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ORIGINAL ARTICLE

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The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in *Arabidopsis npq* and *tt* mutants

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Abstract The *npq1* mutant of *Arabidopsis thaliana* (L.) Heynh. has no xanthophyll cycle due to a lack of functional violaxanthin de-epoxidase. Short-term exposure (< 2 days) of detached leaves or whole plants to the combination of high photon flux density (1,000 μmol m^{-2} s^{-1}) and low temperature (10 °C) resulted in PSII photoinhibition which was more acute in *npq1* than in the wild type. This increased photosensitivity of *npq1* at chilling temperature was attributable to the inhibition of nonphotochemical energy quenching (NPQ) and not to the absence of zeaxanthin itself. In contrast to PSII, PSI was found to be phototolerant to chilling stress in the light in both genotypes. In the long term (10–12 days), PSII activity recovered in both *npq1* and wild type, indicating that *A. thaliana* is able to acclimate to chilling stress in the light independently of the xanthophyll cycle. In *npq1*, photoacclimation involved a substantial reduction of the light-harvesting pigment antenna of PSII and an improvement of photosynthetic electron transport. Chilling stress also induced synthesis of early light-inducedproteins (ELIPs) which, in the long term, disappeared in *npq1* and remained stable in the wild type. In both genotypes, photoacclimation at low temperature induced the accumulation of various antioxidants including carotenoids (except β-carotene), vitamin E (α- and γ-tocopherol) and non-photosynthetic pigments (anthocyanins and other flavonoids). Analysis of flavonoid-deficient *tt* mutants revealed that UV/blue-light-absorbing flavonols have a strong protective function against excess visible radiations. In contrast to the defect in *npq1*, the absence of flavonoids could not be overcome in the long term by compensatory mechanisms, leading to extensive photooxidative and photoinhibitory damage to the chloroplasts. Depth profiling of the leaf pigments by phase-resolved photoacoustic spectroscopy showed that the flavonoid-related photoprotection was due to light trapping, which decreased chlorophyll excitation by blue light. In contrast to flavonoids, the xanthophyll cycle and the associated NPQ seem to be mainly relevant to the protection of photosynthesis against sudden increases in light intensity.

Keywords *Arabidopsis* (chilling stress) · Carotenoid · Chilling stress · Flavonoid · Nonphotochemical energy, quenching · Vitamin E

Abbreviations ELIP: early light-inducible protein · Fv/Fm: maximal quantum yield of PSII photochemistry · LHC(II): light-harvesting chlorophyll-protein complex (of PSII) · NPQ: nonphotochemical energy quenchng · PFD: photon flux density · PS: photosystem · TL: thermoluminescence · Vio: violaxanthin · WT: wild type

Introduction

Oxygenic photosynthesis inevitably generates reactive oxygen derivatives (namely, superoxide radicals, hydroxyl radicals, hydrogen peroxide and singlet oxygen $^1O_2$) and other oxidizing molecules (e.g. $P680^+$, the oxidized form of the reaction-center pigment of photosystem II (PSII; Asada and Takahashi 1987; Niyogi 1999). When the incident light intensity is higher than that required to saturate photosynthesis, the production of those reactive intermediates in photosynthesis can become excessive, leading to oxidative damage to the photosynthetic apparatus and to a reduction in the photosynthetic capacity – a phenomenon termed 'photoinhibition' (Aro et al. 1993).

In order to maintain their normal function under high light stress conditions, chloroplasts have developed a

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panoply of repair and protective systems (Demmig-Adams and Adams 1992; Niyogi 1999). Nonphotochemical energy dissipation (NPQ) is believed to be a major photoprotective mechanism by which light energy absorbed in excess by the light-harvesting chlorophyll-protein complexes (LHCs) of PSII is dissipated as heat (for reviews, see Demmig-Adams and Adams 1996; Horton et al. 1996; Demmig-Adams and Adams 2000). In brief, the rise in the transthylakoid proton gradient in excessive light is thought to result in protonation of specific LHCII polypeptides. Simultaneously, the ΔpH activates the violaxanthin de-epoxidase enzyme, which converts the xanthophyll violaxanthin (Vio) associated with the LHCs to antheraxanthin and zeaxanthin in the so-called xanthophyll cycle. Presumably, phototransformation of Vio and binding of protons to the LHCIIs act synergetically to induce a conformational change that is necessary for thermal energy dissipation. NPQ is a potentially protective mechanism because, by deactivating singlet-excited chlorophylls, it reduces energy delivery to the PSII reaction center and it minimizes generation of $^1O_2$ in the LHCIIs and the PSII reaction center (Demmig-Adams and Adams 2000). On the other hand, the operation of the xanthophyll cycle is probably associated with the release of xanthophyll molecules from the LHCs to the lipid phase of the thylakoid membrane (Rockholm and Yamamoto 1996; Havaux 1998). The transient presence of unbound photoprotective carotenoids in the thylakoid membrane lipid matrix in strong light is believed to be an additional feature of the photoprotective function of the xanthophyll cycle. Although thylakoid membrane lipids are especially susceptible to damage by reactive oxygen species because of the abundance of unsaturated fatty acids, the thylakoid lipid bilayer does not contain antioxidants, except vitamin E (Fryer 1992). Therefore, the light-induced appearance of zeaxanthin in the thylakoid membrane lipid phase in strong light can be considered a protective phenomenon that supplements the action of vitamin E and mitigates lipid peroxidative damage (Havaux 1998).

The recent isolation and characterization of *Arabidopsis thaliana* and *Chlamydomonas reinhardtii npq* mutants have partially confirmed the suggested functions of the xanthophyll cycle and NPQ. The *Arabidopsis npq1* mutant has no functional Vio de-epoxidase and consequently it cannot convert Vio to zeaxanthin in strong light, resulting in a drastic inhibition of NPQ (Niyogi et al. 1998). Following short-term illumination with intense white light, detached *npq1* leaves exhibited enhanced photoinhibition of PSII compared to wild type (WT) leaves (Niyogi et al. 1998). Enhanced lipid peroxidation was also observed in *npq1* leaves, and this phenomenon was directly attributed to the absence of zeaxanthin since the *npq4* mutant, which lacks NPQ but has a normal xanthophyll cycle, was much more resistant to photooxidation than *npq1* (Havaux and Niyogi 1999). The *npq1* mutation was also reported to decrease the tolerance of leaves to $^1O_2$ generated by the photosensitizer eosin (Havaux et al. 2000). However, growth of *Arabidopsis npq1* plants that are acclimated to high light was not appreciably different from that of the WT, which suggests that, in the long term, the xanthophyll cycle is not required for photoprotection (Niyogi et al. 1998). Similarly, the *Chlamydomonas npq1* and *npq4* mutants are able to survive in high light (Niyogi et al. 1997; Niyogi 1999). Possibly, during long-term adaptation, other protective processes can compensate for the defect in *npq1*.

Beside NPQ and zeaxanthin synthesis, plants possess multiple photoprotective mechanisms including adjustment of light-harvesting antenna size, synthesis of stress proteins, accumulation of lipophilic and water-soluble antioxidant molecules, enhancement of scavenging enzymatic systems and accumulation of sunscreen molecules (Demmig-Adams and Adams 1992; Chalker-Scott 1999; Niyogi 1999). In this study, we have examined those mechanisms in *Arabidopsis npq1* plants exposed in the short term and in the long term to excess light energy induced by low temperature. The combination of chilling stress and moderately elevated light intensity is known to be particularly favorable for the induction of both photoinhibition and photooxidation in higher-plant leaves. During cold treatment, the enzymes of the Calvin cycle are slowed so that the incoming light energy funnelled into the electron-transport chain becomes more excessive. This can eventually cause overexcitation of the photosystems and overreduction of the electron carriers, leading to excitation/electron 'leakage' to molecular oxygen and production of activated oxygen species. In the present study, we confirm that loss of the xanthophyll cycle in *npq1* is associated with an increased susceptibility to photoinhibition under short-term photostress at low temperature, but not during long-term photoacclimation. In general, the adaptive responses of *npq1* and WT to long-term exposure to chilling stress in the light were similar except for the chlorophyll-binding proteins, which noticeably decreased in the mutant. We observed also that *A. thaliana* plants exposed to chilling stress in the light accumulated considerable amounts of flavonoid pigments, and the photoprotective role of those non-photosynthetic pigments was studied and compared with that of the xanthophyll cycle pigments with the help of *transparent testa* (*tt*) mutants.

