Antioxidant responses of cucumber (Cucumis sativus) to photoinhibition and oxidative stress induced by norflurazon under high and low PPFDs

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Abstract

Photooxidative damage is exacerbated by norflurazon (NF), which blocks carotenoid biosynthesis. This study examined the influence of photosynthetic photon flux density (PPFD) on the overall responses of both non-enzymatic and enzymatic antioxidants to NF-caused oxidative damage in leaves of cucumber (Cucumis sativus). Seven-day-old cucumber plants were exposed to NF under either low PPFD (30 μmol m⁻² s⁻¹) or high PPFD (300 μmol m⁻² s⁻¹) for 3 days. The NF plants exposed at high PPFD had lower levels of Fv/Fm ratio, quantum yield of electron transport, and 33-kDa protein of photosystem II as compared with the NF plants at low PPFD. In the NF plants, there was a reduction in total chlorophylls and carotenoids except newly formed zeaxanthin in either PPFD. The NF plants at high PPFD resulted in less level of photochemical quenching, qP, and Stern–Volmer quenching, NPQ, than those of the plants at low PPFD, whereas both plants had similar level of non-photochemical quenching coefficient, qN. However, the level of PPFD did not significantly affect the NF-caused induction of antioxidant enzymes including peroxidase, superoxide dismutase, glutathione reductase, and ascorbate peroxidase. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Antioxidant enzymes; Cucumber (Cucumis sativus); Non-photochemical quenching; Norflurazon; Oxidative stress; Xanthophylls

1. Introduction

Photoinhibition and photooxidation can occur when plants are exposed to stress. High light in synergy with other stress factors such as chilling, drought, or low carbon dioxide supply reduces the capacity of photosynthetic systems to utilize incident radiation, leading to a photoinhibition process [1,2]. The photosynthetic electron transport system is the major source of active oxygen species (AOS) in plant tissues [3], having the potential to generate singlet oxygen (¹O₂) and superoxide (O₂⁻), which is favored under downregulation of metabolic pathways. Photosystem (PS) II has long been considered the primary target for photoinhibition [1,2] because PSI is more stable than PSII during strong light treatments [4]. Light-induced inactivation of PSI is suggested to be caused by AOS [5,6]. AOS are eliminated efficiently by an integrated system of non-enzymatic and enzymatic antioxidants that are concentrated in the chloroplast [3]. The capacity of the antioxidative defense system is increased under adverse stress conditions but the imbalance between AOS production and antioxidant defenses ultimately leads to oxidative damage.

The non-enzymatic reductants consist of ascorbate, glutathione, α-tocopherol, carotenoids, and phenolic compounds [7,8]. Among those, xanthophyll cycle-dependent energy dissipation in the
light-harvesting antennae is thought to play an important photoprotective role by mitigating oxidative damage. Non-radiative energy dissipation at PSII is mediated by zeaxanthin and perhaps also by antheraxanthin [9] and is proposed to occur at several sites within or around the PSII reaction center [10]. Researchers have used different terms for energy dissipation, e.g. the quenching coefficient $q_N$ [11] versus Stern–Volmer quenching [9,12] that is referred to as non-photocchemical quenching (NPQ).

In the most powerful source of AOS, chloroplasts, $O_2^-$ that is produced by photoreduction of $O_2$ at PSI and PSII is detoxified by the Mehler-peroxidase pathway [3,13]. The $O_2^-$ is reduced to hydrogen peroxide ($H_2O_2$) by superoxide dismutase (SOD) and then to $H_2O$ by ascorbate peroxidase (APX), which is the key enzyme involved in $H_2O_2$ scavenging. These enzymes, together with monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase (GR), constitute the major defense system against AOS in the chloroplast [3,14]. Additionally, extraplasmic $H_2O_2$ quenching by peroxidase (POD) and catalase (CAT) is also increased in stress responses [15]. A crucial role of these enzymes in protection against oxidative processes has been shown in transgenic tobacco plants overexpressing either Mn-SOD or Fe-SOD [16,17]. In contrast, other studies with transgenic plants suggest that enhancement of a particular antioxidant enzyme does not lead to increased protection [18,19].

