Effects of Light Intensity on Cyclic Electron Flow Around PSI and its Relationship to Non-photochemical Quenching of Chl Fluorescence in Tobacco Leaves

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We tested the hypothesis that plants grown under high light intensity (HL-plants) had a large activity of cyclic electron flow around PSI (CEF-PSI) compared with plants grown under low light (LL-plants). To evaluate the activity of CEF-PSI, the relationships between photosynthesis rate, quantum yields of both PSII and PSI, and Chl fluorescence parameters were analyzed simultaneously in intact leaves of tobacco plants which had been grown under different light intensities (150 and 1,100 µmol photons m−2 s−1, respectively) and with different amounts of nutrients supplied. HL-plants showed a larger value of non-photochemical quenching (NPQ) of Chl fluorescence at the limited activity of photosynthetic linear electron flow. Furthermore, HL-plants had a larger activity of CEF-PSI than LL-plants. These results suggested that HL-plants dissipated the excess photon energy through NPQ by enhancing the ability of CEF-PSI to induce acidification of the thylakoid lumen.

Keywords: Chl fluorescence — Cyclic electron flow — Non-photochemical quenching (NPQ) — Photosynthesis — PSI.

Abbreviations: CEF-PSI, cyclic electron flow around PSI; DPS, de-epoxidation state; Fd, ferredoxin; FNR, ferredoxin-NADP oxidoreductase; FQR, ferredoxin-quinone oxidoreductase; Φ(PSII), quantum yield of PSII; HL, high light; HLHN, high light high nitrogen; HLMM, high light middle nitrogen; Je(PSII), electron flux in PSII; LEF, linear electron flow; LL, low light; LLMN, low light low nitrogen; LLMN, low light middle nitrogen; NDH, NAD(P)H oxidoreductase; NPQ, non-photochemical quenching; PC, plastocyanin; PDF, photon flux density; PQ, plastoquinone; Qh, photochemical quenching; Rubisco, RuBP carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate.

Introduction

Photons absorbed by chloroplasts drive photosynthetic linear electron flow (LEF) in thylakoid membranes. Their energy is stored in the form of NADPH and ATP which are consumed in photosynthetic carbon reduction and photosynthetic carbon oxidation cycles during net CO2 assimilation. The conversion efficiency of photon energy to NADPH and ATP in LEF is high when net CO2 assimilation is limited by the supply of photons; in this case, the quantum yield of PSII [Φ(PSII)] is high. However, when net CO2 assimilation is limited by the carboxylation reaction of ribulose-1,5-bisphosphate (RuBP) catalyzed by RuBP carboxylase/oxygenase (Rubisco), its efficiency is low. As a result, Φ(PSII) decreases and the energy of the absorbed photons is only partly utilized for net CO2 assimilation.

When net CO2 assimilation proceeds at low Φ(PSII), the excess photon energy damages PSII in the thylakoid membranes by stimulating the production of reactive oxygen species (Asada 1996, Asada 1999, Niyogi 2000). To minimize this PSII photoinactivation, plant chloroplasts dissipate excess photon energy as heat, a protective mechanism observed as non-photochemical quenching (NPQ) of Chl fluorescence (Demming-Adams and Adams 1996, Niyogi et al. 1998, Pogson et al. 1998, Niyogi 1999, Yamamoto et al. 1999, Pogson and Rissler 2000, Müller et al. 2001). Under these conditions, violaxanthin de-epoxidase, an enzyme of the xanthophyll cycle, is activated by an acidification of the thylakoid lumen and catalyzes the de-epoxidation of violaxanthin to zeaxanthin.

NPQ of Chl fluorescence is induced by low Φ(PSII) (Miyake et al. 2004, Miyake et al. 2005). For example, when the net CO2 assimilation rate is light saturated, NPQ drastically increases with further increases in light intensity (Miyake et al. 2004). Similarly, lowering the partial pressure of CO2 under light-saturated conditions decreases the net CO2 assimilation rate and induces NPQ (Miyake et al. 2005).

Induction of NPQ requires a pH gradient across the thylakoid membranes. The acidification of the thylakoid lumen is driven by cyclic electron flow around PSI (CEF-PSI; Heber and Walker 1992, Miyake et al. 2004, Miyake et al. 2005). The activity of CEF-PSI increases at high light and/or low partial pressure of CO2 and is positively correlated with the NPQ of Chl fluorescence (Makino et al. 2002, Miyake et al. 2004, Miyake et al. 2005). In this situation, the regeneration rate of NADP+ limits LEF, and the mediators of CEF-PSI, reduced ferredoxin (Fd) and NADPH, accumulate (Mano et al. 1995, Miyake et al. 1995).

The pool size of the pigments of the xanthophyll cycle (zeaxanthin, antheraxanthin and violaxanthin) depends on the light intensity that prevailed during plant growth. Plants grown...
Cyclic electron flow around PSI1820 at high light intensities (HL-plants) have larger pool sizes on a per leaf area basis, compared with low light intensity-grown plants (LL-plants; Demmig et al. 1988, Verhoeven et al. 1997). HL-plants show higher levels of NPQ of Chl fluorescence than LL-plants (Demmig et al. 1988, Verhoeven et al. 1997). These phenomena reflect an acclimatization of HL-plants in which increased irradiation leads to increased production of reactive oxygen.

In the present work, we studied whether tobacco plants regulated CEF-PSI in response to the light intensity. We found that HL-plants showed a large activity of CEF-PSI and a stronger NPQ of Chl fluorescence, compared with LL-plants. We concluded that tobacco regulates the NPQ of Chl fluorescence by modulating the activity of CEF-PSI.

