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ORIGINAL ARTICLE

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Loss of α-tocopherol in tobacco plants with decreased geranylgeranyl reductase activity does not modify photosynthesis in optimal growth conditions but increases sensitivity to high-light stress

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Abstract The enzyme geranylgeranyl reductase (CHL P) catalyses the reduction of geranylgeranyl diphosphate to phytyl diphosphate in higher-plant chloroplasts and provides phytol for both chlorophyll (Chl) and tocopherol synthesis. The reduction in CHL P activity in transgenic tobacco (*Nicotiana tabacum* L.) plants is accompanied by the reduction in total Chl and tocopherol content and the accumulation of geranylgeranylated Chl (Chl_{GG}). The photosynthetic performance and the susceptibility to photo-oxidative stress have been investigated in these transgenic plants. The reduced total Chl content in *Chl P* antisense plants resulted in the reduction of electron transport chains per leaf area without a concomitant effect on the stoichiometry, composition and activity of both photosystems. However, *Chl P* antisense plants were much more sensitive to light stress. Analyses of Chl fluorescence quenching indicated an increased photoinhibitory quenching at the expense of the pH-dependent fluorescence quenching after short illumination (15 min) at moderate light intensities. Prolonged illumination (up to 1 h) at saturating light intensities induced an increased photoinactivation from which the *Chl P* antisense plants could not recover or could only partially recover during a subsequent low light phase. Our data imply that the presence of Chl_{GG} has no influence on harvesting and transfer of light energy in either photosystem. However, the reduced tocopherol content of the thylakoid membrane is a limiting factor for defensive reactions to photo-oxidative stress.

Keywords α-Tocopherol · Carotenoid · Energy dissipation · *Nicotiana* (photo-oxidative stress) · Photo-oxidative stress · Photosynthesis

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Abbreviations Ax: antheraxanthin · Chl: chlorophyll · Chl_{GG}: geranylgeranylated chlorophyll · Chl_{Phy}: phytylated chlorophyll · CHL P: geranylgeranyl reductase · IEF: isoelectric focussing · NPQ: non-photochemical quenching · *q*E: pH-dependent quenching of chlorophyll fluorescence · *q*I: photoinhibitory quenching · *q*P: photochemical quenching · PFD: photon flux density · VAZ: sum of violaxanthin + antheraxanthin + zeaxanthin · Vx: violaxanthin · WT: wild type · Zx: zeaxanthin

Introduction

Photo-oxidative stress is a general and severe problem for all plants, particularly under unfavourable environmental conditions like drought, temperature stress or nutrient limitation. Under such conditions the formation of active oxygen species is increased even at low light intensities (Asada 1994; Foyer et al. 1994). Plants have developed different strategies to control these unavoidable stress conditions. The formation of active oxygen species can be reduced by several mechanisms, e.g. by the dissipation of excess light energy as heat (Demmig-Adams and Adams 1996; Horton et al. 1996; Niyogi et al. 1998), the increase of electron-consuming pathways like photorespiration (Kozaki and Takeba 1996), the water-water cycle (Asada 1999) and cyclic electron flow around photosystem II (PSII; Barber and de las Rivas 1993) or photosystem I (PSI; Heber and Walker 1992). Even more important, however, is the prevention of oxidative damage by active-oxygen scavenging systems consisting of redox substances such as ascorbate (Smirnoff 1996), glutathione (Asada 1994; Foyer et al. 1994), tocopherol (Fryer 1992) and enzymes like ascorbate peroxidase, glutathione reductase, superoxide dismutase (Foyer et al. 1994; Asada et al. 1998; Asada 1999; Niyogi 1999).

While each of these protective compounds essentially contributes to the entire response of the plant to photo-oxidative stress, the relative importance of a single factor

for the vitality of a plant remains unclear. One powerful tool for elucidating the function of each individual compound for the prevention of photo-oxidative damage is the analysis of mutant or transgenic plants with modified levels of a single antioxidant involved in preventing accumulation of active oxygen.

Several mutants with a reduced non-photochemical energy dissipation (so-called *npq* mutants) have been identified in higher plants (Niyogi 1999). Most of them did not show notable differences in comparison to control plants after long-term acclimation to high light (Hurry et al. 1997; Niyogi et al. 1998), indicating that non-photochemical energy dissipation is not generally required for the protection of a plant against photo-oxidative damage. Comparison of different *npq* mutants demonstrated, however, that the xanthophyll cycle contributes to the protection of the photosynthetic apparatus by an additional mechanism, which is different from energy dissipation (Havaux and Niyogi 1999; Havaux et al. 2000).