Photooxidative damage is exacerbated by herbicides, which generate AOS either by direct involvement in radical production or by inhibition of biosynthetic pathways [20,21], as well as atmospheric pollutants and heavy metals [8]. Enhanced activities of antioxidants were associated with resistance to herbicides such as parquat and oxyfluorfen [22,23]. In the present study a potent herbicide norflurazon (NF), which blocks carotenoid biosynthesis by non-competitively binding to phytoene desaturase [24], was used in leaves of *Cucumis sativus*. It eliminates important quenchers of the triplet chlorophyll (Chl) and $^1O_2$, thus initiating photooxidative processes. To assess contribution of each inductive response of antioxidant to overall protective strategies to NF-caused oxidative damage, photochemical efficiency of PSII, composition of photosynthetic pigments, quenching parameters and activities of antioxidant enzymes were determined. The questions of whether the level of photosynthetic photon flux density (PPFD) influences the NF-caused oxidative stress was also examined, and, if so, whether the NF plants at different PPFDs have different capacities to develop antioxidant responses.

2. Materials and methods

2.1. Plant material and growth conditions

Cucumber seeds (*C. sativus* L. cv Summer Long) were sown in vermiculite and transferred after 4 days into synthetic soil in plastic pots. Plants were grown in a controlled environment growth chamber under a temperature of 25°C, a 16-h photoperiod, and a light intensity of 200 $\mu$mol m$^{-2}$ s$^{-1}$ for 3 days. For NF treatment the 7-day-old plants were exposed in a surface application to $15 \mu$M. When the treatment was initiated the first leaves were about to emerge. Following NF application plants were immediately returned to the growth chamber and exposed for 3 days under a 16-h photoperiod with an irradiance of either low PPFD (30 $\mu$mol m$^{-2}$ s$^{-1}$) or high PPFD (300 $\mu$mol m$^{-2}$ s$^{-1}$) at 25°C. Inhibition of carotenoid biosynthesis causes a characteristic bleaching of newly developed leaves. The four treatments employed were: (1) CH, control/high PPFD; (2) NH, NF treatment/high PPFD; (3) CL, control/low PPFD; and (4) NL, NF treatment/low PPFD. The first leaves were used for the measurements of Chl fluorescence, pigment contents and enzyme activities. The experiments were triplicated each with three determinations.

2.2. Chl a fluorescence measurements

In vivo Chl a fluorescence was measured after 5 min dark-adaptation at room temperature using a pulse amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany). Minimal fluorescence yield, $F_0$, was obtained upon excitation with a weak measuring beam from a pulse light-emitting diode. Maximal fluorescence yield, $F_m$, was determined after exposure to a saturating pulse of white light to close all reaction centers. Determination of the quenching components $q_p$ and $q_N$ was conducted by the saturation pulse method and they were calculated as defined by
Schreiber et al. [25]. The quantum yield of electron transport through PSII ($\Phi = \Delta F/F_m$) was calculated according to Genty et al. [26]. Non-photochemical fluorescence quenching was also quantified, as previously done by Bilger and Björkman [12] according to the Stern–Volmer equation, $NPQ = F_m/F_m^{\%} - 1$, where $F_m^{\%}$ is the lowered maximal yield during illumination with photosynthetically active radiation.

2.3. Immunoblot analysis

For the immunoblot of the extrinsic 33-kDa protein of the oxygen-evolving complex in PSII reaction center, the method of Tae et al. [27] was used. The thylakoid membranes isolated from chloroplasts were resuspended in 10 mM NaCl, 50 mM sucrose, and 50 mM sodium phosphate buffer, pH 7.4 and were sedimented at 10,000 × g for 10 min. The pellets were resuspended in the same buffer as mentioned above. The chlorophylls were removed with 80% acetone and the protein pellets were solubilized (1% SDS, 8 M urea, 1% 2-mercaptoethanol, 10.7 mM phosphoric acid). The protein concentrations were measured with the UV spectrophotometric method. Samples of protein to be blotted were electrophoresed in 12% SDS-polyacrylamide gel. The gel was run in 192 mM Glycine, 0.01% (w/v) SDS, and 25 mM Tris–HCl, pH 8.3. Polypeptides were transferred to nitrocellulose paper (pore size: 0.45 µm; Hybond-C, Amersham) with a semi-dry transfer blotter (130-mA constant current, 60 min) (Model TE70, Hoefer Scientific Instruments). The paper was washed in TBS buffer (500 mM NaCl, 20 mM Tris–HCl, pH 7.4), incubated in a sealed plastic bag with 10% milk casein on a rocking shaker (RK1020) for 2 h, removed from the bag, and washed in TBS buffer. After incubating with the antibody in TBS buffer containing 3% bovine serum albumin (BSA) for 2 h, and washing in TTBS buffer containing 0.05% Tween-20, 500 mM NaCl, 20 mM Tris–HCl, pH 7.4, the paper was again incubated with a second antibody [goat antirabbit IgG conjugated with horseradish peroxidase (Bio-Rad)] in TBS containing 3% BSA. The paper was incubated on a rocking shaker for 1 h, washed in TTBS buffer, and stained for 10 min with 0.017% 4-chloro-1-naphthol and hydrogen peroxide in TBS buffer.

