Action Spectrum of Photoinhibition in Leaves of Wild Type and npq4-1 Mutants of Arabidopsis thaliana

Päivi Sarvikas, Marja Hakala, Eija Pätsikkä, Taina Tyystjärvi and Esa Tyystjärvi

Introduction

Light is essential for photosynthesis, but light also damages the photosynthetic apparatus. The most light-sensitive component of the photosynthetic machinery is PSII, and in the following we use the term photoinhibition for light-induced damage to PSII. After photoinhibition, the D1 reaction centre protein is replaced by a new copy in the repair process of PSII. In natural conditions, light stress becomes obvious if the rate of photoinhibition exceeds the rate of repair (for reviews of photoinhibition, see Aro et al. 1993, Melis 1999).

Photoinhibition is caused by both visible and UV light. The rapid photoinhibition and D1 protein degradation under UV light is most probably mediated by light absorption of the manganese (Mn) of the oxygen-evolving complex (OEC) (Barbato et al. 1995, Hakala et al. 2005, Ohnishi et al. 2005). The forms of the absorbance spectra of all Mn$^{3+}$ and Mn$^{4+}$ complexes indicate that if Mn is important for photoinhibition in the UV range, then Mn also contributes significantly to photoinhibition under visible light. We have proposed a detailed mechanism in which photoinhibitory damage is triggered by excitation of an Mn ion of OEC and a subsequent Chl-dependent step is responsible for the final, irreversible inhibition of PSII (Hakala et al. 2005). The Mn hypothesis is supported by the findings that Mn ions dissociate from the OEC during photoinactivation and that the primary photoinhibitory damage can be localized to the OEC under both UV and visible light (Hakala et al. 2005, Ohnishi et al. 2005). In contrast to the Mn hypothesis, most of the previously suggested molecular mechanisms of photoinhibition are related to visible light only. According to the acceptor side mechanism (Vass et al. 1992), visible light-induced photoinhibition is triggered by reduction of the plastoquinone pool, which promotes double reduction, protonation and loss of the primary quinone acceptor ($Q_A$). Singlet oxygen ($O_2^*$) evolves as a side product. In the low light photoinhibition hypothesis (Keren et al. 1997) $O_2^*$ produced in recombination reactions is in itself responsible for the loss of PSII activity. It has also been suggested that the damaging species is $O_2$ produced by illumination of non-haem iron (Jung and Kim 1990) or uncoupled Chls (Santabarbara et al. 2001). The donor side photoinhibition mechanism (Callahan et al. 1986, Chen et al. 1992, Anderson et al. 1998) explains photoinhibition by malfunction of the OEC. If the OEC does not properly reduce the primary electron donor $P_{680}^+$, then $P_{680}^+$ may cause harmful oxidations in PSII.

Photoreceptors of photochemical reactions may be identified by action spectroscopy. In the case of photoinhibition, quantification of the response to different wavelengths is easy, as photoinhibition is known to follow first-order reaction kinetics (Jones and Kok 1966, Tyystjärvi and Aro 1996). Several
action spectra of photoinhibition in isolated thylakoids of higher plants are available: Jones and Kok (1966) from 220 to 700 nm, Renger et al. (1989) from 250 to 350 nm, Jung and Kim (1990) from 360 to 700 nm, Santabarbara et al. (2001) from 640 to 720 nm, and Hakala et al. (2005) from 254 to 700 nm. Recently, Ohnishi et al. (2005) measured the action spectrum of photoinhibition in isolated thylakoids of a thermophilic cyanobacterium from 300 to 700 nm. The action spectra of photoinhibition (Jones and Kok 1966, Renger et al. 1989, Jung and Kim 1990, Hakala et al. 2005, Ohnishi et al. 2005) are in good agreement with each other in the UV region. In the visible range, the action spectrum measured by Jones and Kok (1966) shows clear similarity with the absorbance spectrum of Chl, whereas the spectra of Jung and Kim (1990), Hakala et al. (2005) and Ohnishi et al. (2005) are relatively flat in the visible range and have only a minor peak in the red region. Santabarbara et al. (2001) concluded that the red peak of their detailed action spectrum of photoinhibition resembles the absorbance spectrum of free Chl molecules but not the absorbance spectrum of the antenna Chl of PSII. The in vitro action spectra can be interpreted as showing absorption of Mn$^{3+}$ or Mn$^{4+}$ complexes, but the spectroscopic evidence requires support from other types of experiments since the absorbance spectrum of the OEC is not available and the absorbance spectra of model compounds of the OEC differ substantially from each other in both the UV and visible ranges (Hakala et al. 2005).