Fig. 1  Rate of net CO$_2$ assimilation at intercellular partial pressure of CO$_2$ (Ci) = 20 Pa [A(20)] versus total leaf nitrogen content (total leaf-N, N) (A), and the relationship between A(20) and the net assimilation rate at Ci > 60 Pa [A(>60)] (B) in tobacco leaves. A(20) and A(>60) were estimated from the dependence of the net CO$_2$ assimilation rate on Ci. The plants were grown under different light intensities: closed square, 150 µmol photons m$^{-2}$ s$^{-1}$ (LL-plants); open circle, 1,100 µmol photons m$^{-2}$ s$^{-1}$ (HL-plants). Measurements of the net CO$_2$ assimilation rate were made at a leaf temperature of 25°C, a photon flux density of 1,500 µmol photons m$^{-2}$ s$^{-1}$ and 21 kPa O$_2$. The variety of total leaf-N was generated by applying different amounts of fertilizer (see Materials and Methods). (C) Rubisco content versus total leaf-N, and (D) Chl content versus total leaf-N; symbols as above. See Table 1 for further details.
Results

Photosynthetic characteristics

The net CO₂ assimilation rate [A(20)] at the intercellular partial pressure of CO₂ (Ci) of 20 Pa showed a trend to be significantly related to total leaf nitrogen content (total leaf-N; Fig. 1A, see Table 1). The A(20) as a function of total leaf-N was not significantly different between LL- and HL-plants (Fig. 1A and Table 1). In the present growth conditions for tobacco plants, total leaf-N and the net CO₂ assimilation rate ranged from 50 to 200 mmol N m⁻² and from 5 to 30 µmol CO₂ m⁻² s⁻¹, respectively. The net CO₂ assimilation rate at >60 Pa Ci [A(>60)] also showed a trend to be significantly related to A(20) (Fig. 1B and Table 1). The A(>60) as a function of A(20) was also not significantly different between LL- and HL-plants (Fig. 1B and Table 1). These results suggested that tobacco plants, HL- and LL-plants, grown under 150 µmol photons m⁻² s⁻¹; HL-plants, grown under 1,100 µmol photons m⁻² s⁻¹. See also Fig. 1. Different letters indicate a significant difference tested by analysis of covariance (Sokal and Rohlf 1995) with the sequential Bonferroni test (α < 0.05). The difference between intercepts was tested with a common slope (Sokal and Rohlf 1995).

** p < 0.01.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Independent variable</th>
<th>Plants</th>
<th>Slope</th>
<th>Intercept</th>
<th>Coefficient of determination (r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(20) (µmol m⁻² s⁻¹)</td>
<td>Nitrogen (mmol m⁻²)</td>
<td>LL-plants</td>
<td>0.170a</td>
<td>-3.0a</td>
<td>0.945**</td>
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<tr>
<td></td>
<td></td>
<td>HL-plants</td>
<td>0.147a</td>
<td>0.0a</td>
<td>0.950**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pooled</td>
<td>0.162</td>
<td>-2.3</td>
<td>0.983**</td>
</tr>
<tr>
<td>A(&gt;60) (µmol m⁻² s⁻¹)</td>
<td>A(20) (µmol m⁻² s⁻¹)</td>
<td>LL-plants</td>
<td>1.70a</td>
<td>7.0a</td>
<td>0.913**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HL-plants</td>
<td>1.39a</td>
<td>13a</td>
<td>0.811**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pooled</td>
<td>1.63</td>
<td>7.8</td>
<td>0.963**</td>
</tr>
<tr>
<td>Rubisco (g m⁻²)</td>
<td>Nitrogen (mmol m⁻²)</td>
<td>LL-plants</td>
<td>0.029a</td>
<td>0.68a</td>
<td>0.964**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HL-plants</td>
<td>0.032a</td>
<td>0.8a</td>
<td>0.974**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pooled</td>
<td>0.032a</td>
<td>0.86a</td>
<td>0.979**</td>
</tr>
<tr>
<td>Chl (mmol m⁻²)</td>
<td>Nitrogen (mmol m⁻²)</td>
<td>LL-plants</td>
<td>0.006a</td>
<td>0.09a</td>
<td>0.892**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HL-plants</td>
<td>0.004a</td>
<td>0.12b</td>
<td>0.951**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pooled</td>
<td>0.002a</td>
<td>0.31a</td>
<td>0.254</td>
</tr>
</tbody>
</table>

The Rubisco content per leaf area in HL- and LL-plants, furthermore, tended to be significantly related to total leaf-N (Fig. 1C and Table 1). The Rubisco content as a function of total leaf-N was not significantly different between LL- and HL-plants (Fig. 1C and Table 1). These results implied that in tobacco plants, the net CO₂ assimilation rate below the ambient partial pressure of CO₂ was limited by Rubisco contents, as already reported by Makino et al. (1994) and Makino and Mae (1999).

There was also a pronounced correlation between Chl content per leaf area and total leaf-N (Fig. 1D, see Table 1). However, in contrast to the above parameters, Chl content in HL-plants as a function of total leaf-N was significantly lower than that in LL-plants (Fig. 1D and Table 1). These results also corresponded well with those obtained in rice plants (Makino et al. 1997). The fact that the average ratio of the contents of Chl and nitrogen was lower in HL-plants than in LL-plants indicated that the potentially available photon energy exceeded the photosynthetic demand of HL-plants (Evans 1996). Evidently, the photosynthetic apparatus had been adapted to the high light intensities these plants had experienced during their development (Evans 1996).

Based on the above results, we classified the plants into four groups which differed in photosynthetic performance. HL-plants were divided into high-N and middle-N individuals (HLHN with about 180 mmol N m⁻², and HLMN with about 110 mmol N m⁻², respectively; Table 2), whereas LL-plants were classified as middle-N or low-N (LLMN with about 60 mmol N m⁻², respectively; Table 2). The difference in total leaf-N reflected that in photosynthesis ability, as shown in Fig. 1. Thus, we could see the effect of photosynthesis ability on the regulation of the activity of CEF-PSI among HN-, MN- and LN-plants, beside the effect of growth light intensity (growth PFD).

