and intact chloroplasts (B) of *A. smithii* as a function of assay temperature. Each point is the mean of at least three replicates. The maximum and minimum standard deviations were 6.3 and 3.4, respectively.

resembling the high temperature inhibition of uncoupled nitrite reduction.

DISCUSSION

Temperature response curves of CO_2-dependent O_2 evolution by protoplasts and intact chloroplasts were determined using a concentration of free CO_2 ranging from 160 to 600 μM CO_2, concentrations which suppress photorespiration. The optima and shape of the temperature response curves for CO_2-dependent O_2 evolution at saturating CO_2 by chloroplasts, protoplasts, and leaves are very similar (Fig. 3; Ref. 8). This similarity of response indicates that isolated protoplasts and intact chloroplasts of *A. smithii* may be reliably used to assess the site of high temperature-induced lesions by utilizing membrane-permeable Hill oxidants under conditions which maintain the cell and organelle membrane integrity. An analysis of the supra-optimal temperature responses of photosynthesis in systems of increasing complexity has allowed us to gain additional insight into the causes of high temperature inhibition of photosynthesis in *A. smithii*.

Reduction of nitrite occurs in the chloroplast on the reducing side of PSI, accepting electrons from Fd (7, 19). The reduction of nitrite by intact chloroplasts may be used to estimate the rate of basal noncyclic electron transport imposed by the limitation of the substrate, ADP, to the coupling factor. In the presence of ammonium ion, nitrite reduction may provide an estimate of uncoupled electron transport. Assessment of PSII activity in intact chloroplasts and protoplasts may be accomplished by measuring *p*-benzoquinone-dependent O_2 evolution in the presence of sufficient DBMIB concentration to effectively block electron transport at plastoquinone. The reduction of PGA, on the other hand, requires both ATP and NADPH, thus PGA reduction may serve as an indicator of the generation of both compounds. The comparison of the temperature response of these photochemical reactions in intact chloroplasts to that reported for isolated thylakoids allows us to determine the possible site(s) of high temperature inhibition.

As previously stated, the Q_{10} values for PGA and NO^-_2 reduction were greater than the Q_{10} value for CO_2-dependent O_2 evolution. This indicates that photosynthesis at suboptimal temperatures was limited by neither the rate of electron transport nor the amount of available ATP. The limiting factor(s) at these lower temperatures was not apparent from this study; however, it is possible that the limiting factor at suboptimal temperatures was the flux of carbon through the RuBP carboxylase reaction under reduced photorespiratory conditions as was suggested for the whole leaf of *A. smithii* (8).

Intact chloroplasts exhibited a higher temperature optimum for nitrite reduction (40°C) than for CO_2 and PGA reduction (35°C). It was concluded from this information that noncyclic electron transport was not limiting at temperatures above the CO_2 saturated photosynthetic temperature optimum. Due to the minimal decrease in the rate of nitrite reduction at 45°C, there appears to be sufficient reductant to maintain CO_2-dependent O_2 evolution at 86% of the maximal rate measured in the experiment of Figure 3 (curve B). The resistance of coupled electron transport to supraoptimal temperatures may also be noted in the broad temperature optimum of PGA reduction (Fig. 1). The supraoptimal temperature response of water oxidation and coupled noncyclic electron transport in the intact chloroplast is strikingly similar to that reported by Stidham *et al.* (16) for isolated coupled thylakoids. Both systems maintain near maximal activities in the 30°C to 45°C range with an apparent optimum at 40°C.