2.4. Pigment extraction and analysis

Extraction and HPLC analysis of carotenoids and Chls were done as described previously [28].

2.5. Extraction of soluble protein

Frozen leaves (0.25 g for CAT, GR, and APX; 0.5 g for POD and SOD) were crushed to fine powder in a mortar under liquid N₂. Soluble proteins were extracted by homogenizing the powder in 2 ml of 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA, 1% PVP-40, and 1 mM PMSF. For analysis of APX, the extraction buffer also contained 5 mM ascorbate. Insoluble material was removed by centrifugation at 15,000 × g for 20 min at 4°C, and the supernatant was filtered through filter papers No. 1 (Whatman, Maidstone, UK). Since maintenance of consistent CAT electrophoretic mobility and GR activity was found to require the presence of DTT, an aliquot of each sample was made to 10 mM DTT to be used for CAT and GR zymograms and GR spectrometric assays. For the spectrophotometric assay of SOD, extracts were passed through a PD-10 column (Pharmacia, Uppsala, Sweden).

2.6. Enzyme assays

CAT activity was determined by using a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) according to the method of Natvig [29]. The CAT assay was performed in a 3 ml volume containing N₂-bubbled 50 mM potassium phosphate buffer, pH 7.0, containing 20 mM H₂O₂. POD activity was determined specifically with guaiacol at 470 nm ($\varepsilon = 25.2$ mM cm⁻¹) following the method of Egley et al. [30]. The reaction mixture contained 40 mM potassium phosphate buffer (pH 6.9), 1.5 mM guaiacol, and 6.5 mM H₂O₂ in a 3-ml volume. SOD activity was determined as described by Spychalla and Desborough [31]. The assay was performed at 25°C in a 3-ml volume containing 50 mM Na₂CO₃/NaHCO₃ buffer (pH 10.2), 0.1 mM EDTA, 0.015 mM ferricytochrome C, and 0.05 mM xanthine. APX activity was measured spectrophotometrically by monitoring the decline in A₂₉₀ as ascorbate ($\varepsilon = 2.8$ mM cm⁻¹) was oxidized, using the method of Chen and Asada [32]. The 3-ml reaction volume contained 100
mM potassium phosphate buffer (pH 7.5), 0.5 mM ascorbate, and 0.2 mM H₂O₂ at 25°C. GR activity was measured spectrophotometrically by measuring the decline in A₃₄₀ as NADPH (ε = 6.2 mM cm⁻¹) was oxidized, as described by Rao et al. [33]. The 3-ml assay mixture contained 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM GSSG, and the leaf extract. The assays were initiated by the addition of NADPH at 25°C.

2.7. Native PAGE and activity staining

Equal amounts of protein from plants exposed to different treatments were subjected to 10% non-denaturing polyacrylamide gels at 4°C for 1.5 h with a constant current of 30 mA. After completion of electrophoresis the gels were stained for the enzymatic activities. Catalase activity was detected by incubating the gels in 3.27 mM H₂O₂ for 25 min, rinsed in water, and stained in a solution of 1% potassium ferricyanide and 1% ferric chloride for 4 min [34]. Staining of POD isozymes was achieved by incubating the gels in sodium citrate buffer, pH 5.0, containing 9.25 mM p-phenylene-diamine and 3.92 mM H₂O₂ for 15 min [35]. Gels were stained for SOD isoforms by soaking in 50 mM potassium phosphate, pH 7.8, containing 2.5 mM nitroblue tetrazolium in darkness for 25 min, followed by soaking in 50 mM potassium phosphate, pH 7.8, containing 28 mM nitroblue tetrazolium and 28 μM riboflavin in darkness for 30 min [33]. Gels were then exposed to light for approximately 30 min. Following separation of APX, gels were soaked in 50 mM potassium phosphate buffer, pH 7.8, containing 28 mM tetramethyl ethylene diamine and 2.45 mM nitroblue tetrazolium for 15 min. Gels were stained for GR activity in a solution of Tris–HCl, pH 7.5, containing 10 mg of 3-(4,5-dimethylthiazol-2-4)-2,5-diphenyl tetrazolium bromide, 10 mg of 2,6-dichlorophenolindophenol, 3.4 mM GSSG, and 0.5 mM NADPH in darkness for 1 h [33].