Thus far the action spectrum has not been measured for photoinhibition in vivo from higher plant leaves, although measurements of the general damaging effect of UV light (240–400 nm) on phytoplankton reveal an important contribution of photoinhibition (Boucher and Prezelin 1996). Tyysjärvi et al. (2002) measured the in vivo action spectrum of photoinhibition from cyanobacteria between 365 and 700 nm and concluded that the spectrum is very similar to the action spectra measured from isolated higher plant thylakoids. Greenberg et al. (1989) measured the action spectrum of D1 protein degradation in vivo from Spirodela oligorrhiza and concluded that the spectrum resembles the absorbance spectrum of quinones in UV to blue light transition and the bulk photosynthetic pigments in the visible and far red light regions. However, conclusive evidence exists against the function of the PSII quinones as photoreceptors of photoinhibition (Spetea et al. 1996).

In the present study, we report the action spectrum of photoinhibition measured from intact leaves of wild-type Arabidopsis thaliana and from two mutants, npq1-2 (Niyogi et al. 1998) and npq4-1 (Li et al. 2000). The light-induced component of non-photochemical quenching (NPQ; also referred to as feedback de-excitation) is impaired in the two mutants to different extents. The NPQ mechanism is considered as a protective mechanism against visible light-induced photoinhibition (Li et al. 2002b). Since NPQ would only protect against photoinhibition caused by light absorption by the Chl antenna of PSII, we compared the action spectra of NPQ-less and wild-type plants to calculate the relative importance of Chl as the photoreceptor of photoinhibition at different wavelengths. Furthermore, the action spectra measurements allowed us to draw conclusions about the photoinhibitory importance of different wavelength regions of sunlight.

**Results**

**Action spectrum of photoinhibition in vivo**

We measured the action spectrum of photoinhibition in vivo from young leaves of wild-type and two mutant strains of *A. thaliana*. The npq1-2 strain lacks a functional violaxanthin de-epoxidase and npq4-1 is almost fully NPQ deficient, whereas npq1-2 shows less NPQ than the wild type but more than npq4-1 (Li et al. 2000). In order to inhibit concurrent recovery from photoinhibition, the leaves were treated with lincomycin, an antibiotic that specifically blocks chloroplast-encoded protein synthesis (Mulo et al. 2003). The first-order reaction constant of photoinhibition ($k_{PI}$) was used as a measure of photoinhibition. The in vivo action spectrum (Fig. 1) revealed a strong photoinhibitory response to UV light wavelengths (360 and 254 nm), which tailed into the blue light region. The action spectra of the three strains decreased between 400 and 500 nm with minor shoulders around 450–460 and 480 nm. Between 500 and 700 nm, the action spectra were relatively flat, with

![Action spectrum of photoinhibition in vivo](image-url)
small, indistinct peaks around 530, 650 and 680 nm in the spectrum of the wild type and npq4-1. A wide, shallow peak between 600 and 700 nm was detectable in all spectra. Differences in the heights between the peaks were small in all three spectra.

The photoinhibition experiments were done by illuminating leaves with either monochromatic high-intensity visible light or UV light. The resulting action spectrum is meaningful only if the quantum yield of photoinhibition is independent of photon flux density (PFD) during the measurements. Fig. 2 shows that this condition is fulfilled under both UV light and monochromatic visible light. The $k_{PI}$ values measured under both visible and UV light are lower than those in Fig. 1 because mature leaves were used in the experiments of Fig. 2.

**Protection against photoinhibition by NPQ**

The action spectra of the npq1-2 and npq4-1 mutants had a similar shape to the wild-type spectrum (Fig. 1). All three strains were equally sensitive to photoinhibition between 490 and 530 nm light. When compared with the wild type, both
NPQ-deficient mutants were slightly more sensitive to photoinhibition under all other visible light wavelengths. When the \( k_{\text{PI}} \) values measured at each visible light wavelength were averaged, \( npq1-2 \) was found to be 15% and \( npq4-1 \) 25% more sensitive to visible light photoinhibition than the wild type. The slightly higher sensitivity of the \( npq4-1 \) mutant, compared with both the wild type and \( npq1-2 \), was most clear under blue (420–440 nm) and red (650 and 680 nm) light. The high sensitivity of the \( npq4-1 \) strain at 680 nm extended to the 680–700 nm range. Some differences between the sensitivities of the three strains to UV-induced photoinhibition were also found, but these differences did not show a consistent relationship with NPQ.

