Cold Stress Effects on PSI Photochemistry in *Zea mays*: Differential Increase of FQR-Dependent Cyclic Electron Flow and Functional Implications

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Cold-induced inhibition of CO₂ assimilation in maize (*Zea mays* L.) is associated with a persistent depression of the photochemical efficiency of PSII. However, very limited information is available on PSI photochemistry and PSI-dependent electron flow in cold-stressed maize. The extent of the absorbance change ($\Delta A_{280}$) used for in vivo quantitative estimation of photooxidizable P700⁺ indicated a 32% lower steady-state oxidation level of the PSI reaction center P700 (P700⁻) in cold-stressed compared with control maize leaves. This was accompanied by a 2-fold faster re-reduction rate of P700⁺ in the dark, indicating a higher capacity for cyclic electron flow (CEF) around PSI in cold-stressed maize leaves. Furthermore, the increased PSI-dependent CEF(s) was associated with a much higher stromal electron pool size and 56% lower capacity for state transitions compared with control plants. To examine NADP(H) dehydrogenase (NDH)- and ferredoxin:plastoquinone oxidoreductase (FQR)-dependent CEF in vivo, the post-illumination transitory increase of $F_o$ was measured in the presence of electron transport inhibitors. The results indicate that under optimal growth conditions the relatively low CEF in the maize mesophyll cells is mostly due to the NDH-dependent pathway. However, the increased CEF in cold-stressed plants appears to originate from the up-regulated FQR pathway. The physiological role of PSI down-regulation, the increased capacity for CEF and the shift of preferred CEF mode in modulating the photosynthetic electron fluxes and distribution of excitation light energy in maize plants under cold stress conditions are discussed.

**Keywords:** Cold stress • Cyclic electron flow • P700 • PSI photochemistry • State transitions • *Zea mays* L.

**Abbreviations:** AL, actinic light; AntA, antimycin A; CEF, cyclic electron flow; ETR, electron transport rate; FNR, ferredoxin NADP+ oxidoreductase; FQR, ferredoxin:plastoquinone oxidoreductase; FR, far red; LHC, light-harvesting complex; MT, multiple turnover; NDH, NADP(H) dehydrogenase; NPQ, non-photochemical quenching; PQ, plastoquinone; Q₀ primary electron-accepting quinone in PSII reaction centers; ROS, reactive oxygen species; ST, single turnover; WWC, water–water cycle.

**Introduction**

The general physiological response of maize plants to low temperatures is an inhibition of photosynthetic CO₂ assimilation (Greer and Hardacre 1989, Kingston-Smith et al. 1997, Fryer et al. 1998, Foyer et al. 2002, Savitch et al. 2009). Cold-induced limitations on photosynthesis, i.e. a decrease in the photochemical use of absorbed light energy and the imbalance between the reducing equivalents produced in excess and the consumption capacity of photosynthesis, can induce the production of potentially dangerous reactive oxygen species (ROS) (Baker 1994). This may lead to photoinhibition of photosynthesis (Powles 1984, Baker 1994), and increased susceptibility to photoinhibition has been well established during exposure of photosynthetic organisms to low temperatures in combination with even moderate photon flux densities (Oquist and Martin 1986, Greer et al. 1986, Greer 1990, Osmond 1994). PSII has been identified as the primary target of photoinhibition resulting in photooxidative damage of the D1 reaction center polypeptide of PSII (Krause 1988, Arp et al. 1993, Long et al. 1994). Indeed, decreased abundance and photodamage of the D1 protein have been reported in maize plants exposed to suboptimal temperatures (Nie et al. 1992, Baker 1994, Kingston-Smith et al. 1999, Savitch et al. 2009). It has been suggested that the persistent depression of photosynthetic efficiency in cold-acclimated
maize may also result from the higher proportion of inactive PSII centers and a lower capacity for repair and/or replacement of damaged PSII centers (Fryer et al. 1995).

In addition to PSII photoinhibition, selective inhibition of PSI-related photochemical activities under low temperatures and either high light or moderate/weak illumination in chilling-sensitive plants has been reported (Terashima et al. 1994, Sonoike and Terashima 1994, Ivanov et al. 1998, Sonoike 1999, Scheller and Haldrup 2005). It was demonstrated that exposure of higher plants to low temperatures under moderate to high light results in reduced maximum quantum yield of electron transport through PSI, the pool of photooxidizable reaction center pigment of PSI (P700) and the efficiency of P700 oxidation (Havaux and Davaud 1994, Sonoike and Terashima 1994, Terashima et al. 1994, Ivanov et al. 1998.

Decreased levels of electron paramagnetic resonance (ER)-detectable P700 (Gode et al. 1992, Ivanov et al. 1998) and inhibition of PSI electron transport (Herrmann et al. 1997) were also shown to occur in vivo under high light. Chilling and concomitant oxidative stress have been implicated as major requirements for the PSI photoactivation in vivo (Sonoike 1996).

