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Effect of Chilling on Carbon Assimilation, Enzyme Activation, and Photosynthetic Electron Transport in the Absence of Photoinhibition in Maize Leaves[1]

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The relationships between electron transport and photosynthetic carbon metabolism were measured in maize (*Zea mays* L.) leaves following exposure to suboptimal temperatures. The quantum efficiency for electron transport in unchilled leaves was similar to that previously observed in C_3 plants, although maize has two types of chloroplasts, mesophyll and bundle sheath, with PSII being largely absent from the latter. The index of noncyclic electron transport was proportional to the CO_2 assimilation rate. Chilled leaves showed decreased rates of CO_2 assimilation relative to unchilled leaves, but the integral relationships between the quantum efficiency for electron transport or the index of noncyclic electron transport and CO_2 fixation were unchanged and there was no photoinhibition. The maximum catalytic activities of the Benson-Calvin cycle enzymes, fructose-1,6-bisphosphatase and ribulose-1,5-bisphosphate carboxylase, were decreased following chilling, but activation was unaffected. Measurements of thiol-regulated enzymes, particularly NADP-malate dehydrogenase, indicated that chilling induced changes in the stromal redox state so that reducing equivalents were more plentiful. We conclude that chilling produces a decrease in photosynthetic capacity without changing the internal operational, regulatory, or stoichiometric relationships between photosynthetic electron transport and carbon assimilation. The enzymes of carbon assimilation are particularly sensitive to chilling, but enhanced activation may compensate for decreases in maximal catalytic activity.

Maize (*Zea mays* L.) is one of the most important agricultural crops, but it requires relatively high temperatures for optimal growth and is sensitive to chilling-induced damage (Baker et al., 1983, 1994; Prioul, 1996). Various factors have been implicated in the temperature tolerance of maize leaves, and cold inactivation of pyruvate, Pi dikinase in maize leaves has been demonstrated (Hatch, 1979; Sugiyama et al., 1979; Burnell, 1990). Low-temperature-induced inactivation of CO_2 assimilation may be the metabolic basis for the acute sensitivity of maize leaves to cold-induced photoinhibition (Nie et al., 1992; Massacci et al., 1995; Verheul et al., 1995) and the associated acclimation of the photosynthetic apparatus (Koroleva et al., 1994; Fryer et al., 1995). Chloroplast development and repair are also impaired by cold (Long et al., 1987).

The enzymes of carbon assimilation have been found to be inhibited by exposure to low temperatures in chilling-sensitive plants such as tomato (*Lycopersicon esculentum*) (Sassenrath et al., 1990). Inhibition of Benson-Calvin cycle enzymes was found to closely mirror the chilling-induced inhibition of photosynthesis in tomato (Sassenrath and Ort, 1990; Sassenrath et al., 1990). The pool sizes of FBP and sedohepulose-1,7-bisphosphate increased in tomato leaves during chilling, and these increases appeared to be caused by direct inhibition of FBPase and sedohepulose-1,7-bisphosphatase activities. These are two regulatory enzymes of the Benson-Calvin cycle whose activity is regulated via thioredoxin-mediated reduction of specific sulfhydryl groups. The cold-inhibited enzymes could be extracted from whole tomato leaves and reactivated with DTT (Sassenrath et al., 1990). In this case the enzymes themselves did not appear to be damaged by chilling in the light. In addition, the midpoint potential of the regulatory sulfhydryl on FBPase was not modified (Hutchison, 1994). This led to the conclusion that these regulatory enzymes in the C_3 plants were inhibited in vivo by oxidation of their regulatory thiol groups. Similarly, bean (*Phaseolus vulgaris*), which is also chilling-sensitive, showed a weak inactivation of FBPase following chilling in the light (Holaday et al., 1992). In addition to the thiol-modulated enzymes, other proteins are also affected; for example, Brüggemann et al. (1992) showed that an irreversible decrease in tomato Rubisco activity followed long-term chilling.

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Abbreviations: Chl, chlorophyll; FBP, Fru-1,6-bisphosphate; FBPase, Fru-1,6-bisphosphatase; J_{PSII}, the index of electron transport through PSII and the index of linear electron transport; $J_{PSII(560\ nm)}$, same as J_{PSII} but relating specifically to estimates of J_{PSII} obtained using $\Phi_{PSII(560\ nm)}$; $J_{PSII(660\ nm)}$, same as J_{PSII} but relating specifically to estimates of J_{PSII} obtained using $\Phi_{PSII(660\ nm)}$; LED, light-emitting diode; MDH, malate dehydrogenase; PEPcase, PEP carboxylase; Φ_{PSII}, the quantum yield for PSII photochemistry; $\Phi_{PSII(560\ nm)}$, the quantum yield for PSII photochemistry determined specifically from measurements of chlorophyll fluorescence excited by 560 nm radiation; $\Phi_{PSII(660\ nm)}$, the quantum yield for PSII photochemistry determined specifically from measurements of chlorophyll fluorescence excited by 660 nm radiation.