3. Results

3.1. Chl a fluorescence during photooxidative stress

To confirm the involvement of PSII in the oxidative stress responses, the values of photosynthetic parameters were determined. Exposure of 7-day-old cucumber plants to NF caused substantial photoinhibition of photosynthesis, as indicated by the decline in the photochemical efficiency of photosynthesis. A significant drop in the Fₐ/Fₘ ratio and quantum yield of electron transport through PSII was detected in both NF-treated plants, with almost no quantum yield in NH plants (Table 1). Control plants at either PPFD showed similar values of Fₐ/Fₘ and quantum yield.

The initial fluorescence F₀ was the same in CH and CL plants (Table 1). NH plants exhibited a lower F₀ in contrast to a greater F₀ in NL plants, compared with control plants.

3.2. Quantification of PSII

Quantification of the PSII reaction center protein 33-kDa following NF exposure showed pronounced differences among treatments (Fig. 1). The 33-kDa protein level of CH plants was greater than that of CL plants. Exposure of leaves to NF caused the loss of PSII reaction centers, as indicated by the loss of the extrinsic 33-kDa protein of
Fig. 1. Influence of norflurazon (NF) and photosynthetic photon flux density (PPFD) on the extrinsic 33-kDa protein of oxygen evolving complex in photosystem (PS) II reaction center. The plants were subjected to the same treatments as in Table 1. Treatment notations are the same as in Table 1. M, a 30-kDa molecular weight marker. Measurements were made 3 days after NF treatment. Data represent the mean ± S.E. of three replicates.

Table 2
Effects of norflurazon (NF) exposure at different photosynthetic photon flux densities (PPFDs) on carotenoids (mmol mol⁻¹ Chl a) and Chl pigments (µg g FW⁻¹) of C. sativus

<table>
<thead>
<tr>
<th>Pigments</th>
<th>CH</th>
<th>NH</th>
<th>CL</th>
<th>NL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neoxanthin</td>
<td>25.2 ± 0.7 (99.2)</td>
<td>17.0 ± 1.4 (4.3)</td>
<td>24.3 ± 1.2 (92.4)</td>
<td>15.8 ± 1.5 (15.0)</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>45.8 ± 2.3 (179.8)</td>
<td>60.2 ± 0.3 (15.1)</td>
<td>26.7 ± 1.2 (101.7)</td>
<td>16.9 ± 2.2 (16.0)</td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>2.7 ± 0.2 (10.6)</td>
<td>7.6 ± 0.2 (1.9)</td>
<td>ND</td>
<td>3.3 ± 0.5 (3.2)</td>
</tr>
<tr>
<td>Lutein</td>
<td>58.7 ± 2.3 (231.1)</td>
<td>69.4 ± 3.4 (17.4)</td>
<td>48.8 ± 2.3 (185.8)</td>
<td>41.3 ± 1.3 (39.5)</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>ND</td>
<td>7.8 ± 1.2 (2.0)</td>
<td>ND</td>
<td>6.2 ± 0.3 (5.9)</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>48.1 ± 8.0 (186.6)</td>
<td>2.7 ± 1.0 (0.7)</td>
<td>41.1 ± 2.5 (156.5)</td>
<td>18.9 ± 1.4 (18.1)</td>
</tr>
<tr>
<td>V + A + Z</td>
<td>48.4 ± 2.4 (190.5)</td>
<td>75.6 ± 1.6 (19.0)</td>
<td>26.7 ± 1.2 (101.7)</td>
<td>26.4 ± 1.6 (25.1)</td>
</tr>
<tr>
<td>Chl a+b</td>
<td>1779.8 ± 162.9</td>
<td>1125 ± 9.5</td>
<td>1642.6 ± 26.1</td>
<td>387.9 ± 15.9</td>
</tr>
</tbody>
</table>

*The values in parentheses indicate the pigment contents on nmol per g fresh weight basis. The plants were subjected to the same treatments as in Table 1. Treatment notations are the same as in Table 1. Measurements were made 3 days after NF treatment. ND, not detected. Data represent the mean ± S.E. of three replicates.

the oxygen-evolving complex, with a greater magnitude in NH plants.