Materials and methods

Plant material and growth conditions

Wild-type *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia or Landsberg *erecta*) and the mutants *npq1-2*, *npq4-1*, *ttg-1*, *tt5-1* and *tt3-1* were grown in a phytotron under controlled environmental conditions, as previously described (Havaux and Niyogi 1999). Seeds were obtained from the Nottingham Arabidopsis Seeds Centre (University of Nottingham, UK), except the *npq* seeds, which were kindly provided by Dr. K. Niyogi (University of California, Berkeley, USA). Light stress was imposed by transferring plants aged 5 weeks to a growth chamber at 8 °C/6 °C (day/night, air temperature) and under a high photon flux density (PFD) of

1,000 μmol photons m^{-2} s^{-1}. Leaf temperature, monitored with an infrared thermometer (80T-IR temperature probe and 73III multimeter; Fluke, Everett, Wash., USA), was 10 °C on average. Photosynthetic PFDs were measured with a Li-Cor quantum meter (Li-Cor, Lincoln, Neb., USA). Light stress was also imposed on detached leaves: leaves placed on wet filter paper were exposed to white light (PFD, 1,000 μmol photons m^{-2} s^{-1}) produced by 150-W metal halide lamps (Osram Sylvania, Danvers, Mass., USA) equipped with two infrared suppressor filters (Schott, Mainz, Germany). Leaf temperature was maintained constant at 10 °C. Using a UV radiometer (VLX3 W; Fisher Bioblock Scientific, Illkirch, France), we checked that the light used for the stress treatments did not contain any UV-C and UV-B radiations; the UV-A level was very low (ca. 0.36 W m^{-2}).

Photosynthetic measurements in vivo

Chlorophyll fluorescence emission from the upper surface of the leaves was measured with a PAM-2000 fluorometer (Walz, Effeltrich, Germany), as previously described (Havaux and Davaud 1994; Havaux and Niyogi 1999). The initial level (Fo) of chlorophyll fluorescence was measured with a dim red light modulated at 600 Hz. A 2-s pulse of far-red light was used to determine the true Fo level. The maximal fluorescence level (Fm) was determined with a 800-ms pulse of intense white light. The maximal quantum yield of PSII photochemistry was calculated as $Fv/Fm = (Fm - Fo)/Fm$. The actual quantum yield of PSII photochemistry was measured in leaves illuminated with white light produced by a Schott light source (KL 1500) as $(Fm'–Fs)/Fm'$ where Fs is the steady-state fluorescence level and Fm′ is the maximal fluorescence level in the light. The nonphotochemical quenching of chlorophyll fluorescence (NPQ) was calculated as $(Fm/Fm')–1$.

The activity of PSI was analyzed in vivo by measuring light-induced P700 oxidation using leaf absorbance measurements at ca. 820 nm (Schreiber et al. 1988), as previously described (Havaux and Davaud 1994). The experimental system consisted of an ED-800-T emitter/detector unit (Walz) connected to a Walz PAM-101 system. Maximal rate and maximal extent of P700 oxidation were measured with a high-intensity far-red light (> 715 nm, 40 W m^{-2}) using a storage oscilloscope (Tektronix, Guernsey, Channel Islands, UK).

Photoacoustic spectroscopy of pigments in vivo

Depth-profile analysis of pigments in leaves was done by phase-sensitive photoacoustic spectroscopy as described elsewhere (Narayanan et al. 1997) with a custom-built photoacoustic spectrometer (Havaux 1999). The leaf sample enclosed in the photoacoustic cell was illuminated with monochromatic light selected by appropriate interference filters (bandwidth, 10 nm; Corion, Holliston, Mass., USA) and modulated at 9 Hz. Measurements were performed in the presence of background white light of high PFD (> 4,500 μmol m^{-2} s^{-1}) saturating for photosynthesis, in order to eliminate the photobaric component of the photoacoustic signal due to modulated oxygen evolution (Malkin and Canaani 1994). The in-phase photoacoustic component corresponding to the surface of the sample and the quadrature component (90° out of phase) due to the interior of the sample (mesophyll cells) were recorded simultaneously by a two-phase lock-in amplifier. Normalization of the spectra to constant input light intensity was achieved by using the photoacoustic spectra of carbon black.

Quantification of oxidative stress

Photooxidative damage of the chloroplasts was measured by thermoluminometry with a custom-built apparatus as previously described (Havaux and Niyogi 1999). The leaf sample (leaf disc of 6 mm in diameter) was slowly heated from 25 °C to 150 °C at a rate of 6 °C min^{-1}. Leaf temperature was measured with a K-type thermocouple. Heat-induced luminescence emission was measured with a photomultiplier tube (R376; Hamamatsu Photonics, Shizuoka Pref., Japan). The current from the photomultiplier tube was amplified by an Oriel 70710 transimpedance preamplifier (Oriel, Stratford, Conn., USA). Both leaf temperature and chlorophyll thermoluminescence were recorded by a computer using a DaqPad-1200 data acquisition system (National Instruments, Austin, Tex., USA) and software written by J-M Ducruet (CEA/Saclay). The amplitude of the thermoluminescence band at ca. 135 °C is an index of lipid peroxidation of the thylakoid membranes (Vavilin and Ducruet 1998; Havaux and Niyogi 1999; Havaux et al. 2000).

Determination of photosynthetic pigments

Leaf discs of 6 mm in diameter were frozen in liquid nitrogen before analysis. Pigments were extracted in methanol at 4 °C in dim light. After centrifugation and filtration of the extracts through a 0.45-μm PTFE filter membrane (Alltech, Derfield, USA), pigments were separated by HPLC with a reverse-phase C18 column (Waters Nova Pak, 60 A, 4 μm; 300 mm long, 3.9 mm i.d.; Waters Corp., Milford, Mass., USA) protected by a Waters Bondapak C18 guard column, at a flow rate of 1 ml min^{-1} and with a 50-μl injection volume. The chromatography system consisted of a Waters 600 E system controller and a Waters 486 absorbance detector. Solvent A was methanol:ethyl acetate (68:32, v/v) and solvent B was acetonitrile:methanol:water (72:8:3, by vol.). For the first 10 min, an isocratic elution of 4% solvent A in solvent B was used followed by a linear gradient from 4% to 60% solvent A in solvent B for 2 min. Then solvent A was increased to 70% within 10 s and eluted isocratically for the next 10 min. Finally the column was washed for 6 min with 4% solvent A in solvent B. Pigment concentrations were calculated using standards and published extinction coefficients. Purified zeaxanthin, lutein and β-carotene were obtained from Extrasynthèse (Genay, France). Chlorophyll *a*, chlorophyll *b*, neoxanthin, violaxanthin and antheraxanthin were prepared by TLC with *n*-hexane:isopropanol (100:10, v/v) as the solvent system.

Determination of water-soluble pigments

Flavonoids and anthocyanins were extracted from leaf discs (1 cm in diameter) in acidified methanol (HCl:methanol, 1:99, v/v) as described by Noguès and Baker (2000). Absorption spectra of the extracts were determined after centrifugation using a Cary 50 spectrophotometer (Varian, Palo Alto, Calif., USA). The total UV-absorbing flavonoid content and the anthocyanin content were estimated from absorbances at 350 nm and 530 nm, respectively.

Vitamin E determination

Fresh leaves (300–500 mg)were ground in 1 ml methanol. After sonication and centrifugation, the extract was filtered through a 0.45-μm PTFE filter membrane and analyzed by HPLC with a Varian 5000 liquid chromatograph and an Alltima Peek C18 column (250 mm long, 4.6 mm i.d.; 5 μm; Alltech). Methanol was eluted at a rate of 1 ml min^{-1} and vitamin E was detected by fluorometry (RF-530; Shimadzu Kyoto, Japan) at 330 nm with an exciting light of 290 nm. Peak identification and quantification were done with pure α-tocopherol.

Quantification of LHCII and early light-inducible proteins (ELIPs)

Leaves were frozen in liquid nitrogen before analysis. The procedures for protein extraction, electrophoresis and immunoblotting with rabbit anti-*Arabidopsis* ELIP antibody (dilution of the primary antibody 1:500) and anti-barley LHCIIb antibody (dilution,

1:2,000), as well as quantification, were performed as previously described by Montané et al. (1997). Extracts of total proteins were loaded on the gels. More details are provided in the figure legend. The experiment was repeated independently three times with essentially the same outcome. Equal loading of proteins was checked by protein determination using the Lowry method and, in addition, by Coomassie-staining of a gel of the same preparation.

Results

Photosynthetic electron transport in leaves of WT and *npq1* at 25 °C and 10 °C

The loss of the xanthophyll cycle in the Vio de-epoxidase-deficient mutant *npq1* is associated with a drastic inhibition of NPQ (Niyogi et al. 1998). This is illustrated in Fig. 1A: NPQ measured at 25 °C in mutant leaves was lower than NPQ in WT leaves over a wide range of PFDs except at PFDs lower than about 200 μmol m^{-2} s^{-1}. It is known that, at low PFD, NPQ is mainly due to changes in the light distribution between the two photosystems through the state 1-state 2 transition process, with practically no contribution from the xanthophyll-dependent energetic quenching (Demmig and Winter 1988; Horton and Hague 1988). This can explain the similar magnitude of NPQ in WT and *npq1* leaves in low light. When leaf temperature was decreased from 25 °C to 10 °C, NPQ was markedly amplified in the WT, due to the inhibition of photosynthesis and the resulting increase in the transthylakoid pH gradient. In contrast, low temperature had only a slight effect on NPQ in the *npq1* mutant. Consequently, the difference in NPQ between *npq1* and WT was strongly amplified at 10 °C. For instance, when chilled leaves were exposed to a PFD of 1,000 μmol m^{-2} s^{-1}, NPQ was almost 2 in the WT and only 0.7 in *npq1*. Despite the marked inhibitory effect of the *npq1* mutation on NPQ, the efficiency of photosynthetic electron transport was not affected in *npq1* compared to the wild type at either 25 °C or 10 °C (Fig. 1B). Consequently, high light at low temperature appears to be a good condition for inducing very different NPQ activities in *npq1* and the WT with similar electron-transfer activity.