Despite this, the effects of low temperatures on PSI and PSI-related activities in maize are very limited and somewhat controversial. It has been demonstrated that exposure to low and/or suboptimal temperatures decreased the abundance of the PSI reaction center psaB gene product (Kingston-Smith et al. 1999) and increased the rate of PSI-dependent cyclic electron transport (Ducruet et al. 2005). In other studies, no changes or even an increase in the PSI activity in maize plants subjected to low temperatures was reported (Holá et al. 2007, Kočová et al. 2009).

In the present study, P700 and fluorescence measurements were used to assess the effects of low temperature stress on the efficiency of P700 photooxidation, the contribution of alternative electron transport pathways to P700$^+$ reduction and their effects on the capacity for state transitions in maize plants exposed to cold temperatures. The results in this report indicate that in addition to the well established down-regulation of PSII, cold-stressed maize exhibits a lower photooxidation of PSI, increased capacity for PSI-dependent cyclic electron flow (CEF) and a shift of the NADP(H) dehydrogenase (NDH)-dependent CEF under normal conditions to ferredoxin: plastoquinone oxidoreductase (FQR)-dependent CEF preferred in maize plants exposed to suboptimal temperatures.

### Results

As reported previously (Greer and Hardacre 1989, Foyer et al. 2002, Savitch et al. 2009) exposure of maize plants to a cold temperature of 15°C for 3 d produced 43% lower (0.44 ± 0.01) photochemical efficiency of PSII measured as $F_{v}/F_{m}$ compared with the control plants (0.76 ± 0.01) (Table 1). Concomitantly, the linear electron transport rate (ETR) was also strongly reduced, whereas the excitation pressure ($1 - q_{P}$), which reflects the redox state of the plastoquinone (PQ) pool and the intersystem electron transport chain (Gray et al. 1996, Kramer et al. 2004), and the capacity for non-photochemical quenching (NPQ) were increased in cold-stressed plants. However, low measuring temperature had only a minimal effect on $F_{v}/F_{m}$ indicating that the maximal photochemical efficiency of PSII is not affected in control plants. This indicates that the decreased ETR and increased excitation pressure ($1 - q_{P}$) caused by lowering the measuring temperature is mostly due to thermodynamic constraints on CO$_2$ fixation in control plants (Miskiewicz et al. 2002). In contrast, the lower ETR and higher excitation pressure in cold-stressed plants could be assigned not only to limitations on CO$_2$ fixation, but also to damage in the functional integrity of PSI reaction centers. In addition, the initial (prompt) Chl fluorescence ($F_{0}$) values were also increased by 50% in maize leaves exposed to cold stress (Table 1). This cold stress-induced rise in $F_{0}$ may indicate some structural changes in the photosynthetic apparatus in maize plants exposed to low temperatures (Schreiber and Armond 1978, Ivanov et al. 1992).

Further data concerning the response of the photosynthetic apparatus to cold stress treatment at the level of the supramolecular organization of thylakoid membranes were obtained from 77 K Chl fluorescence measurements (Krause and Weis 1991). Low temperature (77 K) fluorescence emission spectra of maize chloroplasts (Fig. 1) exhibited typical fluorescence maxima characteristic for PSII (685 and 695 nm) and PSI (735–740 nm) emissions (Krause and Weis 1991). Although the emission spectra indicate that the peak positions of various spectral components were identical in both control and cold-stressed plants, thylakoid membranes isolated from cold-stressed maize leaves indicated a lower intensity of PSII-associated emission peaks within the 680–700 nm spectral region and an increased PSI peak as compared with control (Fig. 1A). The fluorescence difference spectra obtained by subtracting the trace of cold-stressed from the trace of control chloroplasts emphasized reproducible differences (Fig. 1B). Three positive contributions peaking at 683, 694 and 718 nm

### Table 1: Photochemical efficiency of PSII ($F_{v}/F_{m}$), electron transport rate (ETR), excitation pressure ($1 - q_{P}$), non-photochemical quenching (NPQ), initial fluorescence ($F_{0}$) and quenching coefficient of $F_{v}$, ($1 - q_{o}$) in cold-stressed maize plants measured at 25°C and cold-stressed (15°C, 3 d) plants measured at 15°C

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (25°C)</th>
<th>Cold-stressed (15°C)</th>
<th>Control (15°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_{v}/F_{m}$</td>
<td>0.762 ± 0.010</td>
<td>0.442 ± 0.014</td>
<td>0.746 ± 0.015</td>
</tr>
<tr>
<td>ETR</td>
<td>107.0 ± 3.5</td>
<td>36.8 ± 3.0</td>
<td>54.7 ± 2.9</td>
</tr>
<tr>
<td>$1 - q_{P}$</td>
<td>0.209 ± 0.010</td>
<td>0.421 ± 0.028</td>
<td>0.539 ± 0.023</td>
</tr>
<tr>
<td>NPQ</td>
<td>0.469 ± 0.059</td>
<td>0.565 ± 0.041</td>
<td>0.877 ± 0.090</td>
</tr>
<tr>
<td>$1 - q_{o}$</td>
<td>1.04 ± 0.01</td>
<td>0.81 ± 0.01</td>
<td>0.90 ± 0.02</td>
</tr>
<tr>
<td>$F_{0}$</td>
<td>324.5 ± 1.6</td>
<td>493.4 ± 5.1</td>
<td>419.8 ± 7.3</td>
</tr>
</tbody>
</table>

Mean values ± SE were calculated from three replicate measurements in three independent experiments ($n = 9$).
were detected. Two negative contributions were also detected at 725 and 742 nm. These differences could be attributed to the lower abundance of PSII-related polypeptides in cold-stressed plants (Savitch et al. 2009) and/or cold stress-induced redistribution of the excitation light in favor of PSI (Baker et al. 1983).