Thick leaves with high Chl content are less sensitive to photoinhibition than thin leaves because the flux of quanta absorbed per PSII decreases with increasing Chl content (Pätsiakk et al. 2002). This protection by Chl occurs independently of the photoreceptor of photoinhibition. Differences in the Chl content of the individual leaves and a weak negative correlation \( r = -0.23 \) between \( k_{\text{PI}} \) and leaf Chl content partly explain the within-data point variation of the \( k_{\text{PI}} \) values of the present study. However, the mean Chl concentrations were similar in all three strains; 15.5 ± 2.8 µg cm\(^{-2}\) in the wild-type, 15.7 ± 3.3 µg cm\(^{-2}\) in \( npq1-2 \) and 15.2 ± 2.4 µg cm\(^{-2}\) in \( npq4-1 \). Thus, the differences in the \( k_{\text{PI}} \) values among the plant strains were not caused by differences in the optical thickness of the leaves.

The differences in the mean visible light \( k_{\text{PI}} \) values of the three strains were apparently related to the low NPQ capacities of \( npq1-2 \) and \( npq4-1 \). To establish a quantitative relationship between NPQ and photoinhibition, we determined how much NPQ was induced during the photoinhibition treatments in each strain using similar lincomycin-treated leaves to those used in the photoinhibition treatments. NPQ was measured after a 6 min period of actinic illumination (450, 550 or 660 nm, as indicated) under the same PFD (1,600 µmol m\(^{-2}\) s\(^{-1}\)) as applied in the photoinhibition treatments. The measurements confirmed that \( npq1-2 \) had 44–50% lower NPQ and \( npq4-1 \) had 67–77% lower NPQ, depending on the wavelength, than the wild type (Fig. 3A). All strains showed the highest NPQ level at 660 nm.

Comparison of the rate constant of photoinhibition \( \left(k_{\text{PI}}\right) \) with NPQ in the three strains showed that \( k_{\text{PI}} \) increased linearly with decreasing NPQ (Fig. 3A). The relationship between \( k_{\text{PI}} \) and NPQ was wavelength dependent, and in all cases the differences in \( k_{\text{PI}} \) values between the wild type, \( npq1-2 \) and \( npq4-1 \) were relatively small, showing that NPQ may vary significantly without having a great impact on photoinhibition. Because the mutant plants retain some capacity for NPQ, we extrapolated the data in Fig. 3A to zero NPQ to obtain an estimate of the full protective capacity of NPQ. The \( k_{\text{PI}} \) values calculated for such a theoretical NPQ-0 plant were slightly higher than the actual \( k_{\text{PI}} \) values of the \( npq4-1 \) mutant. In comparison with the theoretical NPQ-0 plant, NPQ can be interpreted to protect wild-type plants from photoinhibition by 30% at 450 nm, 20 at 550 nm and 10% at 660 nm (Fig. 3B).

In addition to low NPQ values, the \( npq1-2 \) and \( npq4-1 \) mutants had lower quantum yield of PSII electron transport \( \left(\Phi_{\text{PSII}}\right) \) than the wild type (Table 1). All three strains showed the highest \( \Phi_{\text{PSII}} \) values in blue light (450 nm), possibly because blue light is important for stomatal opening (see Short and Briggs 1994). We determined photochemical quenching using the q\( _L \) parameter that assumes energy transfer between PSII centres (Kramer et al. 2004). Depending on the wavelength, the q\( _L \) values of the \( npq1-2 \) strain were 58–62% of the wild-type values, and the q\( _L \) values of the \( npq4-1 \) strain were 38–47% of those of the wild type. Similar differences in photochemical quenching between the mutants and the wild type were found by using the traditional q\( _L \) parameter that assumes direct proportionality between variable fluorescence and the proportion of reduced Q\( _A \) (data not shown). We also estimated the quantum yield of PSII electron transfer per the number of open reaction centres by dividing \( \Phi_{\text{PSII}} \) by q\( _L \). The wild-type

<table>
<thead>
<tr>
<th>Arabidopsis strain</th>
<th>Wavelength (nm)</th>
<th>( \Phi_{\text{PSII}} )</th>
<th>q( _L )</th>
<th>( \Phi_{\text{PSII}}/q_{\text{L}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>450</td>
<td>0.39 ± 0.04</td>
<td>0.40 ± 0.06</td>
<td>1.0 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>550</td>
<td>0.29 ± 0.05</td>
<td>0.26 ± 0.03</td>
<td>1.1 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>660</td>
<td>0.23 ± 0.05</td>
<td>0.19 ± 0.03</td>
<td>1.2 ± 0.11</td>
</tr>
<tr>
<td>npq1-2</td>
<td>450</td>
<td>0.29 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>1.2 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>550</td>
<td>0.24 ± 0.04</td>
<td>0.16 ± 0.04</td>
<td>1.5 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>660</td>
<td>0.18 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>1.7 ± 0.12</td>
</tr>
<tr>
<td>npq4-1</td>
<td>450</td>
<td>0.25 ± 0.06</td>
<td>0.15 ± 0.04</td>
<td>1.6 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>550</td>
<td>0.20 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td>2.1 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>660</td>
<td>0.18 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>2.0 ± 0.08</td>
</tr>
</tbody>
</table>