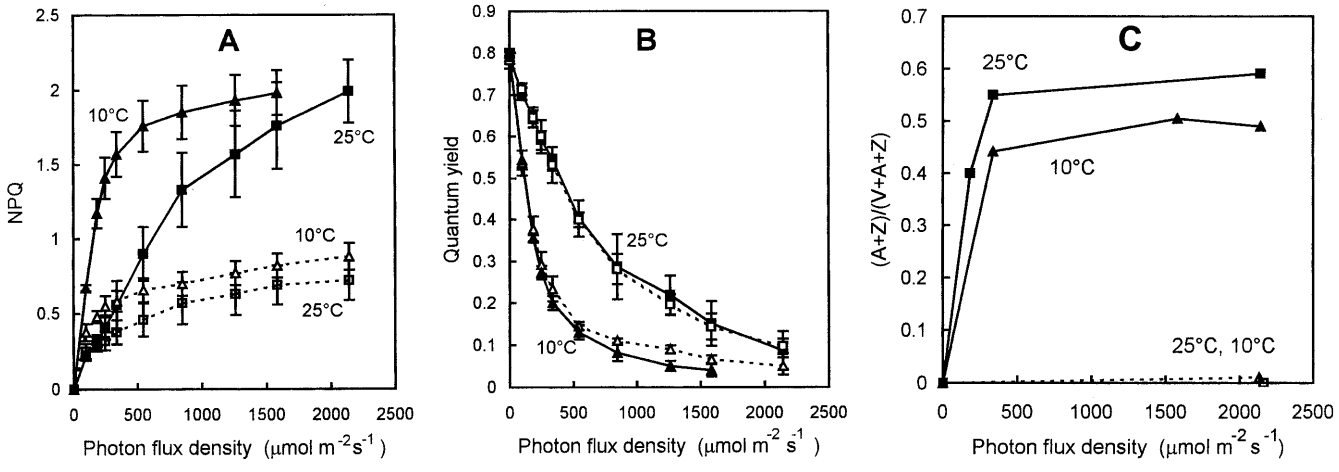
As expected, no significant synthesis of zeaxanthin was observed in *npq1* at 25 °C and 10 °C whereas a large fraction of Vio (ca. 50%) was converted to zeaxanthin in the WT at high PFD (Fig. 1C). The zeaxanthin level in WT leaves appeared to be slightly lower at 10 °C than the zeaxanthin content at 25 °C.

Chilling-induced photoinhibition and photooxidation of detached leaves

Detached leaves were exposed to excess light energy induced by a high PFD (ca. 1,000 μmol m^{-2} s^{-1}) at low temperature (10 °C). This treatment caused a marked inhibition of PSII as indicated by the decrease in Fv/Fm (Fig. 2A). This phenomenon was more pronounced in the *npq1* mutant than in the WT, indicating increased susceptibility of leaves to chilling-induced PSII photoinhibition in the absence of the xanthophyll cycle. The inhibition of NPQ, rather than the absence of the zeaxanthin molecule per se, is involved in the increased photosensitivity of PSII observed in *npq1* since the NPQ-deficient *npq4* mutant, which has a normal xanthophyll cycle (Li et al. 2000), exhibited the same behavior as *npq1* (Fig. 2A, closed rectangles).

When stressed leaves were transferred back to low light at 25 °C, the decrease in Fv/Fm was reversed in both *npq1* and the WT (Fig. 2B). The short-term stress treatment used here did not induce photooxidative damage to the chloroplasts as revealed by chlorophyll thermoluminescence (TL) measurements at high temperature (Fig. 2C). The amplitude of the 135 °C TL band, which is an index of thylakoid lipid peroxidation (Vavilin and Ducruet 1998; Havaux and Niyogi 1999), was similar in the WT and *npq1*, and did not differ from the signal amplitude of control leaves (see time 0 in Fig. 3B).

Fig. 1 Light dependence of NPQ (**A**), the quantum yield for photosynthetic electron transport (**B**), and the (A + Z)/(V + A + Z) ratio (**C**) in leaves of *npq1* (*open symbols*, *dotted lines*) and WT (*closed symbols*, *full lines*) *Arabidopsis thaliana* at 25 °C (*squares*) and 10 °C (*triangles*). Vertical bars indicate SD; $n = 3$. *A* Antheraxanthin, *V* violaxanthin, *Z* zeaxanthin



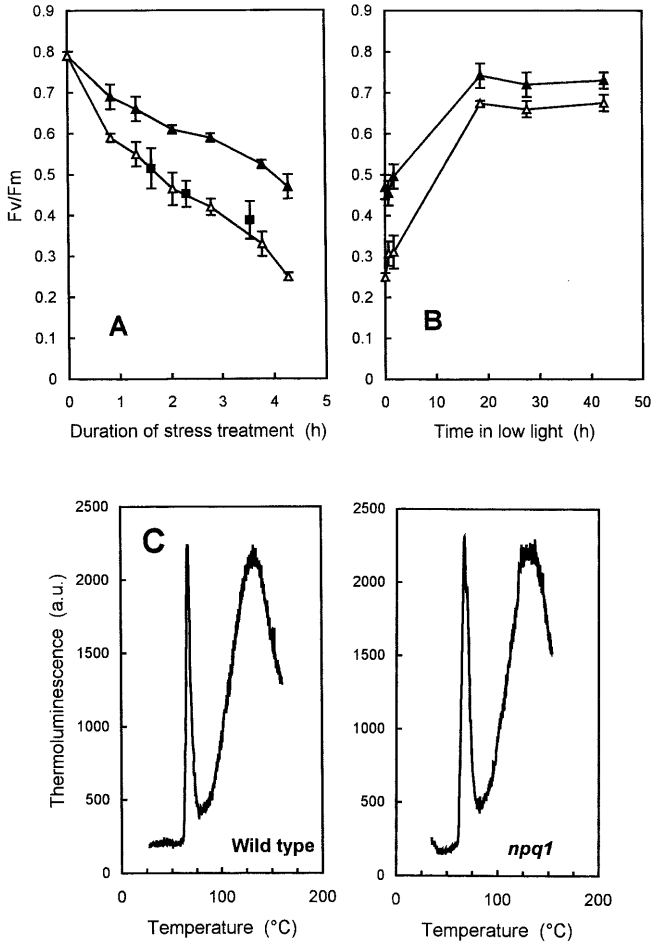


Fig. 2 A, B Changes in Fv/Fm in detached leaves of WT (*closed triangles*), *npq1* (*open triangles*), and *npq4* (*closed squares*) *Arabidopsis* exposed to a high PFD (1,000 μmol m^{-2} s^{-1}) at low temperature (10 °C) (**A**) and during subsequent adaptation to low light at 25 °C (**B**). **C** Chlorophyll thermoluminescence curves of WT and *npq1* leaves exposed for 4.5 h to high light at low temperature. Vertical bars indicate SD; $n = 5$

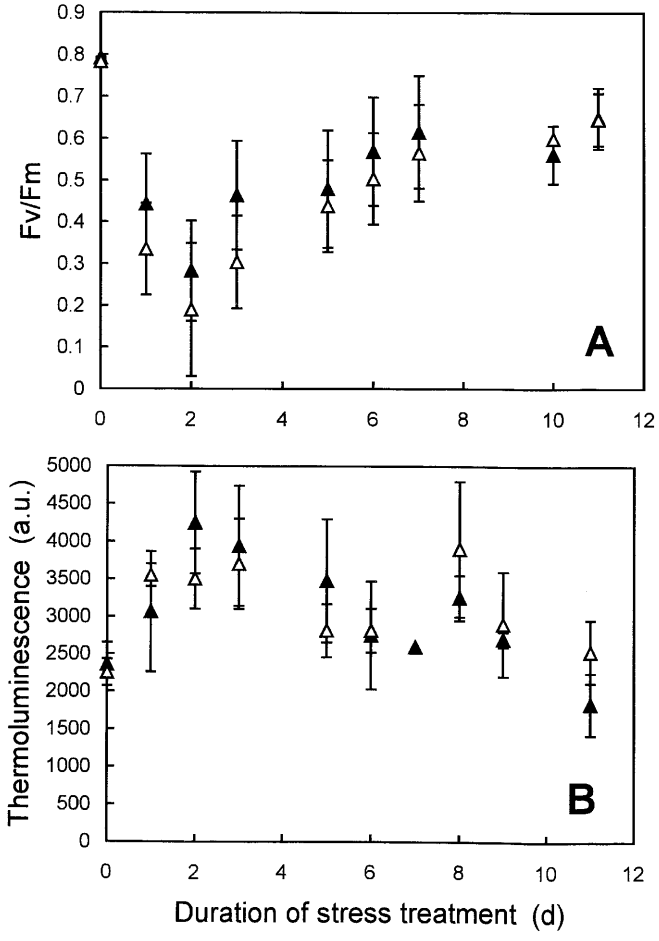


Fig. 3 Photoinhibition of PSII (**A**; as indicated by the decrease in Fv/Fm) and photooxidation (**B**; as indicated by the amplitude of the 135 °C thermoluminescence peak) of *npq1* (*open triangles*) and WT (*closed triangles*) *Arabidopsis* leaves during exposure of whole plants to chilling stress in high light (8 °C/1,000 μmol photons m^{-2} s^{-1}). Vertical bars indicate SD; $n = 3$–6 experiments