Uncoupling conditions enhance the lability of electron transport at supraoptimal temperatures. As seen in Figure 2, curve A, ammonium ion at uncoupling concentrations causes a complete inhibition of NO^-_2-dependent O_2 evolution at 45°C. The same

supraoptimal temperature effect was previously reported by Stidham *et al.* (16) in the *A. smithii* thylakoid system uncoupled by gramicidin and by Bjorkman *et al.* (2) for uncoupled electron transport by chloroplasts from *A. sabulosa* and *Tidestromia oblongifolia.*

The fact that the temperature optimum for PGA-dependent O_2 evolution and saturated CO_2-dependent O_2 evolution coincide at 35°C is significant to the elucidation of the limiting process(es) of photosynthesis at supraoptimal temperatures. The reduction of PGA requires reductant, ATP, and high concentrations of substrate relative to product for the initial PGA kinase reaction. This reaction has a $\Delta F'$ value of +4.5 kcal/mol in the direction of 1,3-bisphosphoglycerate, thus a high ATP/ADP ratio is required for the overall reduction of 3-PGA. As stated earlier, it appears that coupled noncyclic electron transport was not limiting photosynthesis at supraoptimal temperatures. This indicates that the supply of NADPH was not limiting PGA-dependent O_2 evolution nor CO_2-dependent O_2 evolution at supraoptimal temperatures. It would appear that one factor limiting PGA reduction and photosynthesis at supraoptimal temperatures may have been the phosphorylation potential of the stromal compartment. This conclusion is further supported by the reduced photophosphorylation rates of isolated *A. smithii* thylakoids observed at similar temperatures (16).

Reduced photophosphorylation was not the sole determinant of the supraoptimal temperature response of photosynthesis. Maintenance of relatively high rates of PGA-dependent O_2 evolution but relatively low rates of CO_2-dependent O_2 evolution at 45°C (Figs. 1 and 3) indicates that PGA reduction (and presumably photophosphorylation) was more heat stable than CO_2-dependent O_2 evolution by either intact chloroplasts or protoplasts. The reason for the greater instability of CO_2-dependent O_2 evolution cannot be deduced from these experiments. Weis, using data collected from intact chloroplasts and intact leaves, concluded that the state of activation of RuBP carboxylase was reduced at high temperatures (20). Reduced regeneration of RuBP at such temperature could also explain the discrepancy between the PGA reduction and CO_2-dependent O_2 evolution temperature response curves. Stidham *et al.* (16) found that *A. smithii* thylakoids had impaired H^+ uptake at temperatures above 35°C. In intact chloroplasts, this would result in an increase in the stromal H^+ concentration and a decrease in Mg^{2+} concentration. The net result could be to reduce activation of key Calvin cycle enzymes (RuBP carboxylase, fructose, 1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase) and reduce the overall flux through the cycle (11, 21). Alteration of thylakoid membrane proton permeability at supraoptimal temperatures may have been the cause of increased supraoptimal temperature sensitivity of CO_2-dependent O_2 evolution when compared to PGA-dependent O_2 evolution.

PSII-dependent electron transport is coupled to photophosphorylation site II which is apparently free of control by ADP, Pi, and Mg^{2+}. Electron transport through this site is known to be relatively unaffected by the addition of uncouplers (4). Isolation of this segment of the electron transport chain in either protoplasts or intact chloroplasts increases the thermal lability of the water oxidation process (Fig. 4). These data support our previous conclusion that PSII activity is probably the most heat-labile component of the entire electron transport system. It is clear that high temperature lability is associated with uncoupling (Fig. 2) or with electron flow through PSII in the absence of metabolic control (Fig. 4). On the other hand, reaction conditions which allow electron transport through PSII and PSI and maintain a potential for phosphorylation at both coupling sites served to protect the water oxidation process from thermal inactivation.

Loss of water oxidizing activity under uncoupling conditions is attributable to the collapse of the electrochemical proton gradient and an alkalinization of the loculus leading to a loss of PSII function (12). The reduction of *p*-benzoquinone and its derivatives in the temperature range of 15°C to 20°C is known to support ATP synthesis and presumably an electrochemical proton gradient (4, 5, 17), thus the supra-optimal temperature lability of PSII associated electron transport was not expected. It is possible that the interaction of lipophilic class III acceptors with hydrophobic electron donors is more sensitive to high-temperature-induced membrane perturbations. These perturbations could prevent electron flow by enhancing high-temperature-induced membrane disorganization which would increase proton permeability, produce uncoupling and hence inactivation of the water oxidizing system.

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