3.3. Composition of photosynthetic pigments

Table 2 shows that there was a pronounced decline in total Chls with NF, especially under high PPFD. The carotenoid content per unit of Chl a was determined in cucumber plants upon exposure to NF that can produce oxidative stress (Table 2). In CL plants there was an approximately 45% decline in violaxanthin + antheraxanthin + zeaxanthin and also a slight decline in lutein and β-carotene, compared with CH plants, with no difference between the two controls in the quantity of neoxanthin. NH plants had increases in violaxanthin, antheraxanthin, lutein and zeaxanthin compared with CH plants, especially zeaxanthin newly formed, coinciding with a drastic decline in β-carotene. NL plants resulted in lower levels of the xanthophyll cycle pigments and lutein but a higher β-carotene than those of NH plants. However, NL plants also caused a great increase in antheraxanthin and zeaxanthin.

Carotenoid contents on a fresh weight basis were also examined (Table 2). Pigment levels of control plants under both PPFDs were very similar to the profiles of pigments on a Chl a basis. Under high PPFD, NF decreased drastically all pigments except zeaxanthin newly formed. In contrast to Chl a basis, carotenoid levels of NL plants were much greater than those of NH plants when indicated on a fresh weight basis. Particularly, NL plants contained 3-fold more zeaxanthin than NH plants. In low PPFD, antheraxanthin and zeaxanthin were greater in NF plants compared with control, but other pigments were lower.
Fig. 2. Levels of $q_P$, $q_N$ and non-photochemical quenching (NPQ) in leaves exposed to norflurazon (NF) under different photosynthetic photon flux densities (PPFDs). The plants were subjected to the same treatments as in Table 1. Treatment notations are the same as in Table 1. Measurements were made 3 days after NF treatment. Data represent the mean ± S.E. of three replicates. In some cases the error bar is obscured by the symbol.

3.4. Photochemical quenching and energy dissipation

The photochemical quenching ($q_P$), an estimate of the fraction of open PSII centers, was lower in NF plants, with a greater magnitude of decline in NH plants, compared with control plants (Fig. 2). The lowest value of $q_N$ was observed in CL plants. NL plants exhibited much greater level of NPQ than that of NH plants whereas both plants had similar level of high $q_N$. A considerable amount of NPQ existed in control plants at high PPFD, compared with ones at low PPFD. The combined quenching of $q_P$, $q_N$ and NPQ was the lowest in NH plants.

Table 3
Effects of norflurazon (NF) exposure at different photosynthetic photon flux densities (PPFDs) on antioxidant enzyme activities, catalase (CAT) ($\mu$mol O$_2$ min$^{-1}$ mg$^{-1}$ protein), SOD (units mg$^{-1}$ protein) and other enzymes ($\mu$mol min$^{-1}$ mg$^{-1}$ protein)$^a$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CAT</th>
<th>POD</th>
<th>SOD (units mg$^{-1}$ protein)</th>
<th>APX</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH</td>
<td>1.04 ± 0.02</td>
<td>0.063 ± 0.002</td>
<td>84.5 ± 1.7</td>
<td>0.67 ± 0.08</td>
<td>0.100 ± 0.001</td>
</tr>
<tr>
<td>NH</td>
<td>0.48 ± 0.01</td>
<td>0.170 ± 0.003</td>
<td>137.5 ± 16.1</td>
<td>1.06 ± 0.02</td>
<td>0.098 ± 0.003</td>
</tr>
<tr>
<td>CL</td>
<td>1.00 ± 0.06</td>
<td>0.143 ± 0.009</td>
<td>128.8 ± 8.0</td>
<td>0.75 ± 0.05</td>
<td>0.105 ± 0.002</td>
</tr>
<tr>
<td>NL</td>
<td>1.05 ± 0.02</td>
<td>0.164 ± 0.009</td>
<td>124.9 ± 11.8</td>
<td>0.98 ± 0.06</td>
<td>0.105 ± 0.003</td>
</tr>
</tbody>
</table>

$^a$ The plants were subjected to the same treatments as in Table 1. Treatment notations are the same as in Table 1. Measurements were made 3 days after NF treatment. Data represent the mean ± S.E. of three replicates.