In addition to the effects on PSII photochemistry, there are a number of studies indicating that PSI is also affected by chilling temperatures, especially in plants cultivated in temperate and/or warm climates (Sonoike 1999, Allen and Ort 2001, Scheller and Haldrup 2005). The extent of PSI photooxidation (P700⁺) was assessed in vivo by measuring the extent of far-red (FR) light-induced absorbance change at 820 nm (ΔA820) (Schreiber et al. 1988, Klughammer and Schreiber 1991, Ivanov et al. 1998), which reflects the oxidation of P700 to P700⁺. The traces illustrating the FR light-induced P700 transients presented in Fig. 2 and the data summarized in Table 2 indicate 32% lower capacity for P700 photooxidation (ΔA820 in control (A) and cold-stressed (3 d, 15°C) maize leaves (B). After reaching a steady-state level of P700 oxidation (P700⁺) by far-red (FR) illumination, single turnover (ST) and multiple turnover (MT) flash pulses of white saturating light were applied.

Table 2 Effects of cold stress (15°C, 3 d) on the far-red light-induced steady-state oxidation of P700 (ΔA820, P700⁺), intersystem electron pool size (e⁻/P700) (MT AREA/ST AREA), reduction kinetics of P700⁺ (t½ P700⁺) and the effect of DCMU (20 µM) on the white light-induced oxidation of P700 (ΔA DCMU/ΔA) in maize leaves

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control plants</th>
<th>Cold-stressed plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔA820 (P700⁺)</td>
<td>65.8 ± 7.3</td>
<td>45.4 ± 3.2</td>
</tr>
<tr>
<td>e⁻/P700 (MT AREA/ST AREA)</td>
<td>10.2 ± 1.3</td>
<td>14.8 ± 1.1</td>
</tr>
<tr>
<td>t½ P700⁺ (ms)</td>
<td>1096.0 ± 80.2</td>
<td>632.5 ± 56.3</td>
</tr>
<tr>
<td>Stromal e⁻ pool/P700</td>
<td>198.5 ± 24.1</td>
<td>1086.5 ± 102.2</td>
</tr>
<tr>
<td>ΔA DCMU/ΔA (%)</td>
<td>164.1 ± 5.9</td>
<td>119.8 ± 3.0</td>
</tr>
</tbody>
</table>

All measurements were performed at the growth/treatment temperatures of 25 or 15°C, respectively. Mean values ± SE were calculated from four replicate measurements in three independent experiments (n = 12).

Fig. 2 Typical traces of light-induced P700 transients measured as ΔA820 in control (A) and cold-stressed (3 d, 15°C) maize leaves (B). After reaching a steady-state level of P700 oxidation (P700⁺) by far-red (FR) illumination, single turnover (ST) and multiple turnover (MT) flash pulses of white saturating light were applied.

Determination of the apparent size of the electron donor pool to PSI was performed by measuring the flash-induced ΔA820 under steady-state oxidation of PSI by FR light (Asada...
et al. 1992, Asada et al. 1993, Gray et al. 1998). A typical cycle of illumination representing the P700 transients upon application of single turnover (ST) and multiple turnover (MT) saturating pulses of white light are demonstrated in Fig. 2A. The rapid, transient reduction of P700$^+$ to P700 in the presence of FR light in response to ST or MT flashes is attributed to the flow of electrons generated in PSII and the electron transport capacity between PSII and PSI (Asada et al. 1992, Asada et al. 1993). Indeed, the reduction of P700$^+$ induced by ST and MT flashes was completely inhibited in leaf disks infiltrated with a 20 μM solution of DCMU (data not shown). The ST flash of white light caused a partial reduction of P700$^+$ while the MT flash, as expected, resulted in almost complete reduction of P700$^+$ in control plants. However, the relative response of P700$^+$ reduction to ST and MT flashes was much lower in cold-stressed leaves (Fig. 2B) and the estimates of e$^-/P700$ from several independent experiments indicate a 70% larger electron donor pool to PSI (e$^-/P700$) relative to control plants (Table 2). This was associated with a 5.5-fold higher estimate of the stromal electron pool size in cold-stressed relative to control leaves (Table 2).