The light absorption efficiency (p) of leaf, the distribution ratio of light absorbed by the chloroplast to PSI (αI) and PSII (αII, dII), and a parameter of Chl fluorescence (F₅₀/F₅₀) were determined in the above tobacco plants (Table 2), for the evaluation of the electron fluxes in both PSI and PSII (see Materials and Methods). At each growth PFD, the values of p showed a trend to decrease with a decrease in total leaf-N. In particular, the p in HLMN-plants was 83.5% of that in HLHN-plants. The dII in HLN-plants was the lowest in all tobacco plants. However, dI in HLHN-plants was the highest (Table 2). These results suggested that tobacco plants, HLMN-plants, with the lowest value of Φ(PSII) (see Table 3), would distribute the excess light energy absorbed by chloroplasts to PSI more efficiently.
Table 2  Effects of growth light intensity and leaf nitrogen content on p, αII, dII, αI, dl and Fv/Fm in tobacco leaves

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HLHN</th>
<th>HLMN</th>
<th>LLMN</th>
<th>LLLN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth PFD (µmol photons m⁻² s⁻¹)</td>
<td>1,100</td>
<td>1,100</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>N (mmol mol⁻¹) (n = 4)</td>
<td>177a</td>
<td>113b</td>
<td>107b</td>
<td>55c</td>
</tr>
<tr>
<td>p (n = 6)</td>
<td>0.857a</td>
<td>0.719b</td>
<td>0.861a</td>
<td>0.815c</td>
</tr>
<tr>
<td>αII (n = 4)</td>
<td>0.44a</td>
<td>0.30b</td>
<td>0.42a</td>
<td>0.44a</td>
</tr>
<tr>
<td>dII (n = 4)</td>
<td>0.51a</td>
<td>0.42b</td>
<td>0.49a</td>
<td>0.54a</td>
</tr>
<tr>
<td>αI (n = 4)</td>
<td>0.42a</td>
<td>0.42a</td>
<td>0.44a</td>
<td>0.38b</td>
</tr>
<tr>
<td>dl (n = 4)</td>
<td>0.49a</td>
<td>0.58b</td>
<td>0.51a</td>
<td>0.46c</td>
</tr>
<tr>
<td>Fv/Fm (n = 4)</td>
<td>0.817a</td>
<td>0.76b</td>
<td>0.827a</td>
<td>0.816a</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.006)</td>
<td>(0.004)</td>
<td>(0.007)</td>
<td>(0.013)</td>
</tr>
</tbody>
</table>

These effects were analyzed by one-way ANOVA (Sokal and Rohlf 1995). For data presented in the table, a post hoc Tukey HSD test was carried out on the grouped means. HLHN, high light high nitrogen; HLMN, high light middle nitrogen; LLMN, low light middle nitrogen; LLLN, low light low nitrogen. The numbers in parentheses denote the standard deviation. Data were the averages of 4–6 experiments using leaves of tobacco plants from each group. Within the same experiment, values followed by the same letter are not significantly different (P > 0.05).

Photosynthetic activity at ambient partial pressures of CO₂/O₂

The photosynthetic characteristics of the four groups of plants defined above were studied at ambient partial pressures of CO₂/O₂ and at the light intensity to which the plants had adapted during their development (Table 3). The dark respiration rate (Rₙ) was similar in all groups. The net CO₂ assimilation rate (A) was highest in HLHN-plants. Quantum yields of net CO₂ assimilation [Φ(CO₂) = A/(p×growth PFD)] in HL-plants were lower than in LL-plants. Stomatal conductance was lowest in the LLMN group, and Ci was lowest in HLHN-plants. Φ(PSII) in HLMN-plants was the lowest, while the electron flux in PSII, Je(PSII), was highest in HLHN-plants. Je(PSII) resembled A in its response to the light intensity experienced during the growth phase and in its dependence on total leaf-N. NPQ and photochemical quenching (Qp) of HL-plants were higher and lower, respectively, than in LL-plants. Furthermore, the electron flux in PSI, Je(PSI), was highest in HLHN-plants, resulting also in the highest activity of CEF-PSI [Je(CEF-PSI) = Je(PSI) – Je(PSII), see Materials and Methods]. Je(PSI) in HLHN-plants was higher than in HLHN-plants, similar to Je(PSII) and A. Compared with HL-plants, LL-plants showed lower values of A, Je(PSII), Je(PSI) and NPQ, but higher values of Qp. These results implied that the irradiation experienced by LL-plants during their development did not provide photon energy in excess of their photosynthetic capacity.

Photosynthetic activity at CO₂ compensation points

Next, we compared photosynthetic parameters of the four groups at the CO₂ compensation point under the light conditions that had prevailed during plant growth, to study the effect of CO₂ limitation (Table 4). At the CO₂ compensation point and 1,100 µmol photons m⁻² s⁻¹, Je(PSII) in HLHN-plants was significantly higher than in HLHN-plants, which was due to a higher concentration of Rubisco in the former. At 150 µmol photons m⁻² s⁻¹, no difference in Je(PSII) between LLMN- and LLLN-plants was observed (Table 2). This might be due to the fact that at low light intensity, photosynthetic electron transport is limited by the supply of photons. In HL-plants, NPQ tended to increase with decreasing total leaf-N, while Qp tended to decrease. These results indicated that at CO₂-limited conditions, the utilization efficiency of photon energy was lower in HLHN-plants than in HLHN-plants, in which excess photon energy is dissipated as heat, as indicated by the significantly higher NPQ. NPQ was lower and Qp was higher in LL-plants than in HL-plants, suggesting that even at the CO₂ compensation point, these plants were not exposed to the excess photon energy against its utilization. The dependence of Je(PSII) on the light environment during plant development and
its dependency on total leaf-N at the CO₂ compensation point corresponded well to the behavior found in Je(PSII). Je(PSI) in all groups was higher than Je(PSII), indicating that CEF-PSI was operating at the CO₂ compensation point. The difference between Je(PSI) and Je(PSII), i.e. Je(CEF-PSI), in HL-plants tended to be higher than in LL-plants.