Mutants showing deficiency in either vitamin E (Bishop and Wong 1974), ascorbate (Conklin et al. 1997) or glutathione (Cobbett et al. 1998) have been isolated. However, little is known about the consequences of deficiencies in these antioxidants for the photoprotective mechanisms in these mutants. Vitamin E is the collective term for a family of tocopherol derivatives, which are composed of phytyl diphosphate and homogentisate, or tocotrienols, which are composed of geranylgeranyl diphosphate and homogentisate. They are mainly found in envelope and thylakoid membranes of plant chloroplasts, in algae and cyanobacteria (Wise and Naylor 1987). They prevent photo-oxidative deterioration of unsaturated fatty acids, lipids and lipoproteins in the cellular membrane of plants, animals and humans by detoxification of reactive oxygen species (Fahrenholtz et al. 1974; Neely et al. 1988), but are also involved in the modulation of membrane fluidity and permeability (Fryer 1992). Therefore, vitamin E is essential for the protection from environmental stress, chronic diseases and light-induced disorders (reviewed by Fryer 1992).

In plants, the level of all vitamin E derivatives differs quantitatively and qualitatively in each plant species: The ratio between α-tocopherol and chlorophyll (Chl) *a* was 1:10 molecules in spinach (Halliwell 1981), 1:20–30 in cucumber and pea (Wise and Naylor 1987) and 1:40–50 in tobacco (Tanaka et al. 1999). Strong variations in the tocopherol contents were found to depend on the developmental stage of the leaves and on environmental conditions, such as light intensity, temperature, drought, and pollutants (Fryer 1992; Munné-Bosch and Alegre 2000).

Tocopherol-deficiency has been generated in transgenic tobacco plants with reduced activity of geranylgeranyl reductase (CHL P; Tanaka et al. 1999). The CHL P catalyses the reduction of geranylgeranyl diphosphate to phytyl diphosphate in higher-plant chloroplasts. The phytyl diphosphate is required for both Chl and tocopherol synthesis. *Chl P* antisense gene sequences under the control of the CaMV promoter were introduced into tobacco plants (Tanaka et al. 1999). Transgenic plants synthesizing *Chl P* antisense RNA were characterized by reduced levels of endogenous *Chl P* RNA and protein levels, resulting in a slower growth rate, a decreased Chl and tocopherol content in comparison to wild-type (WT) plants, and accumulation of Chl_{GG} (Tanaka et al. 1999). The transgenic phenotype of *Chl P* antisense plants resembles that of the bacteriochlorophyll$_{GG}$ ($BChl_{GG}$)-accumulating *bchP*-deficient mutant of *Rhodobacter capsulatus*, which exhibited much lower photosynthetic growth rates than the WT strain (Bollivar et al. 1994). Since $BChl_{GG}$ in the mutant did not affect energy transfer from the antenna to the reaction centre nor electron-transfer reactions, the lower growth rate was attributed to an increased lability of $BChl_{GG}$-protein complexes (Bollivar et al. 1994).

Our research objectives are focussed on a better understanding of the physiological effects of vitamin E for stress protection in plants. We examined the significance of the tocopherol content for protection of PSII against photoinactivation. As the reduced Chl content and the accumulation of Chl_{GG} did not affect energy transfer and photosynthetic electron transport in *Chl P* antisense plants, we used these plants for studying the consequences (at least in the short term) of reduced tocopherol content on photosynthetic performance and susceptibility to photo-oxidative stress.

Materials and methods

Plant material and growth conditions

Wild-type (*Nicotiana tabacum* L. cv. Samsum NN; IPK Gatersleben) and transgenic tobacco plants were grown in a climate chamber in a 14 h light/10 h dark cycle at a photon flux density (PFD) of about 100 μmol m^{-2} s^{-1} and a temperature of 24 °C. For all experiments, leaves 4 to 7 (counted from the top) were harvested from 6- to 8-week-old plants. Leaves were either used immediately for the experiments or frozen in liquid nitrogen and stored at –80 °C.

Thylakoid preparation

For isolation of thylakoid membranes, three to four leaves were homogenized (Waring blendor, 10 s at maximum speed) in a medium containing 300 mM sucrose, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EDTA and 0.2% (w/v) bovine serum albumin (BSA). After filtration through a nylon mesh (20 μm pore size) chloroplasts were pelleted by 5 min centrifugation at 1,000 *g*. Following re-suspension of the sediment in 50 mM sucrose, 10 mM KH_2PO_4 and 5 mM $MgCl_2$, thylakoid membranes were pelleted by 5 min centrifugation at 2,000 *g*. The pellet was re-suspended in 100 mM sucrose, 10 mM KH_2PO_4, 10 mM $MgCl_2$ and 5 mM KCl and used immediately for measurements of electron transport or stored at –80 °C.