When the FR light was turned off, the kinetics of the subsequent P700$^+$ reduction in the dark are presumed to reflect primarily the rates of cyclic electron transport around PSI (Maxwell and Biggins 1976, Ravenel et al. 1994) and/or the interaction of stromal components with the intersystem electron transport chain (Asada et al. 1992). The rates of P700$^+$ reduction in the dark ($t_{1/2}$P700$^+$red) were 40% faster in maize leaves exposed to cold stress (Table 2), indicating an increased capacity for CEF around PSI and/or increased interaction between stromal electron donors and intersystem electron transport.

The effects of cold stress on the contribution of linear electron transport from PSII to the redox state of P700 was estimated by measuring the extent of steady-state P700 photooxidation induced by white actinic light (AL) in the absence or presence of DCMU (Fig. 4). Under white AL excitation in the absence of DCMU, electrons derived from PSII continuously reduced P700$^+$, resulting in a relatively low steady-state content of P700$^+$ in both control and cold-stressed maize leaves (Fig. 4, solid lines). Preventing the electron donation from PSII in the presence of the PSII inhibitor DCMU caused a 64% increase of the steady-state P700$^+$ (Fig. 4A, dashed line; Table 2) in control plants, suggesting that electrons available for P700$^+$ reduction originated predominantly from PSII and were delivered to P700 via intersystem electron transport. However, cold-stressed maize leaves exhibited a 3.3-fold (19.8%) lower increase of P700$^+$ in the presence of DCMU (Fig. 4B, dashed line; Table 2) compared with the control plants. This indicates that a significant fraction of electrons available for P700$^+$ reduction must have been derived from sources other than PSII and that the contribution of PSII as an electron donor to PSI was limited under cold-stressed conditions.

The apparent lower oxidation level of P700 under FR and the larger intersystem electron donor pool size in cold-stressed maize leaves might also be due to a rapid donation of electrons to P700$^+$ from stromal or cytosolic substrates (Asada et al. 1992, Mi et al. 1992, Asada et al. 1993). Maize mesophyll chloroplasts have been reported to contain a pool of >200

![Fig. 3](#) Representative Western blots of SDS–PAGE-separated polypeptides of mesophyll thylakoid membranes isolated from control (25°C) and cold-stressed (15°C for 3 d) maize leaves probed with antibodies raised against Lhca1–Lhc4, PsaA and FNR. An equal amount of protein (20 μg) was loaded on each lane.
Electrons originating from stromal reductants generated at the acceptor side of PSI that can be donated to the intersystem PQ pool (Asada et al. 1993). Furthermore, the influx of stromal electrons can modulate the redox state of the PQ pool (Hohmann-Marriott et al. 2010). The extent of P700 photooxidation was estimated at the growth temperature of 25°C. Representative traces of five independent experiments are shown.

When oxygen was removed from the measuring media, a fluorescence increase of \( F_o' \) in the absence \( O_2 \) (Fig. 5D), although much lower than that in control plants. This indicates that in cold-stressed plants the higher electron donation into the intersystem electron transport chain is less oxygen dependent and the over-reduction of the intersystem electron transport chain might not originate from respiratory-dependent stromal electron flow.

Eliminating NDH-mediated electron flow from stromal electron donors by inhibiting the NDH complex with \( \text{HgCl}_2 \) (Mi et al. 1992, Ivanov et al. 2000) had a dramatic effect in control plants and almost completely abolished the post-illumination fluorescence increase of \( F_o' \) (Fig. 5E). In contrast, \( \text{HgCl}_2 \) poisoning of cold-stressed leaves had a much lower effect and \( F_o' \) still increased after light-to-dark transition to levels above \( F_o \) (Fig. 5F). This suggests that other pathway(s) for electron flow are also involved in over-reducing the intersystem electron transport chain in cold-stressed leaves. Indeed, inhibiting the antimycin A (AntA)-dependent CEF completely eliminated the electron flow, thus preventing an over-reduction of the intersystem electron transport chain in cold-stressed maize leaves (Fig. 5H).

State 1–state 2 transitions, measured as \( \Delta F \) (Lunde et al. 2000), represent a short-term mechanism to balance the energy distribution between PSII and PSI (Allen et al. 1981, Lunde et al. 2000). In the present study, state transitions were measured in vivo following the differential changes of room temperature Chl fluorescence from PSI by alternatively changing the excitation AL from light preferentially exciting PSII (blue) to light exciting PSI (FR) (Lunde et al. 2000, Ivanov et al. 2006a, Ivanov et al. 2006b). As expected, Chl fluorescence transients (Fig. 6A) in response to the presence and absence of PSI light in maize plants indicated relatively high capacity for state transitions (\( \Delta F = 17.1 \)) when grown under optimal (control) conditions (Table 3). In contrast, cold-stressed plants exhibited 2.2-fold lower capacity for state transitions (Fig. 6B, Table 3) compared with control plants.

Eliminating oxygen as an electron acceptor by displacing it in the presence of nitrogen sharply increases the capacity for state transitions and eliminates the difference in \( \Delta F \) between control and cold-stressed plants. However, while this increase was 2-fold for control leaves, cold-stressed leaves demonstrated a 4.3-fold increase in \( \Delta F \) (Table 3). This indicates that respiratory processes and alternative electron sources to the intersystem electron transport or \( O_2 \)-dependent alternative electron sinks are more involved in modulating the capacity for state transitions under cold-stressed conditions.