3.5. Responses of antioxidant enzyme activities

The influence of NF on the activities of antioxidant enzymes participating in the scavenging of oxidative stress is shown in Table 3. In addition to the three primary enzymes of the Mehler-peroxidase pathway (SOD, GR and APX), we measured activities of CAT and guaiacol-POD. Activity of CAT was much lower in NH plants relative to the other plants, whereas POD and SOD activities in NH, CL, and NL plants were greater when compared with CH plants. APX activity exhibited a considerable increase in relation to NF, but only slight increase in control plants grown at low PPFD. Neither NF nor PPFD level resulted in a discernible change in GR activity among treatments.

The isoform composition of different enzymes was analyzed by native PAGE. Gels stained for CAT revealed no isoform (Fig. 3A), and were in accordance with the induction profile of CAT specific activity, with the lowest activity in NH plants. POD and SOD exist as multiple isoforms (Figs. 3B and 4A). In the profile of POD activity in control plants, four bands (1, 2, 3 and 4) were detected, with staining intensity increased both with NF and with low PPFD (Fig. 3B, lanes b, c and d). NF induced a new band, which has a quite strong staining intensity (Fig. 3B, lanes b and d). The induction of POD isoform 5 was quite NF-specific. Three isoforms were observed when native gels were stained for SOD activity, and no major change was observed in the activity of isoform 3 in the leaves treated with NF (Fig. 4A). SOD isoform 1 was identified as Mn-SOD by its insensitivity to KCN and H$_2$O$_2$, whereas SOD isoforms 2 and 3 were inhibited by both KCN and H$_2$O$_2$, suggesting that they represented Cu,Zn-
Fig. 3. Influence of norflurazon (NF) and photosynthetic photon flux density (PPFD) on the isozyme profiles of catalase (CAT) (A) and peroxidase (POD) (B) of \textit{C. sativus}. The plants were subjected to the same treatments as in Table 1. Non-denaturing activity gels were prepared and run as described in Section 2. Lane a, CH; lane b, NH; lane c, CL; lane d, NL. Treatment notations are the same as in Table 1.

SOD activity. No Fe-SOD isoform was observed in all treatments. Both NF and low PPFD enhanced the intensity of the existing isoform 2, and Mn-SOD also increased slightly. The overall increase of total SOD activity in NF plants and CL plants may result from the increase of isozyme 2. NF enhanced significantly the intensity of the existing APX compared with CH plants, with maximum activity in NH plants (Fig. 4B). However, low PPFD slightly increased the intensity of APX in control plants. A GSSG-specific GR band was present in \textit{C. sativus} (Fig. 4C). The intensities of GR in all treatments remained unaffected by NF or PPFD.

4. Discussion

Exposure of \textit{C. sativus} to NF substantially inhibited photochemical efficiency of photosynthesis, indicated as a decrease in $F_v/F_m$ and quantum yield of PSII electron transport (Table 1). This inhibition could be explained in large part by the photodestruction of plastid components in the deficiency of protecting carotenoids. Both $F_v/F_m$ ratio and quantum yield were significantly different over high and low PPFDs by NF treatments, indicating that the level of PPFD affects structural and functional modifications of the photosynthetic...
apparatus. The relatively small decline in $F_a/F_m$ and quantum yield in NL plants shows that NF-induced photoinhibition was alleviated during NF exposure in lower PPFD. It has been reported that the decrease in $F_a/F_m$ results from change of reaction centers to quenchers by excess light [36]. In NF plants, however, the decrease is probably due to the loss of PSII reaction centers as indicated by the loss of 33-kDa protein of oxygen evolving complex (Fig. 1). The $F_0$ level is known to be affected by environmental stress that causes structural alterations in the PSII complex [36]. The $F_0$ decrease in NH plants (Table 1) might arise from markedly low level of Chls. On the other hand, the $F_0$ increase in NL plants is probably associated not only with low PSII photochemistry ($K_p$) but also with low $K_T$, which is related to excitation energy transfer to non-fluorescent pigments, although the precise reasons for the decline remain to be determined.