The present study was undertaken to evaluate the operational state of the photosynthetic apparatus in maize leaves in vivo (via Chl *a* fluorescence, CO_2 assimilation, and the activities of component enzymes), in chilled and unchilled maize leaves at different irradiances, and to determine the primary site of damage to the photosynthetic apparatus. Chl *a* fluorescence measurements were used to calculate Φ_{PSII}. Comparisons of the relationships between Φ_{PSII} and CO_2 fixation with varying irradiance allow analysis of the operation and regulation of electron transport relative to CO_2 fixation and vice versa. The activity of NADP-MDH can be used as a physiological indicator of the redox state of the stroma of the mesophyll chloroplasts, where it is localized, because of the dependence of the activation state of this enzyme on the electron flow through the Fd-thioredoxin system and the status of the NADPH/NADP pool (Foyer et al., 1992; Foyer, 1993). Similarly, FBPase, which is located in the stroma of the bundle-sheath cells, is a hysteretic enzyme regulated by a supply of reducing equivalents from the electron transport system and by the availability of its substrate, FBP (Harbinson et al., 1989; Foyer, 1993). In conjunction with measurements of enzyme activities, the relationships between electron transport and photosynthetic metabolism can be elucidated for C_4 plants and compared with those observed in C_3 plants.

MATERIALS AND METHODS

All measurements were made on the fully expanded third leaf of maize (*Zea mays* L., line H99) plants (20–25 d old). Maize seeds were germinated in the dark at 25°C on washed vermiculite. Seedlings (5–7 d) were transplanted to 3.5-inch pots. The pots were placed in a growth cabinet maintained at 25°C in the light (1000 μmol m^{-2} s^{-1} PAR, 16-h photoperiod) and at 22°C during the 8-h dark period. Plants were watered automatically twice a day.

Chilling Procedure

At the start of the dark period, plants were placed in a prechilled growth cabinet at 4°C and 350 μmol m^{-2} s^{-1} PAR. Plants were kept at suboptimal conditions for 24 h after which they were returned to optimal growth conditions. Measurements were made on the plants during the first photoperiod after a return to optimal conditions.

Photometric Measurements

Chl fluorescence and light-induced A_{820} were measured as described previously (Harbinson et al., 1989, 1990; Foyer et al., 1992). Modulated Chl fluorescence was excited by radiation from LEDs (HLMP 8150, Hewlett-Packard) screened by a red filter (Walz, Effeltrich, Germany) and an array of green LEDs (peak emission 560 nm) filtered and modulated at a frequency different from that used for the excitation. The intensity of the LED excitation on the leaf surface was 0.8 μmol m^{-2} s^{-1} PAR. The modulated excitation for Chl fluorescence, the broad-band radiation to saturate the reduction of the primary quinone acceptor of the PSII pool, the weak far-red radiation to oxidize the fraction of the primary quinone acceptor pool of PSII that is reduced in the dark, and the actinic radiation were all carried to the leaf chamber by fiber optics. Actinic light was provided by a quartz halogen lamp filtered by near IR and Calflex dichroic mirrors (Balzers, Fuerstentum, Liechtenstein) and a metal film neutral density filter (Balzers) by which the irradiance at the leaf surface could be varied (measured by a quantum sensor, Li-Cor, Lincoln, NE). A GaAsP photodiode (G117, Hamamatsu, Bridgewater, NJ), screened by an RT-830 filter (3 mm thick, Schott, Mainz, Germany), was situated below the leaf and was used to detect the Chl fluorescence produced by both the red and green excitation beams. The composite Chl fluorescence signal was fed to two control demodulators, one of which was used to recover the fluorescence produced by the red excitation and the other to the fluorescence produced by the green excitation beam. Gas-exchange measurements (IR gas analyzer) were made simultaneously with measurements of Chl fluorescence.

The apparatus that was used for these measurements also contained a freeze-clamping system (for photographs and description of chamber assembly, see Foyer, 1993). This allows simultaneous measurements of photosynthetic activity, CO_2 assimilation, and analysis of metabolic status in terms of concurrent enzyme activities. Each data point was collected from a separate plant, enabling the assessment of trends within whole populations of maize seedlings. The variation observed in the figures, therefore, arises from a variation between the leaves within the populations studied. For each data point a single plant was removed from the growth chamber, and the second or third leaf was placed on the leaf chamber and dark-adapted for 5 min. Immediately prior to the start of actinic irradiation, the modulated measuring beam for Chl fluorescence was switched on and the actinic irradiation began. This lasted for between 20 to 30 min, until CO_2 fixation was constant. An intense broad-band actinic beam was then switched on (8000 μmol m^{-2} s^{-1} PAR) to saturate the yield of Chl fluorescence produced by the modulated red-light-measuring beam. From the change in the level of the yield of modulated Chl fluorescence produced by the addition of this intense radiation to the actinic background, the quantum efficiency for electron transport by PSII was calculated according to the method of Genty et al. (1989).