Chilling-induced photoinhibition and photooxidation of whole plants

Intact *Arabidopsis* plants were exposed to a PFD of 1,000 μmol m^{-2} s^{-1} at an air temperature of 8 °C (corresponding to a leaf temperature of about 10 °C). Upon transfer to the new conditions, Fv/Fm noticeably decreased, reaching a low value of ca. 0.3 after 2 days (Fig. 3A). Photoinhibition appeared to be slightly more pronounced in *npq1* than in the WT, in agreement with the experiments on detached leaves. However, we observed a progressive recovery of Fv/Fm during the following days so that Fv/Fm reached a value as high as 0.6 after 10 days in excess light. This partial recovery of the PSII photochemical efficiency was similar in the WT and *npq1*. Clearly, *Arabidopsis* was able to acclimate in the long term to excess light energy at low temperature, independently of the xanthophyll cycle. Figure 3B shows that lipid peroxidation, as reflected by the increase in TL emission, occurred during the first days of the stress treatment. However, the amplitude of the luminescence increase was rather small and there was no significant difference between *npq1* and WT. Again, long-term acclimation to chilling stress in the light was obvious from the progressive lowering of the TL emission. After 10 days in strong light, the amplitude of the 135 °C TL band was similar to that measured before the light treatment, confirming the acclimation of the photosynthetic apparatus to chilling stress in the light in both genotypes.

The activity of PSI was monitored in far-red light (> 715 nm) by leaf absorbance measurements in the near infrared (ca. 820 nm), mainly reflecting changes in the redox state of the PSI reaction-center pigment P700 (Schreiber et al. 1988). Upon illumination with strong far-red light, P700 was rapidly oxidized, within ca. 400 ms, producing a measurable increase in the leaf absorbance at 820 nm (Fig. 4). When a supersaturating fluence rate of far-red light is used (as is the case here, 40 W m^{-2}), information about P700 photooxidation can be obtained by measuring the initial rate of leaf absorbance increase because, under such conditions,

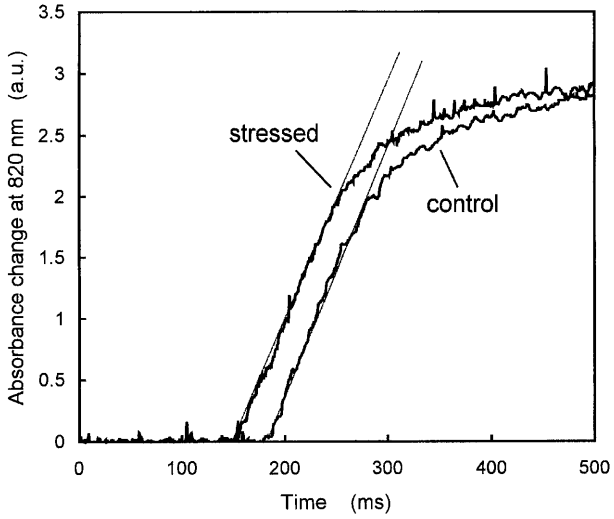


Fig. 4 Kinetics of far-red-light-induced P700 oxidation in leaves of the *npq1* mutant of *Arabidopsis* treated at low temperature and high light (8 °C, 1,000 μmol m^{-2} s^{-1}) for 0 days ('control') or 2 days ('stressed')

$P700^+$ re-reduction will not compete significantly with the oxidation reaction. The amplitude of the absorbance change is a measure of the amount of oxidizable P700. As shown in Fig. 4, 2 days exposure of *npq1* plants to chilling stress had little effect on the amplitude of P700 oxidation and the maximal rate of photooxidation (measured by the slope of the absorbance trace at time 0), indicating that, even in the absence of the xanthophyll cycle, PSI was resistant to chilling-induced photoinhibition, contrary to PSII. Table 1 summarizes the P700 data obtained with WT, *npq1* and *npq4* leaves before and after long-term chilling stress: no or very little inhibition of P700 photooxidation was found in chilled leaves, and no significant difference was found between *npq1*, WT and *npq4*. Photoinduced oxidation of P700 was also analyzed in detached *Arabidopsis* leaves exposed to short-term chilling stress in the light (4 h at 10 °C and 1,000 μmol photons m^{-2} s^{-1}). Again, PSI was found to be phototolerant in the WT. In *npq1*, the maximal rate of P700 photooxidation was somewhat reduced, but this effect appeared to be very small compared to the inhibition of the PSII activity: in this experiment, Fv/Fm fell to 0.34 ± 0.01 and 0.46 ± 0.03 (mean ± SD) in *npq1* and WT, respectively (see also Fig. 2A).

Acclimation of whole plants to high light at low temperature: pigments and vitamin E

As shown in Fig. 5A, the chlorophyll level (per unit leaf area) increased during acclimation to chilling stress in the light in both WT and *npq1*. This pigment increase seemed to be smaller in *npq1* but the difference from the WT was not statistically significant. The chlorophyll *a*/*b* ratio was observed to increase under the stress conditions, particularly in the mutant: from 2.58 ± 0.03 to 2.89 ± 0.13 (mean ± SD) in the WT after 10 days, and from 2.52 ± 0.03 to 3.30 ± 0.14 in *npq1*. The carotenoid level was also affected during acclimation to excess light (Fig. 5B): the total xanthophyll content and the xanthophyll cycle pigment pool were strongly increased. In contrast, β-carotene remained constant (Fig. 5B). The accumulation of xanthophylls was observed in both *npq1* and the WT, but it appeared to be slightly less pronounced in *npq1*. Constancy of β-carotene concentration with substantial increase in the xanthophyll concentration was previously reported in chilling-treated leaves of other plant species (e.g. Koroleva et al. 2000).

The level of the lipophilic antioxidant vitamin E was analyzed during chilling stress in the light (Fig. 5C). There was a small increase in α-tocopherol and γ-tocopherol after 5 days at 8 °C/1,000 μmol m^{-2} s^{-1} in both *npq1* and WT. The accumulation of vitamin E was very marked in the WT after 10 days exposure to the stress conditions. The same phenomenon was observed in *npq1* although it was less pronounced than in the WT (Fig. 5C).

Acclimation of whole plants to high light at low temperature: photosynthetic characteristics

After 10 days exposure to low temperature and high light, the photosynthetic electron transport characteristics of *Arabidopsis* leaves were modified (Fig. 6). Electron transport was less rapidly saturated with increasing PFD (Fig. 6A) compared with electron transport in

Table 1 Far-red light-induced P700 oxidation measured by leaf absorbance changes at 820 nm in *Arabidopsis thaliana* WT, *npq1* and *npq4* leaves exposed for 4 h (detached leaves) or 1, 2 and 10 days (whole plants) to 1,000 μmol photons m^{-2} s^{-1} at 8 °C/10 °C. Strong far-red light (40 W m^{-2}) was used to measure the maximal extent of P700 oxidation ($\Delta Amax/A$) and the maximal rate of P700 photooxidation [$(\Delta A/A)$ s^{-1}]. Data are mean values ± SD of $n = 3$–5 separate experiments

Treatment	Time	$\Delta Amax/A \times 10^{-3}$			$(\Delta A/A)$ $s^{-1} \times 10^{-3}$		
		WT	*npq1*	*npq4*	WT	*npq1*	*npq4*
Control leaves		16.9 ± 0.4	16.1 ± 0.5	17.2 ± 0.7	117 ± 6	128 ± 8	117 ± 11
Long-term stress (whole plants)	1 d	15.3 ± 0.5	16.1 ± 0.5	15.3 ± 0.2	99 ± 6	108 ± 8	96 ± 9
	2 d	15.6 ± 0.3	16.1 ± 0.7	15.7 ± 0.5	100 ± 1	108 ± 9	105 ± 2
	10 d	16.9 ± 1.7	16.5 ± 0.5	17.3 ± 0.7	100 ± 9	98 ± 4	100 ± 4
Short-term stress (detached leaves)	4 h	17.2 ± 0.8	15.6 ± 0.5	16.4 ± 0.7	115 ± 11	100 ± 6	108 ± 6