Chl a fluorescence was measured from young leaves of the wild type and the \( npq1-2 \) and \( npq4-1 \) mutants of *Arabidopsis*. Data are mean values of 4–5 experiments ± SE.
values of $\Phi_{\text{PSII}}/q_L$ ranged from 1.0 to 1.2, suggesting that energy transfer from closed to open reaction centres accounts for 0–17% of PSII electron transport. In the NPQ-deficient mutants, values of $\Phi_{\text{PSII}}/q_L$ were significantly higher than in the wild type, suggesting that up to 50% of electron transport was due to photons absorbed by closed reaction centres (Table 1). This behaviour results partly from the higher proportion of closed PSII centres in the npq1-2 and npq4-1 mutants. In part, the better photosynthetic efficiency of photons absorbed by closed reaction centres in the NPQ-deficient mutants results from their low NPQ. NPQ shortens the lifetime of antenna excitons (Li et al. 2002a), and therefore an exciton in the antenna of a closed reaction centre of an NPQ-deficient mutant has more time to find an open reaction centre than an exciton in the antenna of wild-type PSII.

Comparison of the action spectrum of photoinhibition with potential photoreceptors

The purpose of action spectroscopy is to identify photoreceptor(s) of photochemical reactions. To compare the in vivo action spectrum with the absorbance spectrum of the light-harvesting antenna of PSII in the same experimental system, we measured the excitation spectrum of PSII fluorescence. The excitation spectrum was measured from 410 to 680 nm at 77 K using similar young Arabidopsis leaves and the same narrow band filters as in the photoinhibition measurements. The emission was measured at 695 nm. The excitation spectra of PSII fluorescence (Fig. 4) were found to resemble the action spectrum of PSII activity measured from thylakoids by Boichenko (1998), although the in vivo spectrum shows proportionally higher efficiency of green light. The excitation spectra of PSII fluorescence of the wild type and the npq4-1 mutant were found to be essentially similar, but the npq1-2 strain emitted slightly more between 500 and 650 nm than the other two strains (Fig. 4). Comparison of the in vivo absorbance spectrum of PSII antenna with the in vivo action spectrum of photoinhibition shows, in accordance with earlier results, that the red peak in the action spectrum of photoinhibition can be superficially fitted with the red peak of the absorbance spectrum of the Chl antenna (thick solid line in Fig. 5). However, from red to blue light, the action spectrum of photoinhibition gradually deviated upwards from the excitation spectrum of PSII fluorescence, and finally, below 470 nm, the two spectra could not be fitted together, indicating that the Chl antenna of PSII is not the sole photoreceptor of photoinhibition in visible light. We also compared the action spectrum of
photoinhibition in vivo with the absorbance spectra of model compounds of the oxygen-evolving Mn cluster: Mn$^{4+}$-glucocate (Bodini et al. 1979), [Mn$_{2}$(µ-O)$_{3}$(2,2′:6,2″-terpyridin)$_{3}$]$^{+}$ (Baffert et al. 2002) and [Mn$_{2}$($^{4+}$)µ-O$_{2}$(2,2′:6,2″-terpyridin)$_{3}$]$^{2+}$ (Baffert et al. 2002). These spectra showed general similarity with the action spectrum of photoinhibition, showing high UV absorbance that tails into the visible region (Fig. 5).