Leaf Freezing and Sample Preparation

The irradiated portion of the leaf was removed by forcing a brass cutter, previously chilled with liquid nitrogen, through the leaf such that irradiation was uninterrupted, as described by Foyer et al. (1992). The leaf disc was removed from the apparatus while still frozen and was stored at −80°C until further use.

Enzyme and Metabolite Measurements

NADP-MDH and FBPase were measured by a modification of a method described previously (Harbinson et al., 1990). Frozen leaf discs were ground in liquid nitrogen and

resuspended in 0.5 mL of a buffer containing 0.1 M Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 1 mM EDTA, 20% (v/v) glycerol, and either 1 mM DTT for determination of initial activity or 50 mM DTT for determination of maximal activities. Immediate FBPase and NADP-MDH activities were measured in these extracts. NADP-MDH was measured by following the oxidation of NADP in a 1-mL reaction mixture containing 0.1 M Tris-HCl (pH 7.9), 20 mM $MgCl_2$, 1 mM EDTA, 0.1 mM NADPH, 0.5 mM oxaloacetate, and 50 μL of leaf extract. FBPase activity was measured by the increase in A_{340} of a 1-mL reaction mixture containing 0.1 M Tris-HCl (pH 7.9), 20 mM $MgCl_2$, 1 mM EDTA, 0.2 mM NADP, 1 mM FBP, 17.5 units phosphoglucose isomerase, and 3.5 units of Glc-6-P dehydrogenase. Maximum activation of NADP-MDH and FBPase was measured after a 10-min incubation of the tissue extracted with 50 mM DTT. Rubisco activity was determined by the incorporation of $^{14}CO_2$ into an acid-stable product in a 1-mL reaction mixture containing 50 mM Bicine-NaOH (pH 8.0), 10 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (at 0.5 μCi μmol^{-1}), and 0.33 mM RuBP, as described by Parry et al. (1988). Maximal catalytic activity was determined in dark-adapted samples following incubation (5 min) with 10 mM $NaH^{12}CO_3$. PEPcase activity was determined by the incorporation of $H^{14}CO_3^-$ into an acid-stable product in a 1-mL reaction mixture containing 0.1 M Tris-HCl (pH 7.9), 20 mM $MgCl_2$, 1 mM EDTA, 0.4 mM NADH, 10 mM $NaH^{14}CO_3$ (at 5 μCi μmol^{-1}), 50 units of NADH-MDH, and 0.6 mM PEP, as described by Wong and Davies (1973).

Means and SES were calculated for all of the data. All statistical analyses and curve fitting were performed using FigP software (Biosoft, Cambridge, UK).

RESULTS

CO_2 Fixation

The fixation of CO_2 by unchilled maize leaves increased with increasing irradiance up to the maximum of 1600 $\mu mol\ m^{-2}\ s^{-1}$. At this irradiance CO_2 assimilation approached light saturation (Fig. 1). Following chilling there

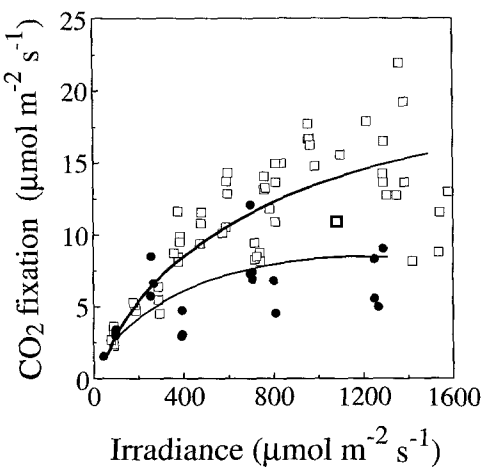

Figure 1. The irradiance dependency of CO_2 fixation for unchilled control (□) and chilled (●) maize plants.