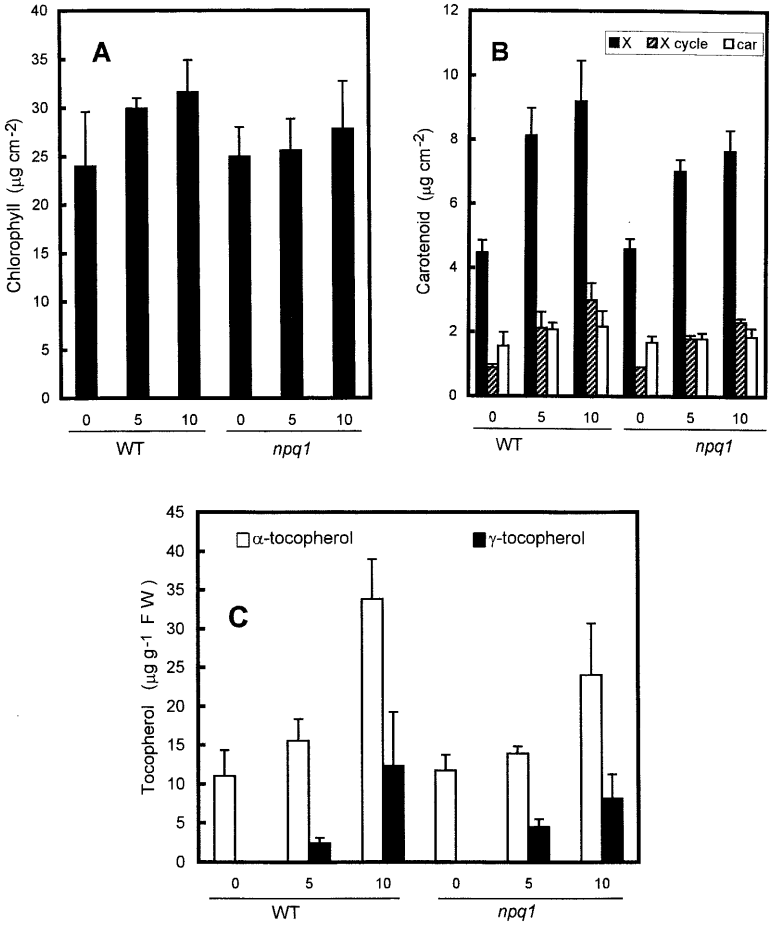


Fig. 5 Chlorophyll concentration (**A**), concentrations of total xanthophylls (*X*), xanthophyll-cycle pigments (*X cycle*) and *β*-carotene (*car*) (**B**), and vitamin E concentration (α-tocopherol and *γ*-tocopherol) (**C**) in leaves of *npq1* and WT *Arabidopsis* grown for 0, 5 or 10 days in strong light at low temperature (8 °C/1,000 μmol photons m^{-2} s^{-1}). Vertical bars indicate SD; $n = 3$–4

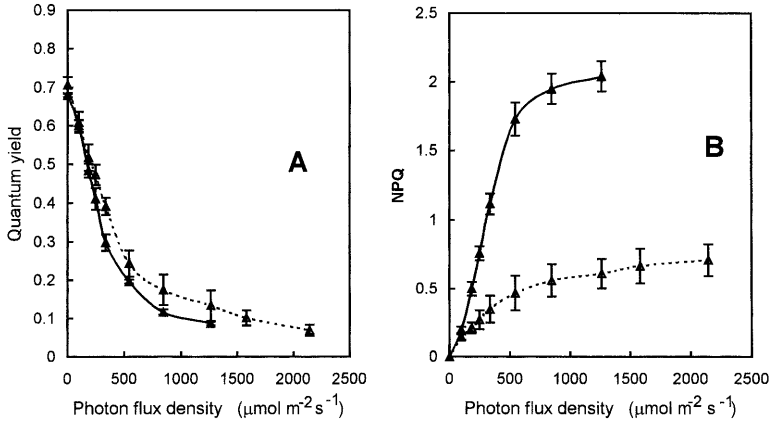


Fig. 6A, B Photosynthetic characteristics in leaves of *npq1* (*open triangles*, *dotted lines*) and WT (*closed triangles*, *full lines*) *Arabidopsis* grown for 8 days in high light and low temperature (8 °C/1,000 μmol m^{-2} s^{-1}). **A** Quantum yield of photosynthetic electron transport. **B** NPQ. Measurements were performed at 10 °C. Vertical bars indicate SD; $n = 3$

control leaves (Fig. 1B, 10 °C experiment). This phenomenon occurred in both genotypes although it seemed that photosynthetic acclimation was better in *npq1*. It is clear from Fig. 6B that acclimation did not involve improvement of NPQ; NPQ remained very inhibited in *npq1*.

Figure 7A shows the level of the major light-harvesting pigment-protein complex of PSII (LHCIIb) before and after exposure to the stress conditions. LHCII remained constant in the WT throughout the stress treatment whereas it significantly decreased in *npq1* at day 10. We have also examined the level of the light-induced stress proteins ELIPs (Fig. 7B). No ELIPs were found under control conditions, as expected. In plants treated for 5 days, a marked accumulation of ELIPs was found in both WT and *npq1*. Clearly, the absence of zeaxanthin synthesis in *npq1* did not interfere with ELIP synthesis. However, after 10 days of stress, ELIPs were stable in the WT but not in *npq1*. In the latter genotype, ELIPs decreased noticeably, and this phenomenon was obviously more pronounced than the decrease in LHCIIb.

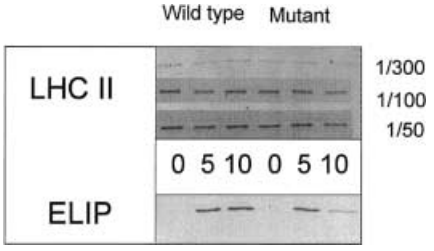


Fig. 7 LHCIIb and ELIP levels in leaves of *npq1* and WT *Arabidopsis* grown for 0, 5 or 10 days at 8 °C/1,000 μmol photons m^{-2} s^{-1}. 20 μg proteins (total protein extract) was loaded on each lane of the gel for ELIP determination and 0.07 μg proteins for LHCII determination (at a dilution of 1/300). This experiment was repeated three times (with different protein concentrations) with the same results

Acclimation of whole plants to high light at low temperature: flavonoids

Arabidopsis plants turned purple when exposed to chilling stress in the light, indicating massive synthesis of anthocyanins in both *npq1* and WT. The change of leaf coloration became visible after 4–5 days of exposure to the stress conditions. We have characterized this anthocyanin accumulation using the photoacoustic technique. One of the most interesting aspects of this technique is the possibility of measuring light absorption at different depths of an opaque sample. When an absorbing sample is illuminated with intensity-modulated light, heat emission resulting from deactivation of the excited species is also modulated, inducing periodic heating of the air layer surrounding the sample. The resulting pressure waves can be measured by a sensitive microphone in the closed photoacoustic cell. However, thermal waves take some time to diffuse to the surface. Consequently, signals from chromophores deeper in the sample will have some phase delay. In contrast, heat emission from the chromophores near the surface of the sample can diffuse quickly to surface; hence phase delay is almost nil. Therefore, the signals from the surface or near surface will be in phase with the light chopping whereas signals from deeper areas will not appear in-phase but contribute more to the quadrature component (90 ° out-of-phase). The in-phase (surface) and quadrature (interior) absorption spectra of the sample can be measured simultaneously by phase-resolved monitoring of photoacoustic photothermal signals. This approach has been effectively used for depth profiling light-absorption characteristics of biological samples, including higher-plant leaves (Nery et al. 1987; Narayanan et al. 1997).

Figure 8A shows the in-phase photoacoustic spectra of a control (green) WT leaf and a light-stressed (purple) WT leaf. It is apparent that light absorption by the epidermis was strongly increased in the green-yellow spectral region (500–600 nm) in stressed leaves, due to the presence of anthocyanins. The amplitude ratio between the photothermal signal measured at 540 nm (Apt-540) and that measured at 670 nm (Apt-670) rose from ca. 1.00 to 1.45 after 10 days in excess light. This phenomenon was not observed in the interior spectrum (Fig. 8B), in agreement with the well-established localization of anthocyanins in the vacuoles of the epidermal or subepidermal cells (Chalker-Scott 1999). Photoacoustic (Table 2) and spectrophotometric (Table 3) measurements indicated that accumulation of anthocyanins in the epidermis was the same in *npq1* and WT leaves. We observed also that absorption in the blue (and probably UV) region was substantially increased in the epidermis of light-treated leaves (Fig. 8A). For instance, the ratio Apt-410/Apt-670 increased from about 1.5 to almost 2 after 10 days exposure of *npq1* and WT *Arabidopsis* plants to the stress conditions (Table 2). Most probably as a result of the increased absorptance