**Photoinhibition in sunlight**

Multiplication of an action spectrum by the emission spectrum of a light source yields a spectrum that shows the relative importance of each wavelength in the reaction under study, if the rate constant of the reaction is directly proportional to the light intensity. Direct proportionality between $k_{PI}$ and photosynthetic photon flux density (PPFD) has been shown for visible light-induced photoinhibition (Tyystjärvi and Aro 1996), and we checked that the $k_{PI}$ of UV-light-induced photoinhibition is also directly proportional to light intensity (Fig. 2A). A multiplication of the action spectrum by the photon number spectrum of sunlight on the Earth shows that under sunlight, 84% of photoinhibition is caused by UV light (Fig. 6). This calculation is only a rough estimate because the UV interpolation was based on just two wavelengths. A similar calculation with the in vitro action spectrum of photoinhibition (Jones and Kok 1966), which is more detailed in the UV region than our action spectrum, shows that if isolated thylakoids were exposed to sunlight, then the contribution of the UV wavelengths to photoinhibitory damage would be 56% (data not shown). Calculations using the in vivo action spectra of the mutant strains predict that the npq1-2 mutant would be equally sensitive as the wild type. We tested the calculation experimentally by exposing lincomycin-treated small leaves of wild type, npq4-1 and npq1-2 to sunlight (PPFD 1,300–1,900 mmol m$^{-2}$ s$^{-1}$). In three independent pairwise comparisons, the $k_{PI}$ values of npq1-2 were 1.06 ± 0.19 and those of npq4-1 were 1.11 ± 0.15 times as high as those of the wild type.

**Discussion**

**Photoreceptors of photoinhibition**

The action spectrum of photoinhibition has been measured previously from visible and UV light regions in vitro (Jones and Kok 1966, Renger et al. 1989, Jung and Kim 1990, Santabarbara et al. 2001, Tyystjärvi et al. 2002, Hakala et al. 2005, Ohnishi et al. 2005). In this study, we report the first in vivo measurement of the action spectrum of photoinhibition from a higher plant. The in vivo action spectrum shows the same general features as the in vitro spectra, with a steady decrease of photoinhibitory efficiency from UV-C light to the blue-green region, and a relatively flat visible region where the average photoinhibitory efficiency is approximately 1% of the photoinhibitory efficiency of UV-C light (Fig. 1). The in vivo spectrum is more flat in the visible region and shows a higher sensitivity to UV-A light than the action spectra measured from isolated thylakoids (Jones and Kok 1966, Hakala et al. 2005, Ohnishi et al. 2005).

The action spectra of photoinhibition, measured in vitro, have been interpreted to show contributions from several different photoreceptors, including the Chls (Jones and Kok 1966, Jung and Kim 1990, Santabarbara et al. 2001), iron–sulfur centres (Jung and Kim 1990) and Mn (Hakala et al. 2005, Ohnishi et al. 2005). The existence of a peak in red light in the action spectrum of photoinhibition is the main argument favouring the importance of Chl as a photoreceptor of photoinhibition. We compared the action spectrum of photoinhibition with the absorbance spectrum of PSII antenna of the same experimental material, adjusting the two spectra so that the red peaks fit optimally (Fig. 5). This comparison indicates that although the red peak of the action spectrum of photoinhibition superficially matches the red peak of Chl absorbance, photoinhibition caused by blue or green light cannot be explained without assuming a strong contribution from another photoreceptor. Comparison of the action spectrum of photoinhibition with the absorbance spectra of the Mn model compounds shows general similarity with a long, flat visible light tail and an increase towards UV, starting in blue light (Fig. 5). This similarity and the other evidence favouring the role of Mn in photoinhibition (Hakala et al. 2005, Ohnishi et al. 2005) strongly suggest that Mn is the second photoreceptor, although no precise match can be demonstrated between the in vivo action spectrum of photoinhibition and the absorbance spectra of the model compounds of the oxygen-evolving Mn cluster. The ratio between the photoinhibitory efficiency of UV light, compared with the

**Fig. 6** The relative photoinhibitory efficiencies of different wavelength regions of sunlight. The normalized product of the photon number spectrum of sunlight and the action spectrum of photoinhibition in vivo in wild-type Arabidopsis (solid line) and the photon number spectrum of sunlight (dotted line). A linear interpolation between data points was used in the UV region, where the in vivo spectrum consisted of only two data points.
photoinhibitory efficiency of visible light, resembles more closely the absorbance spectra of Mn model compounds in the in vitro action spectra (Hakala et al. 2005) than in the in vivo action spectrum (Fig. 5).

The difference between in vivo and in vitro action spectra may partially be caused by the fact that due to their high Chl content, leaves are optically thicker in both visible and UV regions than thylakoid suspensions used in photoinhibition experiments. Because Chl absorbs visible light better than UV light, the protective effect of Chl (Päätikkä et al. 2002) is more important in visible than in UV light. Furthermore, leaves may contain varying amounts of flavonoid pigments that screen visible wavelengths, protecting against photoinhibition specifically in the 500–600 nm wavelength region (Gould 2004). Leaves also show such responses to light quality and intensity that are absent in isolated systems. Strong light induces the movement of chloroplasts towards the anticlinal cell walls (for reviews see Briggs and Christie 2002, Wada et al. 2003), and mutant plants lacking this avoidance response are more susceptible to photodamage than wild-type plants (Kasahara et al. 2002). Because the photoreceptors of chloroplast movements are sensitive to blue and UV light, the movements are expected to lower the relative photoinhibitory efficiency of blue and UV-A light in Arabidopsis leaves, in comparison with action spectra measured from isolated thylakoids. Chloroplasts move at velocities above 1 μm min⁻¹ (Kagawa and Wada 2004) and would thus largely complete the avoidance response in the course of the 20 min illumination treatment, but the extent to which the avoidance response actually affected the action spectra during the 20 min treatment time remains to be studied.