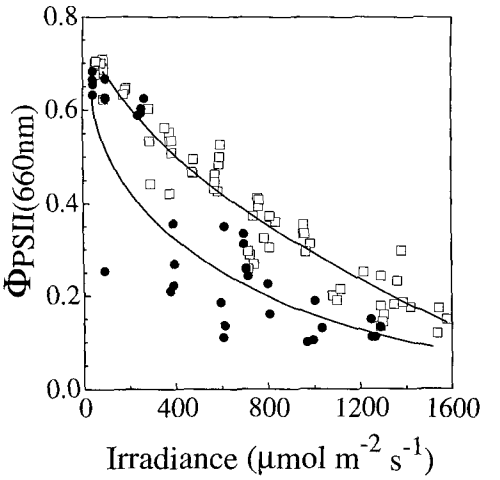

Figure 2. The relationship between $\Phi_{PSII(660\ nm)}$ and irradiance for unchilled control (□) and chilled (●) maize plants.

was no obvious effect on this relationship below about 200 $\mu mol\ m^{-2}\ s^{-1}$, suggesting no effect on the quantum yield of CO_2 fixation under light-limiting conditions and implying the absence of photoinhibition. At irradiances higher than 200 $\mu mol\ m^{-2}\ s^{-1}$ light saturation of CO_2 fixation was observed in the chilled leaves, with a maximal rate of photosynthetic CO_2 assimilation under defined conditions of irradiance in air between 5 and 10 $\mu mol\ m^{-2}\ s^{-1}$. At irradiances greater than 200 $\mu mol\ m^{-2}\ s^{-1}$ a small number of chilled leaves showed rates of CO_2 fixation comparable to that observed in the unchilled leaves. Therefore, it appears that, although the chilling treatment generally resulted in a loss of the maximal rate of photosynthetic CO_2 assimilation under defined conditions of irradiance in air, certain leaves were more resistant to chilling-induced inhibition (Fig. 1). Such a diversity of responses to stress-induced damage is frequently observed within populations of plants.

Quantum Efficiency for Photochemistry by PSII, the Relationship between Linear Photosynthetic Electron Transport and CO_2 Fixation

In unchilled maize leaves Φ_{PSII} decreased with increasing irradiance (Fig. 2). The relationship was clearly not linear, the nonlinearity being most apparent above about 1000 $\mu mol\ m^{-2}\ s^{-1}$. $\Phi_{PSII(660\ nm)}$ generally decreased more with increasing irradiance in chilled leaves than in the unchilled leaves, and the relationship between Φ_{PSII} and irradiance was more conspicuously curvilinear (Fig. 2). The heterogeneity of the response following chilling was greater than in the unchilled maize leaves.

The relationship between $\Phi_{PSII(560\ nm)}$ and $\Phi_{PSII(660\ nm)}$ was linear for both the unchilled and chilled plants (Fig. 3). Notably, the line fitted to the data did not pass through the origin, so, although both $\Phi_{PSII(660\ nm)}$ and $\Phi_{PSII(560\ nm)}$ decreased in parallel, $\Phi_{PSII(660\ nm)}$ was systematically more responsive than $\Phi_{PSII(560\ nm)}$.

The product of Φ_{PSII} and irradiance gives J_{PSII}, a value that is proportional to the rate of photosynthetic electron trans-

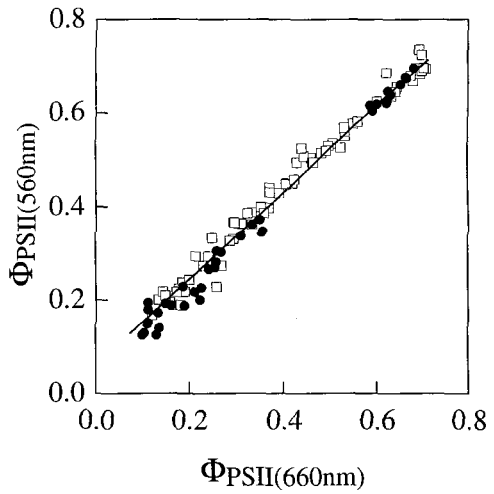

Figure 3. The relationship between $\Phi_{PSII(660\ nm)}$ and $\Phi_{PSII(560\ nm)}$ for unchilled control (□) and chilled (●) maize plants.

port through PSII, although it is not a measure of photosynthetic electron transport through PSII. Accurate determinations of PSII electron transport cannot be made unless the absorbance of light by the photosynthetic pigments and the distribution of excitation energy between PSI and PSII are known. Furthermore, the underestimation of Φ_{PSII} arises because of the contribution of Chl fluorescence from PSI to the initial fluorescence signal. Consequently, the product of Φ_{PSII} and irradiance is best described as an index of linear photosynthetic electron transport. When J_{PSII} was calculated from values of $\Phi_{PSII(660\ nm)}$, the relationship between J_{PSII} and CO_2 fixation was similar for both the unchilled and chilled leaves (Fig. 4a). A nonlinear relationship, with respect to the increasing yield of CO_2 fixation, was apparent as J_{PSII} increased. When J_{PSII} was estimated from $\Phi_{PSII(560\ nm)}$, the relationship between J_{PSII} and CO_2 fixation was also similar in both unchilled and chilled leaves (Fig. 4b) and was linear. Qualitatively, it is evident that the chilled leaves had lower values of both CO_2 fixation and J_{PSII} than did the unchilled leaves (Fig. 4), consistent with the effect of chilling on the CO_2 fixation/irradiance and Φ_{PSII}/irradiance relationships.