Relationship among Je(PSII), Je(PSI), NPQ and Qp at high light and 2 kPa O₂

To elucidate the potential activity of CEF-PSI, responses of Je(PSI), Je(PSII), NPQ and Qp of Chl fluorescence to changes in the partial pressure of CO₂ were studied at 1,100 µmol photons m⁻² s⁻¹ and 2 kPa O₂ (Fig. 2; under these conditions, photorespiration was suppressed). Je(PSII) reflected the net CO₂ assimilation rate (A; Miyake and Yokota 2000).

\[ \text{Je(PSII)} = \alpha \text{II} \times \Phi(\text{PSII}) \times \text{PFD} = 4 \times (A + \text{Rd}) \]

Rd was the day respiration rate (see Materials and Methods). Je(PSI) was plotted against Je(PSII), where typical data were plotted in each group of tobacco plants; Je(PSI) and Je(PSII) were positively correlated in all groups (Fig. 2A, Table 5). Je(PSI) generally was larger than Je(PSII), indicating that CEF-PSI was operating. The dependence of Je(PSI) on Je(PSII) was different between HL- and LL-plants (Fig. 2A and Table 5); while the slopes of the regression lines were the same in HLHN- and HLMN-plants as well as in LLMN- and LLLN-plants, the slopes differed significantly between HL-plants and LL-plants. Thus, at low Je(PSII), Je(PSI) was larger in HL-than LL-plants, but at higher Je(PSII), Je(PSI) became similar in both groups. These results indicated that at a limited rate of LEF, Je(CEF-PSI) was higher in HL-plants than in LL-plants.

Next, we plotted the NPQ of Chl fluorescence against Je(PSII) (Fig. 2B). NPQ in HL-plants was higher than in LL-plants below 50 µmol e⁻ m⁻² s⁻¹ Je(PSII). However, as Je(PSII) increased to about 80 µmol e⁻ m⁻² s⁻¹, NPQ in HLHN-plants decreased to values below those detected in LL-plants. On the other hand, NPQ in HLHN-plants remained at its high level.
Table 4  Effects of growth light intensity and leaf nitrogen content on $\Phi$(PSII), Je(PSII), NPQ, Qp, $\Phi$(PSI) and Je(PSI) at the CO$_2$ compensation point in tobacco leaves

<table>
<thead>
<tr>
<th></th>
<th>HLHN</th>
<th>HLMN</th>
<th>LLMN</th>
<th>LLLN</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Phi$(PSII)</td>
<td>0.32a</td>
<td>0.182b</td>
<td>0.697c</td>
<td>0.61d</td>
</tr>
<tr>
<td>(0.03)</td>
<td>(0.015)</td>
<td>(0.009)</td>
<td>(0.06)</td>
<td></td>
</tr>
<tr>
<td>Je(PSII) (µmol m$^{-2}$ s$^{-1}$)</td>
<td>146a</td>
<td>60b</td>
<td>45.6c</td>
<td>40c</td>
</tr>
<tr>
<td>(14)</td>
<td>(5)</td>
<td>(0.8)</td>
<td>(4)</td>
<td></td>
</tr>
<tr>
<td>NPQ</td>
<td>1.91a</td>
<td>2.30b</td>
<td>0.29c</td>
<td>0.40c</td>
</tr>
<tr>
<td>(0.12)</td>
<td>(0.09)</td>
<td>(0.03)</td>
<td>(0.04)</td>
<td></td>
</tr>
<tr>
<td>Qp</td>
<td>0.53a</td>
<td>0.36b</td>
<td>0.882c</td>
<td>0.83c</td>
</tr>
<tr>
<td>(0.04)</td>
<td>(0.05)</td>
<td>(0.011)</td>
<td>(0.02)</td>
<td></td>
</tr>
<tr>
<td>$\Phi$(PSI)</td>
<td>0.59a</td>
<td>0.387b</td>
<td>0.87c</td>
<td>0.91c</td>
</tr>
<tr>
<td>(0.04)</td>
<td>(0.010)</td>
<td>(0.02)</td>
<td>(0.02)</td>
<td></td>
</tr>
<tr>
<td>Je(PSI) (µmol m$^{-2}$ s$^{-1}$)</td>
<td>287a</td>
<td>179b</td>
<td>54.7c</td>
<td>51.9c</td>
</tr>
<tr>
<td>(17)</td>
<td>(5)</td>
<td>(1.4)</td>
<td>(1.2)</td>
<td></td>
</tr>
</tbody>
</table>

These effects were analyzed by one-way ANOVA (Sokal and Rohlf 1995). For data presented in the table, a posthoc Tukey HSD test was carried out on the grouped means. HLHN, high light high nitrogen; HLMN, high light middle nitrogen; LLMN, low light low nitrogen; LLLN, low light middle nitrogen. The numbers in parentheses denote the standard deviation. Data were the averages of four experiments ($n = 4$) using leaves of tobacco plants from each group. Within the same experiment, values followed by the same letter are not significantly different ($P > 0.05$). Gas exchange, $\Phi$(PSI) and Chl fluorescence were measured simultaneously at the CO$_2$ compensation point, where the net CO$_2$ assimilation rate was zero, obtained by regulating an ambient partial pressure of CO$_2$ at 21 kPa O$_2$ and the growth light intensity.