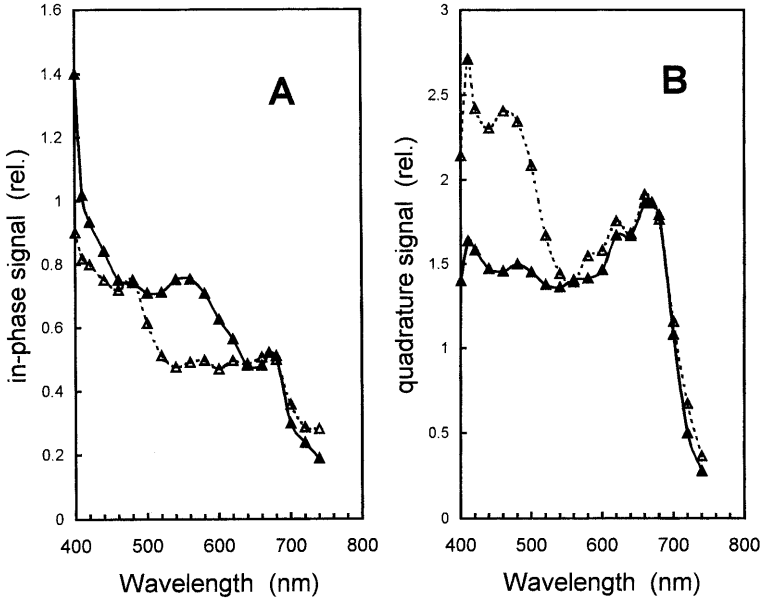


Fig. 8 In-phase (**A**) and quadrature (**B**) photoacoustic spectra of leaves of WT *Arabidopsis* grown at 25 °C/ 250 μmol m^{-2} s^{-1} (*open triangles*, *dotted lines*) or 8 °C/ 1,000 μmol m^{-2} s^{-1} (*closed triangles*, *full lines*) for 10 days. The spectra were normalized at 670 nm

Table 2 Amplitude ratio of the in-phase photothermal signal at 540 nm (*Apt-540*) to the signal at 670 nm (*Apt-670*) and amplitude ratio of the signal at 410 nm (*Apt-410*) to the signal at 670 nm in leaves of *A. thaliana* WT, *npq1*, *tt5*, *tt3* and *ttg* before and after 6 days exposure to chilling stress in high light (8 °C/1,000 μmol m^{-2} s^{-1}). Data are mean values ± SD of $n = 3$ separate experiments (except when indicated in parentheses)

Genotype	Apt-540/Apt-670		Apt-410/Apt-670	
	Before	After stress	Before	After stress
WT	0.93 ± 0.07	1.32 ± 0.08	1.49 ± 0.23	1.86 ± 0.05
npq1	0.85 ± 0.03	1.30 (2)	1.54 ± 0.07	1.95 (2)
tt3	0.88 ± 0.05	1.04 ± 0.07	1.57 ± 0.07	2.07 + 0.47
tt5	0.97 ± 0.01	1.00 ± 0.01	1.46 ± 0.20	1.51 ± 0.13
ttg	0.92 ± 0.05	1.03 ± 0.03	1.61 ± 0.32	2.06 ± 0.23

Table 3 Flavonoid and anthocyanin contents in *A. thaliana* leaves exposed for 6 days to low temperature (8 °C) and high light (1,000 μmol m^{-2} s^{-1}). Anthocyanins and flavonoids are expressed as absorbance cm^{-2} (at 530 nm and 350 nm, respectively). Data are mean values ± SD of $n = 3$ or 4 separate experiments

Genotype	Flavonoids		Anthocyanins	
	Before	After stress	Before	After stress
WT	1.87 ± 0.19	4.50 ± 0.34	0.42 ± 0.06	1.64 ± 0.12
npq1	1.61 ± 0.07	4.34 ± 0.08	0.37 ± 0.02	1.77 ± 0.16
tt3	1.56 ± 0.02	3.26 ± 0.40	0.46 ± 0.02	0.27 ± 0.06
tt5	1.31 ± 0.08	1.23 ± 0.16	0.45 ± 0.03	0.26 ± 0.09
ttg	1.49 ± 0.13	2.61 ± 0.40	0.46 ± 0.03	0.32 ± 0.06

of the epidermis in the blue and green spectral regions, the light absorption in the interior of the leaves was noticeably attenuated at wavelengths lower than ca. 550 nm (Fig. 8B). These changes show the efficient screening function of anthocyanins and other absorbing compounds, leading to a noticeable lowering of light absorption by photosynthetic pigments in the mesophyll tissues.

For technical reasons, it was not possible to examine the photoacoustic characteristics of leaf epidermis in the UV domain. However, the absorbance spectra of flavonoid pigments extracted in acidified methanol clearly show that exposure of *Arabidopsis* to excess visible radiation at low temperature led to a strong accumulation of UV-absorbing compounds (Fig. 9). The magnitude of this accumulation, quantified by the increase in absorbance at 350 nm, was similar in *npq1* and WT (Table 3).

The *Arabidopsis transparent testa* mutants (*tt*) are blocked at different steps of the flavonoid biosynthetic pathway (Koornneef 1990; Shirley et al. 1995). In particular, the *tt5*, *tt3* and *ttg* mutants are unable to synthesize anthocyanin in the shoots. The TT3 gene is the single-copy structural gene of dihydroflavonol-4-reductase (Shirley et al. 1995). The TT5 locus affects an 'earlier' step in the flavonoid synthetic pathway, namely the chalcone isomerase gene. The TTG locus is a regulatory gene that controls the expression of the enzyme dihydroflavonol-4-reductase. The *ttg* mutation

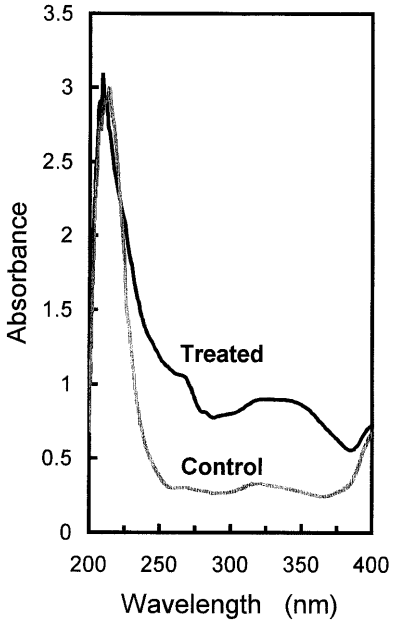


Fig. 9 Electronic absorbance spectra of UV-absorbing pigments extracted in acidified methanol from WT *Arabidopsis* leaves before and after 6 days at 8 °C/1,000 μmol m^{-2} s^{-1}. See *Materials and methods* for more details

has pleiotropic effects with both anthocyanin synthesis and trichome development being affected (Koornneef 1981). From a biochemical viewpoint, the *tt3* and *ttg* mutants differ from *tt5* by the presence of dihydroflavonols and flavonols in the former ones.

As expected, chilling stress in the light did not result in anthocyanin accumulation in the flavonoid mutants as shown by the photoacoustic absorption spectra of their epidermes (Fig. 10). No increase in Apt-540 relative to Apt-670 was found in any of the mutants (Table 2). However, there was a marked difference between the mutants regarding epidermal absorptance at short wavelength. In contrast to *tt3* and *ttg*, *tt5* was unable to increase light absorption in the UV/blue spectral region when exposed to excess light at low temperature (Fig. 10, Table 2). Quantification of anthocyanins and flavonoids confirmed the photoacoustic data (Table 3). Anthocyanins accumulated in the WT and *npq1* but not in the *tt* mutants. The *tt5* mutant was unable to accumulate flavonoids, contrary to *tt3* and *ttg*.

The *tt5* mutant was found to be the most light-sensitive. After 6 days in excess light, PSII was significantly more photoinhibited in *tt5* than in the *ttg* and *tt3* mutants which were only slightly more sensitive to photoinhibition than the WT (Fig. 11A). Incidentally, the ecotypes Columbia and Landsberg *erecta* of WT *A. thaliana* responded similarly to chilling stress in high light. Significant lipid peroxidation, measured by the increase in TL emission at 135 °C, was observed to occur in *tt5* (Fig. 11B). Again, *ttg* and *tt3* were found to be less affected than the latter mutant. One can conclude from the data obtained with the *tt* mutants that flavonoids protect plants from photoinhibition and

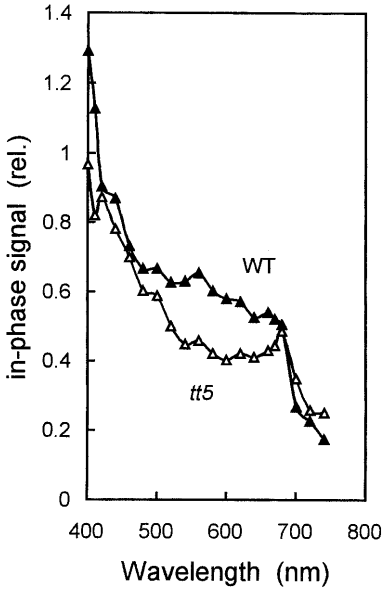


Fig. 10 In-phase photoacoustic spectra of leaves of WT *Arabidopsis* and the anthocyanin-less mutant *tt5* exposed for 6 days to strong light at low temperature (8 °C/1,000 μmol m^{-2} s^{-1}). The spectra were normalized at 670 nm

photooxidation under excess visible radiations, with UV/blue light-absorbing flavonoids (absent in *tt5*) being more efficient in this function than green light-absorbing anthocyanins (absent in all *tt* mutants).