Effect of Chilling Maize on Activities of the Carboxylation Enzymes of the Mesophyll and Bundle Sheath

The activity of Rubisco was measured immediately after extraction (initial activity) and after activation by incubation with Mg^{2+} and CO_2 (total activity). In dark-adapted maize leaves the initial activities were 2.10 (±0.22) μmol min^{-1} mg^{-1} Chl for unchilled plants and 1.11 (±0.12) μmol min^{-1} mg^{-1} Chl for chilled plants. In vitro activation increased the activity to 3.64 (±0.37) μmol min^{-1} mg^{-1} Chl (unchilled plants) and 1.39 (±0.003) μmol min^{-1} mg^{-1} Chl (chilled plants). During the light period the maximal activated Rubisco activity increased to 4.84 (±0.64) μmol min^{-1} mg^{-1} Chl in the unchilled plants and the light-induced increase in the maximal activated Rubisco activity following chilling was 3.56 (±0.30) μmol min^{-1} mg^{-1} Chl. A pronounced effect of the chilling treatment on maize leaves was to increase the Rubisco activation state from 58% in unchilled plants to 80% in chilled plants. This suggests that an increased activation state compensates in part for a decrease in the total Rubisco activity following chilling.

Chilling had only a small effect on the response of Rubisco activity to increasing irradiance. In both chilled and unchilled plants illumination resulted in an increase in initial activity relative to that measured in the dark (Fig. 5a). Lines fitted to the two data sets deviated only at irradiances greater than 500 μmol m^{-2} s^{-1}, the chilled leaves tending to have slightly higher activities than leaves from the unchilled plants.

Similar to Rubisco, PEPcase activity was subject to light activation in maize leaves. In both the chilled and unchilled plants PEPcase activity increased when illumination reached a maximum at an irradiance of approximately 100 μmol m^{-2} s^{-1} (Fig. 5b). Fitted curves revealed that in leaves from unchilled plants increasing irradiance subsequently tended to result in a slight decrease in PEPcase activity. This was not as pronounced in leaves from the chilled plants. It is interesting that the fitted line for the

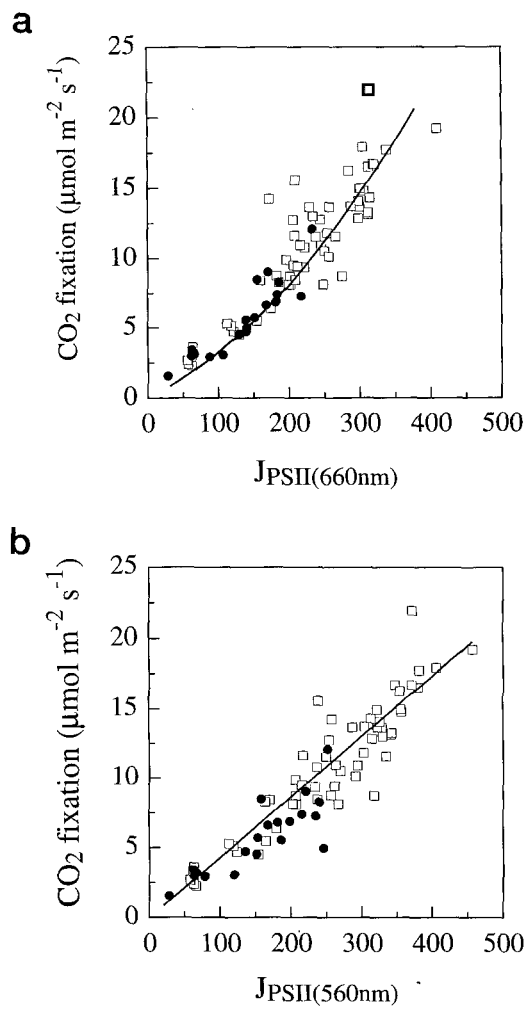

Figure 4. The relationship between $J_{PSII(660\ nm)}$ and CO_2 fixation (a) and $J_{PSII(560\ nm)}$ and CO_2 fixation (b) for unchilled control (□) and chilled (●) maize plants.