**Discussion**

In agreement with our previous report (Savitch et al. 2009) and earlier studies (Greer and Hardacre 1989, Hayden and Baker 1990, Kingston-Smith et al. 1997, Fryer et al. 1998, Foyer et al. 2002), exposure of maize plants to 3 d cold stress caused a significant decrease in the photochemical efficiency of PSII
(Fv/Fm) and the ETRs (Table 1). This was accompanied by a significant relative decrease in the intensity of PSII-related 77 K Chl fluorescence emission peaks compared with the PSI peak in cold-stressed plants (Fig. 1). Similar earlier observations have been discussed in terms of major modification of the structure and/or energetics of LHCII during chilling (Hayden et al. 1986) and/or alterations in the excitation energy distribution between the major Chl–protein complexes of PSII and PSI (state transitions) excessively favoring PSI excitation within the photochemical apparatus of the thylakoid membranes (Baker et al. 1983).

Interestingly, our in vivo measurements of state transitions presented in this study fail to show higher capacity for state transition in cold-stressed plants (Fig. 6, Table 3). On the contrary, the capacity for short-term regulation of the energy distribution between PSII and PSI (Allen et al. 1981, Allen 1992, Kargul and Barber 2008) was 2.2-fold lower in maize exposed to low temperatures. We propose that the observed cold
The measurements were performed under ambient CO2 and O2 or in the presence of only blue light and the maximum fluorescence yield in state 2 (Fm/2) was determined. The authors concluded that decreased abundance and photoinhibitory damage of the D1 protein (Nie et al. 1992, Baker 1994) is one of the major factors for the persistent depression of photosynthetic efficiency in maize plants exposed to suboptimal temperatures (Ortiz-Lopez et al. 1990, Kingston-Smith et al. 1997, Fryer et al. 1998). However, while the PSI efficiency was 42% lower, the linear ETR was decreased 3-fold in cold-stressed maize (Table 1). This implies that additional cold-induced limitations other than PSII are also involved in the >2-fold decrease in CO2 assimilation (Savitch et al. 2009) and ETRs (Table 1).

The present study demonstrates that not only PSII, but also PSI photochemistry is down-regulated, although to a lower extent (32%) in cold-stressed maize (Fig. 2, Table 2). The effects of low temperatures on PSI and PSI-related activities in maize have never been studied in detail, but the lower P700 photo-oxidation reported here is in agreement with the decreased abundance of PSI reaction center psaB gene product reported in maize exposed to suboptimal temperatures (Kingston-Smith et al. 1999). Previous studies have also demonstrated lower PSI photochemistry in photosynthetic organisms exposed to cold stress or acclimated to low temperatures (Ivanov et al. 1998, Savitch et al. 2001, Miskiewicz et al. 2002, Morgan-Kiss et al. 2002). The lower extent of P700 oxidation (P700+) was not accompanied by quantifiable photoinhibitory damage of the PSI complex under the growth stress conditions, although the abundance of the PsA protein complex was 10% lower in cold-stressed plants (Fig. 3) and they were more susceptible to photoinhibition when exposed to high light stress (data not shown). Thus, it may not be photoinhibition of PSI complexes per se (Sonoike 1999, Scheller and Haldrup 2005), but rather PSI acceptor side limitation, i.e. limitation of electron sink pathways such as CO2 assimilation (Kim et al. 2005), that is the major factor for the decrease in P700+ under cold stress conditions in maize.

The altered PSII/PSI stoichiometry would induce highly imbalanced linear electron flow, which may result in an enhancement of cyclic over non-cyclic electron transport (Baker et al. 1983). As expected, the decrease in P700+ was accompanied by faster rates of P700+ reduction in the dark, which is indicative for up-regulation of CEF (Maxwell and Biggins 1976, Ravenel et al. 1994) in cold-stressed maize leaves. Earlier observations have also indicated up-regulated CEF in maize exposed to low temperatures (Ducruet et al. 2005). This up-regulated CEF flow is consistent with the necessity to compensate the impaired linear electron transfer to reach the optimal ATP/NADPH ratio of 3 in NADP-malic enzyme-type C4 mesophyll cells, such as maize (Edwards and Walker 1983, Takabayashi et al. 2005).

PSI-driven CEF produces ATP but no NADPH (Arnon 1959, Bendall and Manasse 1995) via at least two partially redundant NDH-dependent and FQR(PGRS)-dependent, AntA-sensitive cyclic electron transport pathways (for reviews, see Bukhov et al. 2005).
and Carpentier 2004, Shikanai 2007). While the FQR(PGR5)-dependent pathway is a major PSI-driven CEF in C₃ plants (Munekage et al. 2002, Shikanai 2007), the NDH-dependent pathway is the main contributor of the extra ATP required to concentrate CO₂ in bundle sheath cells of C₄ plants (Takabayashi et al. 2005). Indeed, higher abundance of NAD(P)H-dehydrogenase/NAD(P)H-plastoquinone oxidoreductase (NDH complex) was reported in bundle sheath compared with mesophyll cells of C₄ plants (Kubicki et al. 1996, Darie et al. 2005). However, it should be noted that whereas the amount of NDH complex depended on the cell type, the expression of pgr5 transcripts and the accumulation of PGR5 protein was similar in maize bundle sheath and mesophyll cells (Takabayashi et al. 2005).