NPQ occurs in the Chl antenna of PSII, and therefore the efficiency of NPQ in protection against photoinhibition also offers a measure of how important a photoreceptor the Chl antenna is for photoinhibition. The result of the estimation depends on the model of photoinhibition, but if we assume that the PSII electron transfer chain is involved in photoinhibition, then we can assume that photoinhibition is slowed down by NPQ as much as photosynthetic electron transport. A calculation based on experiments with fully developed leaves of Arabidopsis wild type and the npq4-1 mutant showed that approximately one-third of photoinhibition, induced by white visible light, is independent of photons absorbed by PSII antenna (Tyystjärvi et al. 2005). The results of the present study are in agreement with this earlier study, showing that the protective efficiency of NPQ is smaller than would be expected if Chl antenna-dependent photoinhibition was the only mechanism of photoinhibition functioning under visible light.

Effect of NPQ on plant fitness

Comparison of photoinhibition between the wild type and two NPQ-deficient mutants of Arabidopsis indicates that the maximum protective efficiency of the NPQ mechanism against visible light-induced photoinhibition is relatively small (Fig. 3), in agreement with earlier studies (Grasses et al. 2002, Li et al. 2002b, Tyystjärvi et al. 2005). The small magnitude of the NPQ effect also explains why NPQ-deficient plants do not show symptoms of chronic photoinhibition when grown under high light in the laboratory (Niyogi et al. 1998, Havaux and Niyogi 1999, Li et al. 2000, Niyogi et al. 2001). Because the rate constant of photoinhibition is directly proportional to PFD (Tyystjärvi and Aro 1996), 9% higher sensitivity to photoinhibition, as we calculated for npq4-1 compared with the wild type under sunlight, is equivalent to exposure of npq4-1 to 9% more intense light. Such a small increase in growth light intensity would not be expected to cause any symptoms of photoinhibition when the concurrent repair of PSII functions. Experiments confirmed the result of this calculation. The reason for the small difference in the calculated response to sunlight is that the photoinhibitory efficiency of sunlight’s UV component, against which NPQ does not provide protection, is four times higher than the contribution of the visible part of sunlight’s spectrum (Fig. 6). This result confirms earlier conclusions (Vass et al. 1992, Turscanyi and Vass 2000, Larkum et al. 2001) that damage to photosynthesis contributes significantly to the overall damage caused by UV light to plants. The very high photoinhibitory efficiency of UV light may be related to the young age and growth conditions of the leaf material used in the present study. The in vitro action spectrum of photoinhibition (Jones and Kok 1966) would suggest that more than half of the photoinhibitory efficiency of sunlight is due to the UV part. UV-absorbing compounds, especially flavonoids and hydroxycinnamic acids, but also anthocyanins, synthesized by plants grown under natural sunlight (Cockell and Knowland 1999, Day and Neale 2002) are expected to lower the photoinhibitory efficiency of the UV part of sunlight in comparison with plants grown under artificial illumination.

Even though protection by NPQ is a minor effect, NPQ-deficient plants consume more resources to repair PSII after photoinhibition, which may in part explain why NPQ-deficient mutant plants are less fit in field conditions than wild-type Arabidopsis (Kühlheim et al. 2002). It should also be noted that NPQ protects against damaging reactions other than photoinhibition, e.g. against oxidative damage (Demming-Adams and Adams 1992, Horton et al. 1996, Niyogi 1999), and that NPQ may be an important factor in the regulation of the balance between the two photosystems (Peterson and Havir 2001). Our results also show that the in vivo photosynthetic electron transfer rate was slower in the young leaves of NPQ-deficient mutants than in the wild type (Table 1). These additional effects may turn out to be more important than photoinhibition in lowering the fitness of NPQ-deficient plants in the field.