Discussion

In the present work, we tested the hypothesis that plants grown at high light intensities (HL-plants) had a more active CEF-PSI than plants adapted to low irradiation (LL-plants). This hypothesis was deduced from the following facts (Miyake et al. 2004, Miyake et al. 2005): at high light and/or low CO$_2$, where $\Phi$(PSII) is low, the activity of CEF-PSI relative to that of LEF is enhanced. This enhanced CEF-PSI is positively correlated with the NPQ of Chl fluorescence. Thus, CEF-PSI appears to contribute to the induction of NPQ by stimulating the formation of $\Delta$H across thylakoid membranes. Furthermore, compared with LL-plants, HL-plants showed higher NPQ (Demmig-Adams and Adams 1992, Demmig-Adams and Adams 1994, Demmig-Adams and Adams 1996). Therefore, HL-plants were expected to have a higher activity of CEF-PSI for the induction of NPQ. In fact, we found that HL-plants grown at 1,100 µmol photons m$^{-2}$ s$^{-1}$ had a larger activity of CEF-PSI compared with LL-plants grown at 150 µmol photons m$^{-2}$ s$^{-1}$ (Tables 3, 4 and 6, Fig. 2A). We compared CEF-PSI activity of plants adapted to high or low light intensities under the following three sets of conditions. First, under atmospheric CO$_2$/O$_2$ and an irradiation intensity equaling that experienced during their development, HL-plants showed higher Je(PSII) and Je(PSI) and a higher activity of CEF-PSI than LL-plants (Table 3). Furthermore, HLHN-plants had higher values of Je(PSII), Je(PSI) and CEF-PSI, as compared with HLMN-plants. Secondly, under the same illumination as above but at a CO$_2$ concentration below the compensation point, HL-plants also showed higher activities of Je(PSII), Je(PSI) and CEF-PSI than LL-plants (Table 3). HLHN-plants had a more active CEF-PSI than HLMN-plants. These results indicated that under the conditions given, the activity of LEF was always very high. Therefore, to elucidate the dependence of CEF-PSI on LEF, we applied a third set of conditions in which the CO$_2$ partial pressure was varied at 2 kPa O$_2$ and 1,100 µmol photons m$^{-2}$ s$^{-1}$ (Table 6). Plots of Je(PSII) versus Je(PSI) indicated that CEF-PSI correlated positively with LEF (Fig. 2A). Furthermore, HL-plants apparently had a higher activity of CEF-PSI than...
Two main pathways for electron flow in CEF-PSI are proposed to exist in higher plants (Asada et al. 1993, Mi et al. 1995, Bukhov and Carpentier 2004):

(I) PSI→Fd→FQR→Cyt b₆/f→PQ→Cyt b₆/f→PC→PSI (Fd, ferredoxin; FQR, Fd-quinone oxidoreductase; PQ, plastoquinone; PC, plastocyanin)

(II) PSI→Fd→FNR→NADPH→NDH→PQ→Cyt b₆/f→PC→PSI [FNR, Fd-NADP oxidoreductase; NDH, NAD(P)H oxidoreductase].

Pathway I is mediated by FQR and is inhibited by an antibiotic, antimycin A (Arnon et al. 1954, Arnon 1959, Arnon et al. 1967, Arnon and Chain 1975). Pathway II is mediated by NDH (Mi et al. 1992a, Mi et al. 1992b). We showed that the activity of CEF-PSI increased under conditions in which the regeneration rate of NADP⁺ limited LEF, for example at high light and/or low CO₂ partial pressure (Miyake et al. 2004, 2004).

LL-plants at low rates of LEF, which probably promotes the NPQ of Chl fluorescence at low Φ(PSII).

Fig. 2 (A) Plot of Je(PSI) versus Je(PSII). (B) NPQ of Chl fluorescence versus Je(PSII). (C) Qp of Chl fluorescence versus Je(PSII). Responses of Je(PSI), Je(PSII), NPQ and Qp to changes in CO₂ partial pressure were analyzed at a leaf temperature of 25°C, a photon flux density of 1,100 µmol photons m⁻² s⁻¹ and 2 kPa O₂. Typical data were plotted for each group of tobacco plants (open circle, HLHN-plants; closed circle, HLMM-plants; closed square, LLMN-plants; open square, LLLN-plants; see main text for definition of group characteristics). See Table 5 for further details on the regression analyses.
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Miyake et al. (2005). Under these conditions, the reduced forms of Fd and/or NADPH accumulate, leading to the activation of either pathway of CEF-PSI (Mano et al. 1995, Miyake et al. 1995). In the present work, we observed that the activity of CEF-PSI in HL-plants was higher than in LL-plants (Fig. 2A, Table 6). This may be due to an increase in the concentration or the activity of a compound that limits the rate of CEF-PSI, which might be Fd, FQR or NDH. In fact, transformed tobacco plants overexpressing Fd in chloroplasts showed an increased electron donation to PQ in thylakoid membranes (data not shown).

In HL-plants, differences in $\Phi$(PSII) affected the dependence of NPQ and Qp on the activity of LEF [Je(PSII); Fig. 2]. The dependence of Qp on Je(PSII) in HLHN-plants having a large $\Phi$(PSII) was identical to that observed in LL-plants (Fig. 2C). On the other hand, below 150 $\mu$mol e$^{-}$m$^{-2}$s$^{-1}$ Je(PSII), NPQ was larger in HLHN-plants than in LL-plants. These results suggested that a high activity of CEF-PSI in HLHN-plants promoted the rate of NPQ.

In HLMN-plants, which showed the lowest $\Phi$(PSII) of all groups, Qp reached the largest values, indicating that PQ was shifted to the oxidized state due to the limited activity of LEF. Furthermore, the NPQ of Chl fluorescence at 80 $\mu$mol e$^{-}$m$^{-2}$s$^{-1}$ Je(PSII) was almost the same in HLMN- and LL-plants. However, its value increased with decreasing LEF and approached the values detected in HLHN-plants. These findings indicated that the potential activity of NPQ in HLMN-plants corresponded to that in HLHN-plants.