The results presented in this study clearly indicate a differential response to inhibitors specific for the NDH- and FQR(PGR5)-dependent cyclic electron transport pathways in control and cold-stressed maize leaves (Fig. 5). While a post-illumination increase of F₅₀ in plants acclimated to optimal temperature was sensitive mostly to the inhibitor of the NDH complex, cold-stressed plants were sensitive predominantly to AntA, a specific inhibitor of the FQR(PGR5) pathway. It has been suggested that in addition to its major role in catalyzing the reduction of NADP⁺ in the linear electron flow, FNR could be involved in the reverse electron flow from NADPH to ferredoxin (Jin et al. 1994, Friedrich et al. 1995) and may be involved in modulating the activity of PSI-dependent CEFs (Bojko et al. 2003). The localization of FNR at Cyto/f (Clark et al. 1984; Zhang et al. 2001) also implies its role in cyclic electron transport. The observed 24% increase in the relative abundance of FNR (Fig. 3) supports the suggestion of up-regulated ferredoxin-dependent (FQR) CEF in cold-stressed maize leaves. Thus we report for the first time a shift between two alternative CEF pathways in maize mesophyll cells exposed to cold stress conditions.

The physiological significance of the observed shift of the preferred CEF mode of operation from NDH-dependent under normal conditions to PGR5 dependent under cold stress conditions would be important in balancing the photosynthetic electron fluxes and distribution of excitation light energy under contrasting temperature regimes. It has been suggested that although the two CEF pathways are partially redundant, each of them may fulfill different functions: the NDH-dependent pathway may serve predominantly as a safety valve that prevents over-reduction of the stroma, while the PGR5-dependent PSI CEF is essential and mostly involved in maintaining the correct production ratio of ATP/NADPH (Shikanai 2007). During growth and development of maize plants under optimal temperature conditions the PQ pool remains preferentially oxidized because the rate of consumption of photosynthetic electrons through metabolic sinks (carbon fixing reactions) keeps pace with the rate at which PSII undergoes charge separation to reduce the PQ pool. Under these conditions the linear photosynthetic electron flow from PSII (water splitting) to PSI (NADP⁺ generation) within the photosynthetic apparatus of mesophyll cells dominates and is sufficient to sustain the optimal energetic requirement for CO₂ assimilation (Edwards and Walker 1983, Takabayashi et al. 2005). In addition, two partially redundant PSI-dependent cyclic electron transport pathways, NDH dependent and FQR(PGR5) dependent, might also be involved. Our results suggest that the electron pool size available from stromal components in maize mesophyll cells is much larger than in C₃ plants (Asada et al. 1993, Table 2) and although cyclic electron transport activity is low in maize under normal growth conditions the NDH-dependent pathway predominates and would be involved in preventing over-reduction of the stroma by channeling excess electrons to the intersystem electron transport.

Exposure of maize plants to cold stress results in lower demand for electrons required for carbon fixing reactions. Under cold stress conditions imposing limitations at the acceptor site of PSI, the excitation pressure over PSI increases and the PQ pool becomes predominantly reduced. The excess electrons can be diverted from the linear electron flow and utilized through the Mehler–ascorbate peroxidase pathway [the water–water cycle (WWC)] (Asada 1999) and other oxygen-dependent electron sinks generating ROS and/or re-circulated by the NDH- and/or FQR-dependent PSI-dependent cyclic electron transport pathways. The back electron flow over-reduces the primary electron acceptor of PSII (Qₐ), resulting in decreased PSI photochemistry (ΦPSI), increased non-photochemical quenching (ΦNPQ) and photo-damage of the reaction center protein D1 of PSII. The ROS generated at the acceptor side of PSI can also cause photodamage of the reaction center heterodimer PsAF/B of PSI. The restricted linear electron transport (Fig. 2, Table 1) and the altered stoichiometry between PSII and PSI (higher PSI/PSII ratio) is a prerequisite and would require increased CEF in supplying the additional ATP (Hatch 1987) needed for effective, although down-regulated CO₂ assimilation in plants exposed to low temperatures. The fact that the increased CEF in cold-stressed maize plants is sensitive mostly to AntA, but not to NDH-specific inhibition (Fig. 5) indicates that in contrast to control plants, the up-regulated CEF is preferably FQR(PGR5) dependent, while the NDH pathway is probably marginally involved in supplying additional ATP in maize mesophyll cells exposed to low temperatures. In addition, the Mehler–ascorbate peroxidase pathway (Mehler reaction) or the WWC has been shown to serve effectively as an additional electron sink in maize plants (Ivanov and Edwards 1997, Ivanov and Edwards 2000) and this pathway is up-regulated in cold-stressed maize plants (Savitch et al. 2009). The increased activity of the WWC (Mehler reaction) helps in oxidizing the PQ pool for the turnover of CEF under cold stress conditions, when the requirement for PQ pool oxidation increases, i.e. the increased capacity of the WWC guarantees the higher activity of CEF (Miyake 2010). Considering that a strong cause–effect relationship between state transitions and the switch between linear electron flow and CEF has been established (Finazzi et al. 2002), we suggest that the decreased capacity for state
transitions in cold-stressed maize plants corresponds to the up-regulation of AntA-sensitive FQR(PGR5)-dependent CET.