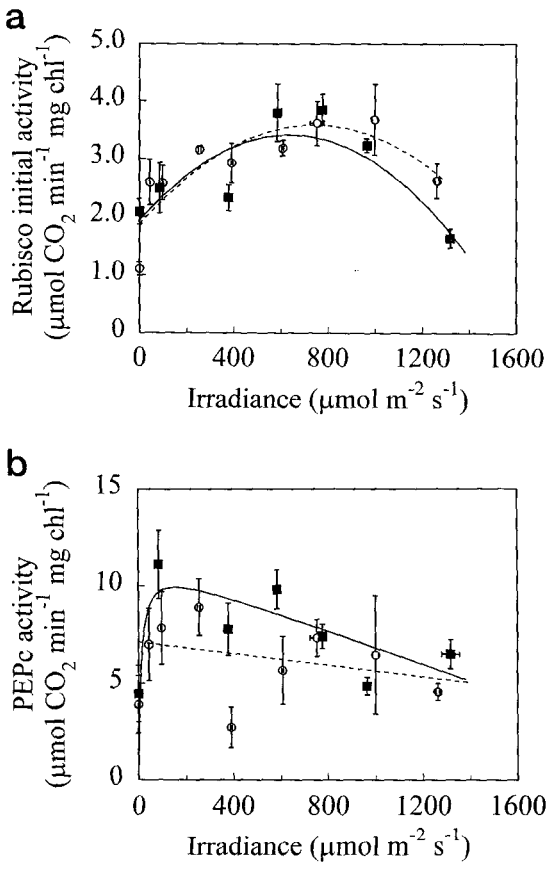

Figure 5. The relationship between Rubisco activity and irradiance (a), and PEPcase activity and irradiance (b) for unchilled (■) and chilled (○) maize plants.

chilled plants decreased below that of the unchilled control plants, suggesting that the PEPcase activity of the chilled leaves was less activated for a given irradiance than in the unchilled leaves. Only at higher irradiances were measured PEPcase activities similar for a given irradiance.

Effect of Chilling on Thiol-Regulated Stromal Enzymes

In dark-adapted plants the maximal catalytic activities (activated by incubation with DTT) of both FBPase and NADP-MDH were low. FBPase activities were 0.95 (±0.10) and 0.73 (±0.42) μmol min^{-1} mg^{-1} Chl for the unchilled and chilled leaves, respectively. NADP-MDH activities were 1.27 (±0.74) and 1.41 (±0.81) μmol min^{-1} mg^{-1} Chl for the unchilled and chilled leaves, respectively. For both enzymes, the initial activity in dark-adapted leaves represented approximately 5% of the total catalytic activity.

Even in low light (less than 100 μmol m^{-2} s^{-1}) FBPase activity was approximately 20-fold of that measured in dark-adapted leaves. In unchilled plants FBPase activity was relatively constant between 50 and 1500 μmol m^{-2} s^{-1} (Fig. 6a), corresponding to 40% of the maximal activation (1.22 μmol min^{-1} mg^{-1} Chl). In chilled plants, however, the maximum extractable activation was achieved at an irradiance of 150 μmol m^{-2} s^{-1}, after which the measured activity showed a progressive decrease. In contrast to the light response of PEPcase, FBPase activity in leaves of the chilled plants exceeded that of the unchilled controls over the range of irradiances used, and at higher irradiances similar activities were observed.

NADP-MDH activity increased markedly with increasing illumination, up to a maximum value at 750 μmol m^{-2} s^{-1} (Fig. 6b). At very low irradiances, the NADP-MDH activity of leaves from the chilled plants was slightly increased compared with the unchilled plants; the increased variability following chilling did not allow a more precise interpretation. Taken together, these results suggest that the stromal compartments of the mesophyll and bundle-sheath chloroplasts are more reduced following chilling in maize leaves. The variability of the data collected is probably intrinsic to maize. The activation states observed for FBPase (80%) and NADP-MDH (30%) extracted from unchilled maize were comparable to those previously observed with pea leaf extracts at similar irradiances (Harbinson et al., 1990).

DISCUSSION

The chilling-induced decrease in the capacity for CO_2 fixation observed in these experiments was not associated with any photoinhibition, but was paralleled by a pronounced loss of Φ_{PSII} with increasing irradiance. The chill-