Table 5  Coefficient of determination between the dependent variable [Je(PSI) or Qp] and the independent variable [Je(PSII)] in tobacco leaves grown under different light conditions and with different nitrogen content in the leaves

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Independent variable</th>
<th>Plants</th>
<th>Slope</th>
<th>Intercept</th>
<th>Coefficient of determination ($r^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Je(PSI) ($\mu$mol m$^{-2}$ s$^{-1}$)</td>
<td>Je(PSII) ($\mu$mol m$^{-2}$ s$^{-1}$)</td>
<td>HLHN</td>
<td>1.28a</td>
<td>100</td>
<td>0.991**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLMN</td>
<td>1.11a</td>
<td>110</td>
<td>0.988**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LLMN</td>
<td>1.53b</td>
<td>27</td>
<td>0.997**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LLLN</td>
<td>1.41b</td>
<td>35</td>
<td>0.990**</td>
</tr>
<tr>
<td>Qp</td>
<td>Je(PSII) ($\mu$mol m$^{-2}$ s$^{-1}$)</td>
<td>HLHN</td>
<td>0.0030a</td>
<td>0.030</td>
<td>0.997**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLMN</td>
<td>0.0053b</td>
<td>0.060</td>
<td>0.995**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LLMN</td>
<td>0.0030a</td>
<td>0.005</td>
<td>0.999**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LLLN</td>
<td>0.0030a</td>
<td>0.001</td>
<td>0.999**</td>
</tr>
</tbody>
</table>

HLHN, high light high nitrogen; HLMN, high light middle nitrogen; LLLN, low light low nitrogen; LLMN, low light middle nitrogen. See also Fig. 2. Different letters indicate significant difference tested by analysis of covariance (Sokal and Rohlf 1995) with the sequential Bonferroni test ($\alpha < 0.05$).

Table 6  Effects of growth light intensity and leaf nitrogen content on NPQ, Qp and Je(PSI) at about 50 $\mu$mol e$^{-}$m$^{-2}$s$^{-1}$ of Je(PSII) in tobacco leaves

<table>
<thead>
<tr>
<th></th>
<th>HLHN</th>
<th>HLMN</th>
<th>LLMN</th>
<th>LLLN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Je(PSI) ($\mu$mol m$^{-2}$ s$^{-1}$)</td>
<td>50a</td>
<td>49.6a</td>
<td>50.2a</td>
<td>50a</td>
</tr>
<tr>
<td></td>
<td>(0.7)</td>
<td>(1.2)</td>
<td>(1.7)</td>
<td>(2)</td>
</tr>
<tr>
<td>NPQ</td>
<td>2.61a</td>
<td>2.54a</td>
<td>1.98b</td>
<td>2.12b</td>
</tr>
<tr>
<td></td>
<td>(0.08)</td>
<td>(0.09)</td>
<td>(0.10)</td>
<td>(0.06)</td>
</tr>
<tr>
<td>Qp</td>
<td>0.160a</td>
<td>0.345b</td>
<td>0.159a</td>
<td>0.160a</td>
</tr>
<tr>
<td></td>
<td>(0.005)</td>
<td>(0.005)</td>
<td>(0.004)</td>
<td>(0.003)</td>
</tr>
<tr>
<td>Je(PSI) ($\mu$mol m$^{-2}$ s$^{-1}$)</td>
<td>174a</td>
<td>167a</td>
<td>99b</td>
<td>100b</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>(5)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
</tbody>
</table>

These effects were analyzed by one-way ANOVA (Sokal and Rohlf 1995). For data presented in the table, a posthoc Tukey HSD test was carried out on the grouped means. HLHN, high light high nitrogen; HLMN, high light middle nitrogen; LLLN, low light low nitrogen; LLMN, low light middle nitrogen. Numbers in parentheses denote the standard deviation. Data were averages of four experiments ($n = 4$) using leaves of tobacco plants from each group. Within the same experiment, values followed by the same letter are not significantly different ($P > 0.05$). Gas exchange, $\Phi$(PSII) and Chl fluorescence were measured simultaneously at about 50 $\mu$mol e$^{-}$m$^{-2}$s$^{-1}$ of Je(PSII) obtained by regulating an ambient partial pressure of CO$_2$ at 2 kPa O$_2$ and 1,100 $\mu$mol photons m$^{-2}$s$^{-1}$.
Within the range of Je(PSII) observed, Qp was higher in HLMN-plants than in the other groups (Fig. 2). On the other hand, NPQ was low, except at very low Je(PSII), in HLMN-plants. These results might reflect the acclimatization of plants to high light intensities which result in relatively low Φ(PSII), especially in plants with low total leaf-N, as shown in the present work. Moreover, under high light conditions, the photosynthetic electron transport systems are saturated with electrons, leading to the production of reactive oxygen species (Asada 1999). This can be deleterious for the plant, which may be the reason why HLMN-plants show a large Qp, not a large NPQ. The present results appear in line with the idea that the dissipation of excess photon energy observed as NPQ of Chl fluorescence is a short-term response of plants to conditions inducing low Φ(PSII), whereas the maintenance of PQ in the oxidized state is a long-term response.

Since the correlation between Qp and Je(PSII) was similar in HLHN-, LLMN- and LLLN-plants which differed significantly in their leaf-N contents (Fig. 2C, Table 5), the increased Qp detected in HLMN-plants did not appear to result from N shortage.

The molecular mechanisms giving rise to an increased Qp relative to Je(PSII) as observed in HLMN-plants are unknown. In HLMN-plants, the excitation efficiency of PSII might have been low, or that of PSI might have been high, compared with plants of the other groups. Intriguingly, dII was lowest and dI highest in HLMN-plants (Table 2). The regulation of dI and dII is driven by state transitions (Allen 1992, Bellaﬁore et al. 2005); the detailed mechanisms remain to be resolved.

HL-plants showed a higher NPQ of Chl fluorescence at low activity of LEF than LL-plants (Fig. 2B). One of the reasons may be that HL-plants also showed a higher activity of CEF-PSI, as discussed above. However, there might be another possible explanation, if the pool sizes of pigments of the xanthophyll cycle would have been larger in HL- than in LL-plants (Demmig et al. 1988, Demmig-Adams and Adams 1996, Verhoeven et al. 1997, Logan et al. 1998, Cheng et al. 2000). Plants adapted to high light intensities tend to have larger pool sizes and a higher de-epoxidation state (DPS), as compared with shade plants. It is noteworthy that the pool size of xanthophyll cycle pigments and DPS are positively correlated with the NPQ of Chl fluorescence (Demmig-Adams and Adams 1992, Demmig-Adams and Adams 1994, Demmig-Adams and Adams 1996, Demmig et al. 1996). Therefore, both the activity of CEF-PSI and the amount of pigments of the xanthophyll cycle increase when plants are exposed to conditions in which Φ(CO₂) is lowered. These stress responses enhance the NPQ of Chl fluorescence and contribute to the suppression of the production of reactive oxygen in the PSI reaction center, i.e. photoinhibition.