Materials and Methods

Plant material and growth conditions

Zea mays L. plants (inbred line CK44) were grown in a controlled environment under a 16 h photoperiod at a 25 °C/20 °C (day/night) temperature regime, 75% humidity and a photon flux density of 500 μmol m⁻² s⁻¹. Plants were grown in plastic pots (190 mm diameter × 190 mm depth, 1 plant per pot) containing approximately 3,600 cm³ total volume of soil. Greenhouse soil was composed of top soil, peat moss, pumice and perlite in a ratio of 4:4:1 (by vol.). Plants received Peters® 20:20:20 (N:P:K) plus micronutrients water-soluble fertilizer at the recommended rate of 100 g 100 l⁻¹ in irrigation water. For cold stress, 3-week-old plants were placed for 3 d under a 15 °C/15 °C (day/night) temperature regime and the same photoperiod and irradiance as control grown plants. All measurements were performed on the middle portion of the fourth fully developed leaves of 3-week-old plants 8 h after the onset of the photoperiod.

Modulated Chl fluorescence

Chl a fluorescence of dark-adapted (35 min) control leaves and cold-stressed maize plants was measured using a PAM 101 Chl fluorescence measuring system (Heinz Walz GmbH) (Schreiber et al. 1986) as described previously (Ivanov et al. 1995). The photochemical (qP) and non-photochemical (qN) Chl fluorescence quenching parameters were calculated when the steady-state F₁ level was reached. The nomenclature of van Kooten and Snel (1990) was used for the parameters of Chl fluorescence. PSI’ excitation pressure’ or the relative reduction state of PSII at the growth temperature and growth irradiance was measured as 1 − qP (Huner et al. 1996).

The reduction state of the PQ pool was measured following the post-illumination transient increase of Chl fluorescence at the F₁ level (Asada et al. 1993, Mano et al. 1995). Chl fluorescence was measured at the corresponding growth/stress temperatures of 25 or 15 °C, respectively, at ambient CO₂ and O₂ conditions using a PAM 101 Chl fluorescence measuring system (Heinz Walz GmbH) (Schreiber et al. 1986) as described previously (Ivanov et al. 1995). The photochemical (qP) and non-photochemical (qN) Chl fluorescence quenching parameters were calculated when the steady-state F₁ level was reached. The nomenclature of van Kooten and Snel (1990) was used for the parameters of Chl fluorescence. PSI’ excitation pressure’ or the relative reduction state of PSII at the growth temperature and growth irradiance was measured as 1 − qP (Huner et al. 1996).

Low temperature (77 K) Chl fluorescence

Mesophyll thylakoid membranes from control and cold-stressed maize leaves were isolated according to Darie et al. (2005). Thylakoid membranes were suspended in a buffer containing 35 mM Tricine (pH 7.8), 0.3 M sorbitol, 7 mM NaCl and 3.5 mM MgCl₂.

77 K fluorescence emission spectra of isolated thylakoids were produced using a liquid nitrogen device attached to a PTI Fluorometer (Model LS-1, Photon Technology International Inc.) equipped with a red-sensitive photomultiplier tube (Type R928P, Hamamatsu Photonics) as in Ivanov et al. (2006a). Samples were dark adapted for 30 min at the corresponding temperature and quickly frozen in liquid nitrogen in the presence of 30% glycerol before the measurements. Corrected fluorescence emission spectra were recorded from 600 to 800 nm. Chl fluorescence was excited at 436 nm. Excitation and emission slit widths were 4 nm. The Chl concentration in the probe was 5–10 μg ml⁻¹. Background emission due to the medium was subtracted from all corrected fluorescence emission spectra.

SDS–PAGE and immunoblotting

Mesophyll chloroplasts were isolated according to Darie et al. (2005). The purity of the mesophyll chloroplasts and the lack of contamination by the bundle sheath chloroplast fraction were evaluated by the estimation of Rubisco activity using a spectrophotometric method described earlier (Sharkey et al. 1991).

Thylakoid membranes for SDS–PAGE from control and cold-stressed maize leaves were isolated as described earlier (Krol et al. 1999, Savitch et al. 2009). Thylakoid preparations were solubilized in a 60 mM Tris–HCl (pH 7.8) buffer containing 1 mM EDTA, 12% (w/v) sucrose and 2% (w/v) SDS to achieve an SDS : Chl ratio of 20 : 1. Solubilized samples containing an equal amount of protein (20 μg lane⁻¹) were separated on a 15% (w/v) linear polyacrylamide gel using a Mini-Protean II apparatus (Bio-Rad).