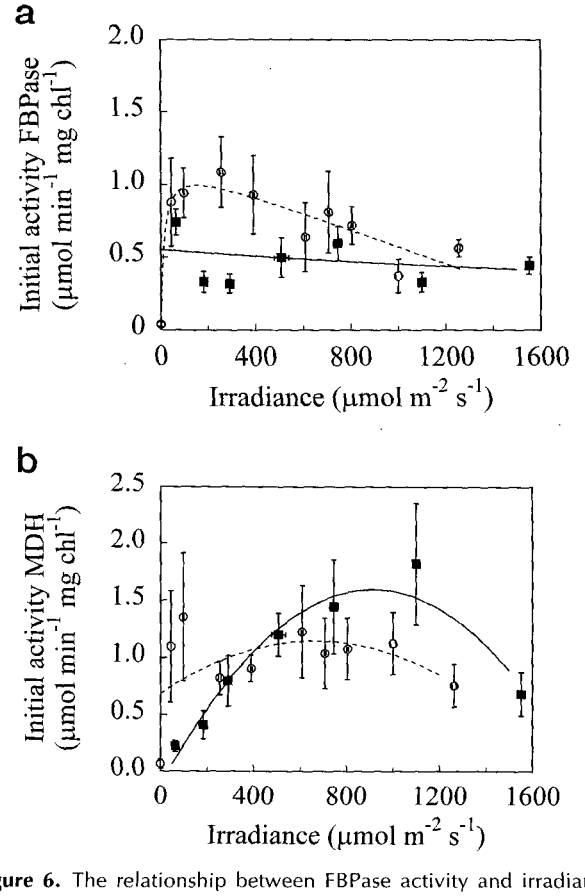

Figure 6. The relationship between FBPase activity and irradiance (a), and NADP-MDH activity and irradiance (b) for unchilled (■) and chilled (○) maize plants.

ing treatment did not induce a stoichiometric diversion of reducing equivalents away from CO_2 fixation to alternative electron acceptors, such as molecular O_2. These data show that the basic operation of C_4 photosynthesis remains qualitatively unaltered by the chilling treatment.

The measured relationships between either $J_{PSII(660\ nm)}$ or $J_{PSII(560\ nm)}$ and CO_2 fixation suggest that the effectiveness of electron transport in driving CO_2 assimilation increases as the rate of electron transport increases. The differences between the J_{560}/CO_2 assimilation relationship and the J_{660}/CO_2 relationship are not attributable to methodological problems but arise from physiological phenomena. At high Φ_{PSII} values, both 560 and 660 nm excitation wavelengths give identical Φ_{PSII} estimates. However, it is clear that at low Φ_{PSII} values measurements at 560 and 660 nm excitation wavelengths give different Φ_{PSII} estimates. The simplest explanation for this discrepancy is that the 560 nm excitation penetrates the leaf more deeply than does the 660-nm measuring beam (Nishio et al., 1994). Since the 660 nm excitation beam is more strongly absorbed by the leaf, this estimate of Φ_{PSII} will reflect the Φ_{PSII} of the upper layers of the leaf, which experience a higher irradiance than the lower leaf layers. The values obtained from the lower leaf layers will make a greater contribution to the overall estimate of Φ_{PSII} obtained when using the more penetrating 560 nm radiation. Since the excitation radiation is absorbed by the leaf to some degree, the choice of excitation wavelengths will therefore inevitably result in measurements of Φ_{PSII}, which are biased to some degree. The measurement of CO_2 fixation has no such bias. CO_2 fixation from any part of the leaf contributes equally to the total CO_2 fixation rate measured. The relative contribution of different layers of the leaf to the overall rate of CO_2 fixation will, however, be influenced by the penetration of actinic light (Nishio et al., 1994). This implies that the relationship between photosynthetic electron transport, derived from measurements of Chl fluorescence and CO_2 fixation, will be determined not only by the number of reducing equivalents required per CO_2 fixed, but also by the choice of the measuring wavelength used to excite Chl fluorescence and the actinic wavelengths used to drive photosynthesis. This possibility has major implications for the use of Chl fluorescence measurements as a quantitative tool with which to measure or analyze photosynthesis in vivo.

In optimal conditions the overreduction of the stroma is prevented by coordination of synthesis of ATP and NADPH with rates of use in carbon metabolism (Foyer et al., 1990, 1992). The differential compartmentation of photosynthetic and metabolic processes in maize leaves means that each compartment may perceive stress differently. The evidence presented here shows that the enzymes of the C_3 cycle are inhibited by chilling, since maximal extractable Rubisco activity was decreased by about one-third following the chilling treatment. Maximal extractable FBPase activity was also decreased following chilling. The loss of these enzymes after chilling in the dark indicates that the low-temperature treatment has induced changes in the enzyme proteins, such as decreased transcription, enhanced degradation, or, in the case of Rubisco, increased binding of inhibitors (Gutteridge et al., 1986; Edmondson et al., 1990; for discussion, see Portis, 1992). In the light, however, initial Rubisco activities of both chilled and unchilled plants were similar and show the same light response. Chilling therefore damages the Rubisco protein per se. The measured chilling-induced decrease in "activated" Rubisco activity was present in both the light and in the dark. In the light a metabolic compensation for this decrease was evident, the activation state (initial rate of Rubisco activity: maximum rate of activity after activation with Mg^{2+} and CO_2) of Rubisco in the chilled plants was increased relative to the unchilled controls following chilling. Hence, a greater proportion of the Rubisco active sites exists in the carbamylated form in chilled plants, indicative of up-regulation of metabolism in response to the continued functioning of the electron transport chain.