HLMN-plants showed a particularly low value of Fv/FM (Table 2), probably due to the sustained high NPQ of Chl fluorescence (Verhoeven et al. 1999, Öquist and Huner 2003). When evergreen species such as Scotch pine and Euonymus kiautschovicus expand their leaves in winter when net CO₂ assimilation is suppressed, the DPS in the xanthophyll cycle is increased in these leaves irrespective of the light intensity illuminating the leaves. As a result, Fv/FM in the dark is kept low (Verhoeven et al. 1998, Öquist and Huner 2003). In the sustained NPQ of Chl fluorescence, the formation of ΔpH across the thylakoid membranes in the dark might be driven by NDH-dependent chlororespiration for the activation of violaxanthin de-epoxidase (Öquist and Huner 2003). Furthermore, the npq-2 mutant of Arabidopsis which lacks zeaxanthin epoxidase accumulates zeaxanthin even in the dark and shows a lowered value of Fv/FM compared with the wild type (Dall’Osto et al. 2005). These results suggested that HLMN-plants might also accumulate zeaxanthin in the dark.

In general, decreases in Fv/FM indicate an inactivation of PSII (Hikosaka et al. 2004, Hirotsu et al. 2005), which results in a decreased net CO₂ assimilation rate at high Ci, because this rate is limited by the rate of RuBP regeneration which is driven by photosynthetic electron flow. However, in the Fv/FM range from 0.75 to 0.80, no decrease in the net CO₂ assimilation at low or high Ci was observed (Hikosaka et al. 2004, Hirotsu et al. 2005), indicating that Fv/FM shifts within this range do not reflect the activity of PSII. Furthermore, in the plot of A(>60) against A(20) (Fig. 1B), no differences between the regression lines for HL- and LL-plants were observed. This fact indicates that neither HL- nor LL-plants suffered from photoinhibition in our experiments.

Materials and Methods

Plant growth conditions

Tobacco plants (Nicotiana tabacum cv. Xanthi) were grown from seeds under standard air-equilibrated conditions with 16 h·8 h daylight–night cycles at 25 and 22°C, respectively, and 50–60% relative humidity. PFDs were adjusted to 150 (low-light treatment) and 1,100 µmol photons m⁻² s⁻¹ (high-light treatment). Seedlings were kept in 0.5 dm³ pots containing commercial peat-based compost, and were watered daily. Plants were fertilized with 1,000-fold diluted Hyponex 8–12–6 (Hyponex Japan, Osaka, Japan) either three times a week, once a week or once in 3 weeks. All measurements described below were made 3 weeks after sowing when the fifth to tenth leaves were fully expanded.

CO₂ fixation, Chl fluorescence and P700⁺ absorbance

For measurements of photosynthetic parameters and collection of leaves, tobacco plants were transferred to a dark room 4 h after the start of the light period. After the incubation of tobacco in the dark room for about 60 min, CO₂ fixation (gas exchange) and Chl fluorescence were measured simultaneously. P700⁺ absorbance was measured sequentially after Chl fluorescence measurement. All measurements were repeated at least three times using three different plants. The measurements of the leaf attached to the plant were done over a leaf area of 6 cm². The basal system of gas exchange was adopted as previously detailed by Miyake and Yokota (2000), except that LI-6400 (Li-Cor, Lincoln, NE, USA) was used as IRGA. Leaf temperature was adjusted to 25.0 ± 0.5°C. The mixture of gases was saturated with water vapor at 16 ± 0.1°C, which corresponded to 1.825 kPa. Irradiance was provided by a halogen lamp (KL-1500; Walz, Effeltrich, Germany)
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to the leaf chamber through the glass fiber optics that were linked to a PAM Chl fluorometer that is described below.

Chl fluorescence was measured with the PAM Chl fluorometer through the same fiber optics. The steady-state fluorescence yield ($F_S$) was monitored continuously and a 1,000 ms pulse of saturating light was supplied at intervals of 60 s to determine the maximum variable fluorescence ($F_{v’}$). The $\Phi_{PSI}$ at steady state was defined as ($F_{M’} - F_s’$) as proposed by Genty et al. (1989). NPQ and $q_p$ of Chl fluorescence were calculated as ($F_{M’} - F_s’$) according to Bilger and Bjorkman (1994) and as ($F_{M’}’ - F_s’’$) according to Oxford and Baker (1997), respectively. $F_{v’’}$ was the minimum Chl fluorescence yield with maximum opening of all PSI reaction center Chl P680 in the light at steady state.

The absorbance of P700$^+$ was measured with the same PAM Chl fluorometer by exchanging the Chl fluorescence detector unit for an ED-P700DW-E emmiter–detector unit (Walz, Effeltrich, Germany) (Backhausen et al. 1998, Holstegref et al. 2003). The amplitude of full P700 oxidation was measured in the dark for each leaf before illumination was started. In darkness, P700 is in its reduced state, and full oxidation of P700, [P700]$^{−}$, was achieved by illumination with far-red light (>700 nm), which excites only PSI. The oxidation of P700, [P700]$^{+}$, was monitored by the change in the $A_{10−1600}$, during illumination, the same amount of oxidizable P700 should be available, unless the PSI electron acceptors are already in their reduced state and cannot accept more electrons. During illumination, the fraction of reduced [reduced P700] or PSI acceptor ($A^+$) is determined by short saturating light pulses, which give full oxidation of P700, followed by a ‘dark pulse’, which yields fully reduced P700. The difference between the P700 amplitude in the light and the far-red-induced amplitude determined in the dark-adapted leaf must be attributed to $A^+$. The $\Phi_{PSI}$ was calculated as described by Klughammer and Schreiber (1994), $\Phi_{PSI} = [\text{reduced P700}]/[\text{P700}]_{\text{dark}}$.