Discussion

Protective functions of the xanthophyll cycle against chilling stress and high light in the short term and in the long term

The xanthophyll cycle protects PSII against photoinactivation when plants grown in low light are suddenly transferred to excess light energy. This was shown previously in *npq1* leaves exposed to very high PFD at 25 °C (Niyogi et al. 1998; Havaux and Niyogi 1999), and is confirmed here in detached and attached *npq1* leaves exposed to excess light energy induced by chilling stress at moderately elevated PFD (Figs. 2A, 3A). In this context, one can also refer to previous work by Demmig-Adams et al. (1989) who showed that *Rhizophora mangle* leaves that contained high zeaxanthin levels and were in a low fluorescence state after a pre-illumination treatment were more tolerant to chilling-induced reduction of the PSII photochemical efficiency than untreated leaves. In the work presented here, we have also shown that the increased susceptibility of *npq1* to photoinhibition at low temperature is attributable to the inhibition of NPQ, and not to the absence of zeaxanthin per se, since the NPQ-deficient mutants *npq4* and *npq1*, which differ by the presence or the absence of zeaxanthin, respectively (Li et al. 2000), behaved quite similarly under chilling stress in the light (Fig. 2A). In previous work, we showed that the zeaxanthin molecules synthesized in strong light had a direct protective effect, distinct from NPQ, against photooxidative damage of the chloroplasts (Havaux and Niyogi 1999; Havaux et al. 2000). Although low temperature is considered to favor photooxidation, this phenomenon is mainly observed in chilling-sensitive plant species, such as maize or cucumber, and much less in chilling-resistant ones (Wise 1995). *Arabidopsis thaliana* belongs to the latter class of plants and therefore it is not surprising that little lipid peroxidation occurred in this species at 8 °C/10 °C and 1,000 μmol photons m^{-2} s^{-1} and that the 'direct' antioxidant function of zeaxanthin was not observed in this study.

In whole plants, chilling-induced loss of PSII activity was transitory, with maximal photoinhibition being measured after 2 days exposure to 8 °C/1,000 μmol photons m^{-2} s^{-1} (Fig. 3A). PSII recovered during the subsequent days, both in the WT and in *npq1*. Long-term adaptive changes were also visible in the TL emission signals and in the light-dependence curve of photosynthetic electron transport, particularly in *npq1*: the lipid-peroxidation-related TL band decreased progressively to the initial luminescence level measured before stress (Fig. 3B), and the photosynthetic electron transport was less rapidly saturated with increasing PFD in acclimated

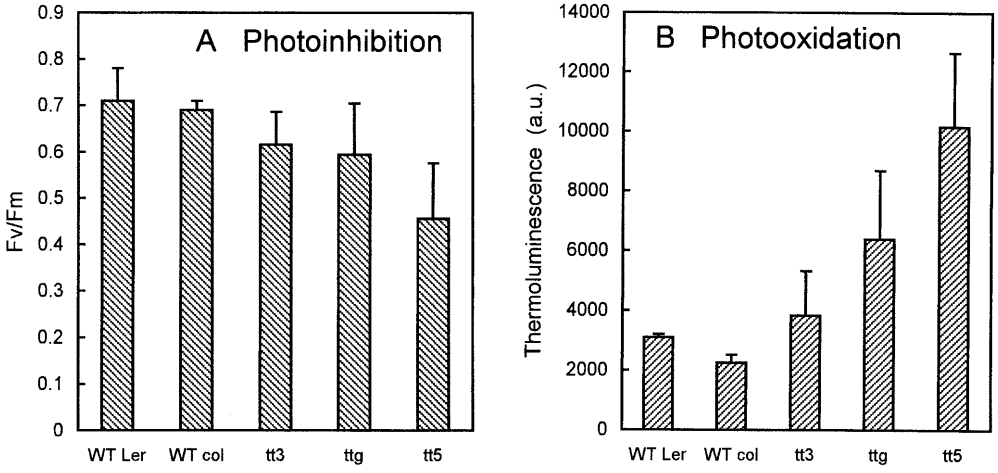


Fig. 11 PSII photoinhibition (**A**; as indicated by the decrease in Fv/Fm) and photooxidation (**B**; as monitored by the 135 °C-TL peak amplitude) in leaves of WT *Arabidopsis* [Columbia (*Col*) and Landsberg *erecta* (*Ler*) ecotypes] and the anthocyanin-less mutants *tt3, tt5, ttg* after 6 days in strong light at low temperature (8 °C/1,000 μmol m^{-2} s^{-1}). Vertical bars indicate SD; $n=4$

leaves than in control leaves (Fig. 6A vs. Fig. 1B). Consequently, one can conclude that *A. thaliana* is able to acclimate in the long term to chilling stress in the light and that this acclimation is independent of the xanthophyll cycle. Moreover, photoacclimation of *npq1* plants at low temperature did not involve improvement of NPQ (Fig. 6B).

PSII did not recover completely during acclimation to chilling stress in the light, with Fv/Fm reaching a value close to 0.6 after 10–12 days, although linear electron transport was not inhibited compared to the corresponding activity in control leaves (Figs. 1B, 6A). The maintenance of a permanently reduced Fv/Fm could be a protective mechanism (Huner et al. 1993) and it could be due to sustained energy dissipation in PSII as previously observed in various plant species acclimated to chilling temperature (e.g. Adams et al. 1995). However, this phenomenon was not directly mediated by zeaxanthin and/or antheraxanthin since Fv/Fm was similar in *npq1* and WT leaves in the acclimated state.

Low temperature has been shown to induce a preferential photoinhibition of PSI in some plant species (Havaux and Davaud 1994; Terashima et al. 1994). However, leaf absorbance measurements at ca. 820 nm did not reveal any significant damage to PSI in chilled WT *Arabidopsis* leaves (Table 1). Moreover, the absence of the xanthophyll cycle in *npq1* was not accompanied by a substantial increase in the sensitivity of PSI to chilling-induced photoinhibition. Consequently, the photoprotective action of NPQ seems to be rather specific to PSII, although the photoinduced conversion of Vio to zeaxanthin is known to occur in both photosystems (Thayer and Björkman 1992). Similarly, we observed in previous work (Havaux et al. 2000) that, under different experimental conditions from those used here, pigment destruction in photodamaged *npq1* leaves preferentially affected PSII.

From our data and previous studies on *npq* mutants, it is apparent that the xanthophyll cycle and the associated NPQ have a significant protective function when low-light-grown leaves are suddenly exposed to excess light energy. The latter condition is found, for example, in canopies where leaves can be exposed repetitively to high intensity sunflecks (Logan et al. 1997; Barradas et al. 1998). This protection is much less obvious in the long term when plants acclimate to high excitation energy conditions. However, one cannot exclude the possibility that the xanthophyll cycle can also provide protection against long-term photostress under different conditions from those used in this work. Under more-extreme light-stress conditions (very high PFD with long photoperiod), the absence of the xanthophyll cycle in *npq1* plants resulted in irreversible photooxidation occurring in mature leaves in the time scale of several days (Havaux et al. 2000). However, photooxidative damage was not found in young, developing leaves in the center of the leaf rosette, suggesting that, even under those conditions, *npq1* plants are able to acclimate and survive. Possibly, the lack of zeaxanthin and NPQ was compensated in *npq1* by some adaptive changes in the photosynthetic apparatus.

Photosynthetic pigments and pigment-binding proteins during acclimation of WT and *npq1* to chilling stress in the light

One of the goals of this study was to determine and compare the physiological changes involved in the acclimation of WT and *npq1 Arabidopsis* plants to low temperature and high light. Growth of WT plants at 8 °C/1,000 μmol photons m^{-2} s^{-1} caused a strong increase in the xanthophyll level (Fig. 4B) and a less pronounced increase in the chlorophyll content (Fig. 4A). As a result, the carotenoid-to-chlorophyll ratio rose from 0.25 to 0.36. Although the pigment changes seemed to be slightly less pronounced in *npq1*, the augmentation of the carotenoid/chlorophyll ratio was similar to that measured in the WT (ca. 0.35). In excess light, the lifetime of the singlet excited state of chlorophyll increases, leading to the formation of triplet chlorophyll, which can generate $^1O_2$ by reaction with ground-state triplet oxygen. In contrast, carotenoids are able to quench $^1O_2$ and also they can react directly with triplet chlorophyll, thus avoiding $^1O_2$ production (Krinsky 1979). Therefore, an increase in the carotenoid content relative to the chlorophyll content is a potentially protective change and, in fact, this pigment change was observed previously in various plant species exposed in the long term to excess light energy (e.g. Adams et al. 1995).