Immunoblotting was performed by transferring the proteins from the SDS–polyacrylamide gel to nitrocellulose membranes (0.2 μm pore size, Bio-Rad). The membranes were probed with antibodies raised against Lhca1, Lhca2, Lhca3, Lhca4 (Agrisera, Sweden) PsaA and FNR. Dilutions used were 1 : 2,000 for Lhca1, 1 : 2,000 for Lhca2, 1 : 1,000 for Lhca3, 1 : 1,000 for Lhca4, 1 : 1,000 for PsaA and 1 : 500 for FNR. Densitometric scanning and analysis of X-ray films from each replicate immunoblot was performed with a Hewlett Packard Scanjet 4200C desktop scanner and ImageJ 1.41o densitometry software (Wayne Rasband, National Institute of Health, USA, http://rsb.info.nih.gov/ij).
P700 measurements

FR light-induced oxidation of P700 (P700\textsuperscript{+}) was determined in vivo in leaves of control and cold-stressed maize plants at the growth/stress temperatures of 25 or 15°C under ambient O\textsubscript{2} and CO\textsubscript{2} conditions using a PAM-101 modulated fluorometer (Heinz Walz GmbH) equipped with a dual-wavelength emitter–detector ED-P700DW and PAM-102 units (Klughammer and Schreiber 1991) as described in detail by Ivanov et al. (1998). FR light (λ\textsubscript{max} = 715 nm, 10 W m\textsuperscript{-2}, Schott filter RG 715) was provided by an FL-101 light source. The redox state of P700 was evaluated as the absorbance change around 820 nm (ΔA\textsubscript{820–860}) due to the cation radical (P700\textsuperscript{+}) on the adaxial side of the leaf in a custom-designed cuvette. Alternatively, P700 oxidation was induced by white AL (500 μmol photons m\textsuperscript{-2} s\textsuperscript{-1}) pool size in the absence or presence of 20 μM DCMU.

The transient reduction of P700\textsuperscript{+} signal after application of ST and MT flashes of white saturating light was used for estimation of the apparent intersystem electron (e\textsuperscript{−}) pool size (Asada et al. 1993, Ivanov et al. 1998). MT saturating flashes (50 ms) and ST saturating flashes (halfpeak width 14 μs) were applied with the XMT-103 and XST-103 power/control units (Heinz Walz GmbH), respectively. The complementary areas between the oxidation curve of P700 after ST and MT excitation and the stationary level of P700\textsuperscript{+} under FR illumination represent the ST and MT areas, respectively, and were used for estimation of the functional pool size of intersystem electrons on a P700 reaction center basis which was determined as: e\textsuperscript{−}/P700 = MT area/ST area (Asada et al. 1992).

For estimation of the functional pool size of electrons that can be donated to P700\textsuperscript{+} from stromal sources, the complementary areas between the stationary level of P700\textsuperscript{+} under illumination with FR light and the oxidation curves of P700 by FR after an MT flash (MT area), and after the illumination with AL white light (500 μmol photons m\textsuperscript{-2} s\textsuperscript{-1}; AL area) were determined as described earlier (Asada et al. 1992, Savitch et al. 2001) using the following expression:

\[
\text{Stromal } e^-\text{pool/P700} = (\text{MT area/ST area}) \times ([\text{AL area/MT area} - 1])
\]

State transitions

State 1 and state 2 transitions in control and cold-stressed maize leaves were estimated at the corresponding growth temperatures of 25 and 15°C, respectively, as described by Lunde et al. (2000) and Ivanov et al. (2006b), using the PAM-101 Chl fluorescence measuring system equipped with a red light source consisting of a lamp with a Corning 4–96 filter and an FR light provided by a FL-101 light source (λ\text{max} = 715 nm, 10 W m\textsuperscript{-2}, Schott filter RG 715). State 2 was induced by exposing dark-adapted leaves to blue light (80 μmol photons m\textsuperscript{-2} s\textsuperscript{-1}) favoring PSII for 20 min. FR light favoring PSI was superimposed to induce state 1 and after 20 min the maximum fluorescence yield in state 1 (F\textsubscript{m1}) was determined. The FR light was then turned off and after 20 min in the presence of only blue light the maximum fluorescence yield in state 2 (F\textsubscript{m2}) was determined. The relative change in state transition (ΔF) was calculated as: ΔF = [(F\textsubscript{m1} – F\textsubscript{m2})/F\textsubscript{m1}] × 100 where F\textsubscript{m1} and F\textsubscript{m2} designate maximum fluorescence in the presence of PSI light (state 1) and state 2, respectively (Lunde et al. 2000).

Statistical analysis

The data presented are the means of three independent biological experiments, with 3–10 replicates for each experiment (±SE). The number of replicates for each measurement is provided in the table descriptions and figure legends.

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References

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