**Estimation of electron fluxes in both PSI and PSII**

$\Phi_{PSI}$ and $\Phi_{PSII}$, were defined, as follows:

\[
F(PSI) = \text{Je(PSI)}/(\alpha_{I} \times PFD)
\]

\[
F(PSII) = \text{Je(PSII)}/(\alpha_{II} \times PFD)
\]

Je(PSI) and Je(PSII) were the electron fluxes in PSI and PSII, respectively. $\alpha_I$ and $\alpha_{II}$ were the distribution ratio of light illuminating the leaf to PSI and PSII, respectively. If the intensity of light illuminating the leaf was 1,

\[
l = p + \beta.
\]

$p$ was the ratio of light absorbed by chloroplasts and $b$ was that not absorbed in the leaf. $b$ contained the reflectance and transmittance of light in the leaf. Furthermore, if the intensity of light absorbed by chloroplasts was 1,

\[
l = dl + dlII.
\]

dl and dlII were the distribution ratio of light absorbed by chloroplasts to both PSI and PSII, respectively. Therefore, $\alpha_I$ and $\alpha_{II}$ were expressed as, follows:

\[
\alpha_I = p_{I} \times dl
\]

\[
\alpha_{II} = p_{II} \times dlII
\]

The value of $\alpha_{II}$ was determined under non-photorespiratory conditions, where Je(PSII) was expressed from the stoichiometry of the Calvin cycle, as follows: Je(PSII) = $\alpha_{II} \times \Phi(PSII) \times PFD = 4 \times (A + Rd)$, where $A$ was the net CO$_2$ assimilation rate and $Rd$ was the day respiration rate (Genty et al. 1989, Miyake and Yokota 2000, Rusuksa et al. 2000, von Caemmerer 2000, Makino et al. 2002, Miyake et al. 2004). Rd was estimated from curves of A versus Ci obtained at various PFDs, as described by Brooks and Farquhar (1985). We obtained the constant value of $\alpha_{II}$ under any light intensity and Ci at 2 kPa $O_2$. (Miyake et al. 2004).

The ratio of light absorbed by chloroplasts in tobacco leaves, $p$, was determined with an LI-1800 spectroradiometer and the 1800–12S integrating sphere attachment (Li-Cor Inc., Lincoln, NE, USA). For each leaf, both a reference scan and a sample scan of reflectance or transmittance were made from 400 to 700nm at 1 nm intervals. The sample scan was divided by its corresponding reference scan, and integrated over the wavelength range to obtain the average reflectance or transmittance (Chen and Cheng 2003). The $p$ was calculated as: $1 – \text{reflectance – transmittance}$.

From the values of $\alpha_{II}$ and $p$, $\alpha_I$ was calculated as $\alpha_I = p - \alpha_{II}$. From the following equations, we estimated both Je(PSI) and Je(PSII), from the measured values of $\Phi(PSI)$ and $\Phi(PSII)$ in tobacco leaves.

\[
\text{Je(PSI)} = \alpha_{I} \times \Phi(PSI) \times PFD
\]

\[
\text{Je(PSII)} = \alpha_{II} \times \Phi(PSII) \times PFD
\]

The electron flux in CEF-PSI [Je(CEF-PSI)] was estimated from the difference between Je(PSI) and Je(PSII), Je(CEF-PSI) = Je(PSI) – Je(PSII) (Miyake et al. 2005).

Furthermore, we calculated the values of both dl and dlII from the above estimated values of $p$, $\alpha_I$ and $\alpha_{II}$.

The constant value of $\alpha_{II}$ indicated that $\alpha_{II}$ was also constant. The constant ratio of $\alpha_{I} / \alpha_{II}$ showed dl / dlII to be constant. From these results, the change in dl / dlII by state transition was negligible. In state transition, the reduction of the PQ pool increased dl / dlII (Allen 1992, Finazzi et al. 1999, Haldrup et al. 2001, Wollman 2001, Finazzi et al. 2002). However, in the present work, even though state transition occurred, it would not contribute to the enhancement of CEF-PSI at high light and/or low CO$_2$.

The apparent quantum yield of the net CO$_2$ assimilation at steady state ($\Phi(\text{CO}_2)$) was estimated as $A/(p \times PFD)$, where $A$ was the net CO$_2$ assimilation rate and PFD was the photon flux density illuminating the leaf.

**Measurements of Rubisco, leaf nitrogen and Chl**

The amounts of Rubisco and total leaf nitrogen were determined on the same leaves as used for the gas exchange studies (Makino et al. 1988). After the photosynthetic measurements, the leaf was quickly cut off and its fresh weight and leaf area were measured; then the leaf was immediately homogenized in 50 mM Na-phosphate buffer (pH 7.5) containing 10 mM dithiothreitol (DTT) and 12.5% (v/v) glycerol at a ratio of leaf to buffer of 1 : 7 (g : ml) using a chilled mortar and pestle with acid-washed quartz sand (0.30 g). Total Chl was determined in this homogenate (Makino et al. 1992).

A portion of 100 μl of this homogenate was weighed and subjected to Kjeldahl digestion. Total leaf-N was determined by the method of Hind (1993), with the SuperKjel 1200/1250 System (ACTAN, Tokyo, Japan). Also, instead of the above method, the Nitro-Ace System (EYELA, Tokyo, Japan) was used for the determination of total leaf-N (Yasuhara and Nokihara 2001). The remaining homogenate was centrifuged at 40,000×g for 15 min at 0–4°C. The amount of Rubisco protein in the supernatant was determined spectrophotometrically by formamide extraction of Coomassie brilliant blue R-250 after SDS–PAGE, as described in Makino et al. (Makino et al. 1985, Makino et al. 1986).
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