The most striking difference observed between stressed *npq1* and WT leaves was related to the size of the light-harvesting chlorophyll antenna of PSII. Photoacclimation of *npq1* leaves was associated with a noticeable increase in the ratio of chlorophyll *a* to chlorophyll *b* and a strong reduction in the LHCIIb level quantified by immunodetection (Fig. 7). The reduction in PSII antenna size was accompanied by a modification of the light saturation curve of electron transport through PSII (Fig. 6A), with electron flow being less rapidly saturated with increasing PFD. Those effects were not observed in the WT. This differential response of the WT and *npq1* suggests that efficient energy dissipation through the NPQ process in WT leaves allowed PSII to keep large chlorophyll antennae, even when excitation energy was high. In contrast, the inhibition of NPQ in *npq1* was compensated by a strong reduction of the PSII light-harvesting pigment system, in order to avoid excessive excitation pressure on PSII and subsequent photoinhibition. As a consequence, *npq1* chloroplasts became somehow 'specialized' to high light conditions whereas WT chloroplasts were more 'flexible', keeping large chlorophyll antennae for optimal light harvesting in limiting light conditions and exhibiting high NPQ capacities for dissipation of excess excitation energy in high light.

The early light-inducible proteins (ELIPs) possess a high sequence homology with the LHCIIs but differ

from those proteins by their smaller size and transient appearance (Adamska 1997). ELIP synthesis is triggered by conditions of excess light energy such as high PFD and/or low temperature (Montané et al. 1997). The physiological functions of the ELIPs are still unclear. It has been suggested that they could be transient chlorophyll-binding proteins that function as scavengers for chlorophyll molecules during turnover of pigment-binding proteins (Adamska 1997; Adamska et al. 1999). Alternatively, ELIPs could function in dissipation of a surplus of light energy (Braun et al. 1996; Montané et al. 1997; Krol et al. 1999). This view is supported by the fact that ELIPs can also bind carotenoids, particularly xanthophylls (Adamska et al. 1999). A functional interaction between zeaxanthin and ELIPs (or its algal homolog Cbr) has been proposed (Levy et al. 1993; Braun et al. 1996; Montané et al. 1997; Krol et al. 1999). The protective function of ELIPs in high light is supported by the finding that, in barley, the ELIP level is well correlated with the capacity for PSII activity restoration after a photoinhibitory light treatment (Bei-Paraskevopoulou and Kloppstech 1999).

The stress conditions used in this study were very favorable for ELIP synthesis in *Arabidopsis* as shown by the Western blots presented in Fig. 7. The absence of zeaxanthin in *npq1* did not interfere with ELIP synthesis, as shown by the similar accumulation of ELIPs in the WT and *npq1* after 5 days of stress. However, in the long term (10 days), ELIPs decreased and almost disappeared in *npq1*, but not in the WT. This observation is somewhat surprising. If ELIPs have a photoprotective function, one would expect ELIPs to accumulate in *npq1* chloroplasts in order to compensate for the absence of the xanthophyll cycle and NPQ. Possibly, zeaxanthin synthesized in strong light binds to ELIPs (Levy et al. 1993; Krol et al. 1999) and stabilizes them, e.g. by deactivating the excited states of the chlorophyll molecules bound to the protein. Alternatively, because of the strong reduction in the LHCII level in *npq1* (Fig. 7), it is possible that chlorophyll scavenging during LHCII turnover became less necessary leading to a down-regulation of ELIPs. Irrespective of the exact reasons for the ELIP disappearance in *npq1*, this phenomenon did not seem to impair the acclimation process to chilling stress in the light, raising some questions about the protective nature of ELIPs.

Vitamin E

Apart from carotenoids, plants possess a series of other $^1O_2$ quenchers such as vitamin E and anthocyanins. In plants, the lipophilic antioxidant vitamin E (of which α-tocopherol is the major component) is located exclusive in the lipid phase of the thylakoid membranes where it acts as a $^1O_2$ quencher and has a role in controlling membrane fluidity and stability (Fryer 1992). In WT and *npq1 Arabidopsis* plants exposed to chilling stress in the light, α- and γ-tocopherols were observed to accumulate to high levels (Fig. 5C). Compared to carotenoids, tocopherols are less active in $^1O_2$ quenching (DiMascio et al. 1990) and, in *Arabidopsis* leaves, they are present in lower quantities (compare Fig. 5A with Fig. 5C, taking into account that the leaf specific weight is 0.25 mg FW mm^{-2}). However, whereas carotenoids are largely bound to proteins, tocopherols are free in the lipid matrix of the membrane (Havaux 1998). Because of its unique localization, vitamin E probably has a prominent role in the protection of the chloroplastic membrane against lipid peroxidation. In addition, α-tocopherol is able to terminate lipid peroxidation chain reactions (Fryer 1992). Consequently, it is likely that vitamin E accumulation during chilling stress in the light reinforced the resistance of the thylakoid membranes to photooxidation. However, this phenomenon was not influenced by the zeaxanthin level since no significant difference was found between *npq1* and WT with respect to the chilling-induced increase in the tocopherol concentration. This contrasts with the behavior of *npq1* leaves exposed to very high PFD at the growth temperature (25 °C) where the absence of zeaxanthin was associated with a higher level of vitamin E than that measured in the WT (Havaux et al. 2000).

Non-photosynthetic pigments

Anthocyanins are water-soluble flavonoid pigments which are localized in the vacuoles of cells in or just below the epidermis (Chalker-Scott 1999). This peculiar localization was confirmed in *Arabidopsis* leaves by our photoacoustic measurements (Fig. 8): a marked absorption band peaking at ca. 540 nm appeared with stress in the surface photoacoustic spectrum but not in the interior spectrum. Comparable accumulation of anthocyanins in the leaf epidermis was found in *npq1* and WT plants exposed to low temperature (Tables 2, 3). Whereas anthocyanins are usually considered as protective agents against the harmful effects of UV radiation, little attention has been paid to their involvement in the defense against damage caused by visible radiations. Anthocyanins are believed to be sunscreens, and there are some data suggesting that anthocyanins provide protection against PSII inhibition and photosynthetic pigment bleaching by light trapping (Gould et al. 1995; Smillie and Hetherington 1999). Accordingly, we observed that light absorption by mesophyll cells in chill-treated *Arabidopsis* leaves was noticeably reduced in the blue-green spectral region compared to the red wavelengths (Fig. 7B). This reduced light absorption was due to anthocyanins (ca. 540 nm) and also to blue-light-absorbing compounds. The increase in leaf epidermis absorptance in the blue spectral region was detected in all genotypes except *tt5*, suggesting that flavonols and/or dihydroflavonols were responsible for this phenomenon. Although flavonols and dihydroflavonols are known mainly as UV absorbers, quercetin and kaempferol, which are both absent in *tt5*

have a strong absorption band in the blue spectral region, at ca. 420 nm (Mabry et al. 1970; Li et al. 1993). Those UV/blue light absorbers seem to be more important for photoprotection than anthocyanins, at least under our light-stress conditions. Indeed, the absence of those compounds in the *tt5* mutant was associated with an increased sensitivity to lipid peroxidation and photosystem destruction, whereas the loss of anthocyanins in *tt3* did not result in significant lipid photooxidation and photoinhibition compared to the WT (Fig. 11). Incidentally, the flavonoid mutant *tt5* appeared to be much more photosensitive than the xanthophyll-cycle mutant *npq1* (compare Fig. 11 with Fig. 3). Whereas *Arabidopsis* exposed to chilling stress in high light was able to compensate for the defect in *npq1*, the absence of flavonoids in the *tt5* mutant could not be fully overcome by compensatory changes and resulted in increased photooxidation of the leaves.

In conclusion, *A. thaliana* responds to excess light energy under chilling stress by accumulating carotenoid and flavonoid pigments as well as lipophilic antioxidants. The simultaneous accumulation of those photoprotectants is probably an important component of the acclimation to chilling stress, which reduces photooxidative stress in the chloroplasts. Actually, we were unable to detect any significant lipid peroxidation in acclimated leaves using the TL method. The loss of the xanthophyll cycle in the *npq1* mutant did not preclude the adaptive changes in the concentration of antioxidants. In addition, the *npq1* mutation did not lead to an increased photodestruction of the photosynthetic apparatus in plants acclimatizing in the long term to chilling stress in the light. Either the Vio cycle has no protective function during long-term exposure of plants to excessive light energy or the absence of zeaxanthin and the resulting inhibition of NPQ can be compensated by some adaptive mechanisms. One of these mechanisms has been identified in this work, namely the reduction of the light-harvesting pigment system of PSII. In contrast, *A. thaliana* was not able to compensate for the absence of flavonoid pigments absorbing UV/blue radiations. Previously, flavonoids were recognized as UV-B protectants (Li et al. 1993; Landry et al. 1995). This study has shown that flavonoids also protect chloroplasts against destruction induced by visible light, presumably by a screening effect. In the long-term, flavonoids appeared to be more effective photoprotectors than the xanthophyll-cycle carotenoids. The latter pigments seem to be relevant to the protection of plants against damaging effects of short-term photostress rather than long-term exposure to excess light energy.

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