The decrease in $F_a/F_m$ is also due to the formation of a new quencher of excitation energy, the xanthophyll cycle pigment zeaxanthin. Less amount of violaxanthin + antheraxanthin + zeaxanthin accounts for less requirement for $q_N$ and NPQ in CL plants due to low photoinhibitory stress, compared with CH plants (Table 2 and Fig. 2). The treatment with NF caused a significant accumulation of antheraxanthin and zeaxanthin, but a concomitant reduction in $\beta$-carotene on a Chl $a$ basis and in Chls with a greater magnitude under high PPFD (Table 2). The profile of pigment levels on a fresh weight basis in NF plants was obviously different from that of Chl $a$ basis, with an overall reduction in the other pigments except zeaxanthin. The newly formed zeaxanthin in the presence of carotenoid biosynthesis inhibitor (Table 2) confirms the possibility that zeaxanthin plays a role in the photoprotective mechanism [9,28]. A decline of photochemical efficiency in NF plants is associated with a decrease in $q_p$ and an increase in $q_N$ (Table 1 and Fig. 2). A lower NPQ of NH plants, as compared with NL plants, exhibited a close correlation with level of antheraxanthin and zeaxanthin on a fresh weight basis, not per Chl $a$ content. Interestingly, the irradiance environment during NF-caused oxidative stress dramatically affects zeaxanthin-related NPQ, not $q_N$. In contrast to $q_N$, NPQ is proportional to the effective rate constant for energy dissipation in the antennae as well as the concentration of quenching centers [37]. The results provide evidence that energy dissipation through the xanthophyll cycle does not completely explain for the acclimatory process of the plants to oxidative stress caused by NF. Thus the plants necessitate other components of dissipative process.

Antioxidant enzymes are likely to play a considerable part of defense mechanism against NF-induced oxidative stress. CAT takes part in an efficient protective role to oxidative stress [38], however, in this study NH plants exhibited a significant decline in CAT activity (Table 3), consistent with reports of CAT inactivation in leaves exposed to high light [39,40]. The increase in the activities of POD, SOD and APX might occur against oxidative stress or serve to compensate for low CAT activity (Table 3). The ascorbate-glutatione cycle has been known to be activated under oxidative stress conditions [38]. However, GR, a rate-limiting enzyme in the $H_2O_2$-scavenging cycle [41], was not altered in all treatments by different PPFDs (Table 3), suggesting that the functioning of the cycle was not activated efficiently in NF plants. The Mehler-peroxidase pathway has been postulated to cause not only $q_p$, but also the build-up of $\Delta pH$ and consequent zeaxanthin formation [11,13]. Considering similar levels of the antioxidant enzymes except CAT in NH and NL plants, the response of these enzymes to NF may not depend on the level of PPFD. The increase of APX activity appears to be NF-dependent, but the activities of POD and SOD are considerably increased even in CL plants (Table 3).

The induction of individual isozymes to NF is interpreted as a response to augmented AOS generation. A membrane-bound scavenging system within chloroplasts, that is functionally linked to PSI, has been suggested to intercept the toxic oxygen species very close to the site of formation [16]. NF-induced SOD activity is mainly due to increased expression of Cu,Zn-SOD-2 isoform, and also to Mn-SOD isoform to some extent (Fig. 4A). Cu,Zn-SOD, which is associated with the PSI complex, results in a great increase of its mRNA level upon NF-induced photooxidation [42], and another up-regulated antioxidant, Mn-SOD is suggested to confer protection against NF [43]. NF caused also a prominent increase in APX staining activity in NF plants compared with control (Fig. 4B), indicating a possible protective role of these
enzymes to oxidative stress. Both NF and PPFD exert differential effects on the expression of the multiple forms of POD (Fig. 3B). The NF-treated plants were capable of synthesizing a new isofom of POD, which could be considered as a response to NF-caused oxidative damage. The results suggest that enzymatic removal of H2O2 by POD and APX is the dominant pathway during NF-induced enzymatic scavenging system.

In the NF-treated leaves of C. sativus, the detoxification of AOS is undertaken through functionally interrelated antioxidant mechanisms. The qP and ΔpH-dependent qN through the Mehler-peroxidase pathway, in addition to zeaxanthin formation, take part in mitigating oxidative stress induced by NF. NF-induced photooxidation in combination with high PPFD causes severe photoinhibition, which may arise from an insufficient capacity to dissipate excess excitation energy through qP and NPQ. The plants, however, seem to develop a component of photoinhibitory quenching, qI, which is related to light-dependent alterations in the PSI reaction center complex and frequently indicated as a decrease in Fv/Fm [36]. Interestingly, the level of PPFD influences qP and NPQ in response to NF, not the induction of antioxidant enzymes. This might be explained by the fact that PSI is more stable than PSII upon strong light [4] and that the AOS-scavenging enzymes of the chloroplast may serve to protect PSI.

References