For many years it has been known that chilling inhibits the enzymes of the carboxylation phase of the C_4 cycle (Hatch, 1979; Sugiyama et al., 1979; Burnell, 1990). Extractable activities of PEPcase were lower in chilled maize leaves than in unchilled controls. PEPcase activity in chilled plants in vivo may be even less than observed in vitro because the substrate PEP will be diminished due to chilling damage to the cold-labile PPi-dikinase (Sugiyama et al., 1979; Burnell, 1990). The data suggest that the production and carboxylation of PEP are the most temperature-sensitive steps of photosynthesis in maize leaves.

Inhibition of CO_2 assimilation is the result of inactivation of both the C_4 and the C_3 cycles in maize leaves. The enzyme proteins per se appear to be damaged during chilling, since full activity cannot be restored by activation treatments. The effect of chilling in maize leaves is therefore clearly distinct from that observed in tomato (*Lycopersicon esculentum*) (Sassenrath et al., 1990). Relevant to this may be the recently observed effects of chilling on the circadian regulation of transcription (Martino-Catt and Ort, 1992). In tomato low- temperature treatment delays the progress of the circadian clock regulating the transcription of certain nuclear-encoded genes, including the Chl *a/b*-binding protein of PSII and Rubisco activase (Martino-Catt and Ort, 1992). Essentially, the clock stops for the duration of the chilling treatment and resumes upon rewarming. Unfortunately, the affected rhythms are then out of phase with the actual time of day. In chilling-tolerant species such as spinach (*Spinacea oleracea*), such rhythms do not appear to be affected by a low-temperature treatment (Ort et al., 1989). Chilling must therefore perturb the normal turnover of proteins. Furthermore, proteins are damaged by oxidation during exposure to stress and proteases are inhibited by oxidants; thus, damaged proteins with impaired functional activities may accumulate (Stadtman and Oliver, 1991). In maize seedlings exposed to 4°C pronounced accumulation of oxidized proteins was found. Whether this was caused by protease inactivation or decreased protease gene expression was unresolved (Prasad, 1996).

NADP-MDH is modulated by reversible thiol reduction via the Fd-thioredoxin system. Low-NADP-MDH activities in darkened leaves indicate that the mesophyll stroma is

relatively oxidized. NADP-MDH activity increases as the flux of reducing equivalents through PSI increases with increasing irradiance. In air a linear relationship has been demonstrated between the activation states of both NADP-MDH or FBPase, and the flux of electrons through the photosystems (Harbinson et al., 1990). A progressive increase in the redox state of the stroma with increasing irradiance occurs in maize, in agreement with previous observations in pea leaves (Foyer, 1992; Foyer et al., 1992). At high irradiances, NADP-MDH activity tends to decrease, indicating a limitation of noncyclic electron flow. In contrast to NADP-MDH, FBPase activity is regulated by both the supply of reducing equivalents and substrate. In maize PSII is entirely mesophyll located, with PSI being distributed between the mesophyll and bundle sheath. The activation state of FBPase in maize nevertheless mirrors the activation in C_3 plants (Foyer et al., 1990; Harbinson et al., 1990). Cooperativeness between compartments means reducing equivalents are sufficiently available in the bundle sheath for FBPase activation. This supply is not perturbed by chilling, suggesting that the plasmodesmata and membrane-transport systems continue to function despite the chilling treatment. Whereas the incorporation of HCO_3^- into organic acids is perturbed by chilling, the supply of NADPH by the mesophyll electron transport chain is unchanged from that in unchilled plants. Differences in the activation state of FBPase may be due to changes in the FBP supply.

Enzyme stability per se may be the critical factor relating to chilling perturbations to the mesophyll and bundle-sheath stromal enzymes. Unchilled plants show similar activation characteristics to those previously reported for pea in response to increasing irradiance (Harbinson et al., 1990; Harbinson and Foyer, 1991). Chilled maize leaves show remarkably little change in response to irradiance, although somewhat higher activities are detected in chilled, rather than in unchilled, plants at low irradiances. This would suggest that after chilling the stroma is more reduced at low irradiance than at high irradiances. The results presented here suggest that co-regulation between the electron transport chain and CO_2 assimilation in maize is a robust process. Indeed, the increased activation of enzymes following chilling suggests that the system senses a decreased capacity for CO_2 assimilation and attempts to redress the balance via an increased activation of component enzymes.

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