In summary, our results show that the action spectrum of photoinhibition in vivo is basically similar to the action spectrum of photoinhibition in vitro, indicating that the same reactions occur in both. Our earlier suggestion that photoinhibition has a photoreceptor that is not excited via the Chl antenna of PSII (Hakala et al. 2005) is supported by the finding that NPQ protects against visible light-induced photoinhibition less effi-
ciently than it would protect if photoinhibition was fully dependent on photons absorbed by PSII antenna. The high importance of blue and UV-A light in the in vivo action spectrum of photoinhibition is in accordance with our earlier suggestion that this photoreceptor is Mn. It is also apparent that a Chl-dependent reaction is involved. Further studies on the mechanism of photoinhibition are required to find out whether the different photoreceptors function in series as suggested by us (Hakala et al. 2005, see also Ohnishi et al. 2005) or whether several different photoinhibition mechanisms function simultaneously.

Materials and Methods

Plant material and growth conditions

*Arabidopsis thaliana* L. mutant strains used in this study have been described earlier (Nyogi et al. 1998, Li et al. 2000). Wild-type (Col-0), npq1-2 and npq4-1 plants were grown for 35–45 d under the light regime of 8 h light/16 h dark. PPDF was 100 µmol m⁻² s⁻¹, relative air humidity 70% and temperature 22°C. Young, approximately 1 cm long leaves were used for the experiments.

Photoinhibition treatments

Prior to illumination, the leaves were incubated overnight with their petioles in 2.3 mM lincomycin. For visible light (400–700 nm) treatments of *Arabidopsis* leaves, light from a 300 W high-pressure ozone-free xenon lamp (Oriel Instruments, Stratford, CT, USA) was focused with a pair of fused silica lenses and filtered through narrow band filters (half-band width 7–12 nm) (Corion, Holliston, MA, USA). During the treatment, the leaf was gently pressed on a moist paper, kept at 20°C, and the illuminated area (0.34 cm²) was defined with a mask. The illumination at each visible light wavelength was done under a PFD of 1,600 µmol m⁻² s⁻¹ for 20 min. PFD was measured with a photometer calibrated for its spectral response (LiCor, Lincoln, NE, USA). UV light (365 and 254 nm) was produced by an ENF-280C quartz lamp (Spectronics, Westbury, NY, USA). The half-band widths of the 365 and 254 nm peaks were 38 and 3 nm, respectively, and the illumination lasted for 8 and 5 min, respectively. The PFD of UV light was measured actinometrically according to Hatchard and Parker (1956) and the PFD values were 207 µmol m⁻² s⁻¹ at 365 nm and 174 µmol m⁻² s⁻¹ at 254 nm.

Photoinhibition under natural sunlight was tested with lincomycin-treated leaves similar to those used in the action spectra measurements. The leaves were exposed to natural sunlight in Turku during three sunny days (PPFD 1,300–1,900 µmol m⁻² s⁻¹, temperature 25–30°C) for 10–17 min, adjusting the length of the treatment according to the PPDF, and thereafter thylakoids were isolated from the treated leaves and from control leaves, and oxygen evolution activity was measured from the isolated thylakoids. The relative sensitivity of each mutant strain to photoinhibition was calculated by dividing the *k*<sub>P</sub> value of the mutant by the *k*<sub>P</sub> value of the wild type, obtained from the same experiment.

Thylakoid isolation and measurement of oxygen evolution

The illuminated area of the *Arabidopsis* leaf was cut out and ground in a small mortar in 60 ml of ice-cold thylakoid extraction buffer (40 mM HEPES-KOH, pH 7.4; 1 M glycinebetain; 0.33 M sorbitol; 1 mM EDTA; 10 mM MgCl₂) and suspended in 960 ml of PSIIs measuring buffer (40 mM HEPES-KOH, pH 7.6; 0.33 M sorbitol; 5 mM MgCl₂; 5 mM NaCl; 1 M glycinebetain; 1 mM KH₂PO₄; 5 mM NH₄Cl). Oxygen evolution was measured with a Hansatech oxygen electrode (King’s Lynn, UK) in saturating light with 0.5 mM 2,6-dimethylenbenzoquinone as electron acceptor. Unilluminated control samples were isolated in the same way.

The *k*<sub>P</sub> was calculated by fitting the light-induced decrease in oxygen evolution activity according to the first-order reaction kinetics

\[
A(t) = A(0)\exp(-k_P t)
\]

where *t* is time and *A* is the number of active PSII centres. The *k*<sub>P</sub> values measured after treatments under UV light were additionally divided by the PFD of the treatment and multiplied by 1,600 µmol m⁻² s⁻¹, to normalize the *k*<sub>P</sub> values for comparison with the visible light values obtained after treatments at 1,600 µmol m⁻² s⁻¹. This normalization procedure does not change the *k*<sub>P</sub> values measured under visible light. The light response curve of photoinhibition under both UV wavelengths and at 660 nm was measured from lincomycin-treated mature leaves of wild-type *A. thaliana* using the same light sources and filters as in the action spectra measurements, but varying the PFD of the photoinhibitory illumination.

Chlorophyll content

The Chl concentrations of the thylakoid samples were determined after the oxygen evolution activity was measured. The sample was gathered from the oxygen electrode cuvette and centrifuged for 5 min at 10,000xg. The pellet was diluted in buffered 80% acetone, filtered through an acetone-resistant filter (Millipore, Millipore Corporation, Billerica, MA, USA), and the Chl content was determined according to Porra et al. (1989). The Chl concentrations of the leaves were determined in *N,N*-dimethylformamide (Inskeep and Bloom 1985).

Emission spectrum of the sun

The photon number spectrum of sunlight on Earth was calculated from an energy spectrum provided by NASA (http://science.nasa.gov/headlines/images/sunbathing/sunspectrum.htm). The photoinhibitory contribution of each wavelength of sunlight was calculated by multiplying the sun’s photon number spectrum by the action spectrum of photoinhibition. Linear interpolation was used in the UV region where we had only two data points in the action spectrum.

Excitation spectrum of PSII fluorescence

The excitation spectrum of Chl a fluorescence (the absorbance spectrum of PSII antenna) was measured at 77 K from *Arabidopsis* leaves between 410 and 680 nm. Light from a projector lamp was first filtered through a 700 nm low-pass filter (Corion), condensed with two quartz lenses and filtered through a narrow band filter from the same filter set as was used for the action spectra of photoinhibition. Each series of emission spectra (410–680 nm) was measured from the same leaf with a diode array spectrophotometer (S2000, Ocean Optics Inc., FL, USA). The PFD of the actinic light at each wavelength was determined with a wavelength-calibrated LiCor photometer, and the PFD values were 1–16 µmol m⁻² s⁻¹, depending on the excitation wavelength. The excitation spectrum of PSII fluorescence was plotted from the emission at 695 nm after subtracting a low baseline and dividing by the PFD. The measurement was repeated with three leaves at each wavelength.

Non-photochemical quenching

NPQ was determined by fluorescence measurements at room temperature. Before the measurements, 1–2 h dark-adapted leaves were gently pressed on moist paper and attached to a cuvette with a 6 mm diameter mask. The fluorescence induction curves were measured with a PAM-101 fluorometer (Walz, Effeltrich, Germany). First, the basic fluorescence level (F<sub>i</sub>) was measured under a weak measuring beam. A saturating white light pulse (2 s; 5,000 µmol m⁻² s⁻¹) was
used to obtain maximum fluorescence ($F_{m}'$, measured after dark adaptation; $F_{m}'$, measured during continuous illumination). After the saturating pulse, actinic light (450, 550 or 660 nm, defined by the same filters as used for the action spectra of photoinhibition; 1,600 μmol m$^{-2}$ s$^{-1}$) was switched on for 6 min, after which $F_{m}'$ was induced with a saturating flash. In order to determine $F_{0}'$, a 0.5 s of darkness. The $F_{0}'$ beam was switched off and the leaf was given 2 s of far red light (>700 nm) and subsequently illuminated with five 0.5 s pulses of the measuring beam. The measuring beam pulses were separated from each other by 0.5 s of darkness. The $F_{0}'$ value was defined by extrapolating the fluorescence level at the top of each measuring beam pulse to the point when the actinic light was switched off. NPQ ($[(F_{m}' - F_{m})/(F_{m}' - F_{0}' )]$ (Bilger and Björkman 1990), $\Phi_{psII}$ ($F_{m}' - F_{p}/F_{m}'$) (Genty et al. 1989), and the photochemical quenching parameters $q_{p} [\Phi_{psII} - F_{m}]/(\Phi_{psII} - F_{p})$ (Schreiber et al. 1986) and $q_{p} (q_{p}F_{p}/F_{m})$ (Kramer et al. 2004) were calculated from the fluorescence data. $F_{m}$ is the fluorescence value measured under continuous illumination.

Acknowledgments

We thank Professor Krishna K. Niyogi from the University of California for generously providing us with the npq1-2 and npq4-1 seeds of A. thaliana. Mari Tikkanen is thanked for assisting in the experiments. This work was financially supported by Academy of Finland, by Finnish Cultural Foundation and by Turku University Foundation.

References


(Received September 7, 2005; Accepted January 4, 2006)