Electron transport measurements

The activities of both photosystems and linear electron transport were measured with a Clark-type oxygen electrode under saturating illumination with red light ($\lambda > 630$ nm). Thylakoids equivalent to 10 μg Chl ml^{-1} were used for all measurements. The activity of PSII

($H_2O \rightarrow$ 1,4-benzoquinone) was measured in a medium containing 330 mM sorbitol, 40 mM Hepes-NaOH (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$, 2 μM gramicidin D, 5 mM NH_4Cl and 1 mM 1,4-benzoquinone. Linear electron transport ($H_2O \rightarrow K_3[Fe(CN)_6]$) was measured in the same medium using 0.5 mM $K_3[Fe(CN)_6]$ as terminal electron acceptor instead of 1,4-benzoquinone. The activity of PSI was determined in an assay containing 40 mM Tricine-NaOH pH 8.0, 5 mM $MgCl_2$, 60 mM KCl, 1 mM NaN_3, 1 μM 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) and 100 U ml^{-1} superoxide dismutase (1 U inhibits the auto-oxidation of pyrogallol at 50%). 1 mM ascorbate and 0.5 mM 2,3,5,6-tetramethyl-*p*-phenylenediamine (TMPD) were used as electron donor and 25 μM methyl viologen as electron acceptor.

Pigment analysis

Pigment analysis was carried out by reversed-phase HPLC as described elsewhere (Färber et al. 1997). For pigment extraction, leaf discs were frozen in liquid nitrogen and disrupted in a mortar under addition of 1 ml acetone. After a short centrifugation, pigment extracts were filtered through a 0.2-μm membrane filter and either used directly for HPLC analysis or stored for up to 2 days at –20 °C.

Fluorescence measurements

Room-temperature Chl fluorescence was measured using a pulse-amplitude-modulated fluorimeter (PAM; Walz). The formation and relaxation of photochemical (*q*P) and non-photochemical quenching (NPQ) of Chl fluorescence were recorded according to established protocols (Quick and Stitt 1989; Walters and Horton 1991). Actinic illumination was varied between 100 and 1,200 μmol photons m^{-2} s^{-1} and light-saturated pulses (4,000 μmol photons m^{-2} s^{-1}) were given at intervals of 120 s. The relaxation kinetics of NPQ in the dark were used for the calculation of the two components of NPQ, energy-dependent quenching (*q*E) and photoinhibitory quenching (*q*I). The fast-relaxing component (within the first 10 min of dark relaxation after light treatment) was assigned to *q*E and the slowly relaxing component (after 10 min of dark relaxation) to *q*I. The quenching component *q*T, which is assigned to state transitions (Quick and Stitt 1989; Walters and Horton 1991) and usually relaxes with intermediate kinetics, was found to be negligible under these experimental conditions. The quenching parameters NPQ, *q*E and *q*I were calculated with a Stern-Volmer-type equation as described by Thiele et al. (1997).

Measurements of CO_2 assimilation

Assimilation of CO_2 in intact leaves was determined by infrared-gas-exchange analysis using the CMS 400 minicuvette system (Walz) in combination with a BINOS-100/4P gas analyser. A light-saturation curve of CO_2 assimilation was determined after leaf exposure for 20 min at PFDs between 0 and 2,000 μmol m^{-2} s^{-1} at a temperature of 24 °C and 70% humidity. The data (one datum point per minute) were analysed with the DIAGAS analysis program. The flow rate of the gas stream was adjusted to 1,500 ml min^{-1}.

Results

Pigment composition and tocopherol content

The analyses were performed with three different *Chl P*-antisense plants designated lines 6, 10 and 20. These three lines were selected from a set of 70 transgenic lines and initially characterized (Tanaka et al. 1999). Lines 6 and 10 showed a drastic reduction in growth rate and green pigmentation. The Chl composition of the different plants reflects the CHL P activity. The total Chl content per leaf area (Table 1) coincides with earlier results (Tanaka et al. 1999) although the Chl reduction in lines 6 and 10 was less pronounced than previously published. Our data also confirm the previous correspondence between the level of CHL P expression in the antisense tobacco lines and the proportion of Chl_{GG}: lines 6 and 10 contained about 40% Chl_{GG} of total Chl and line 20 only 7% Chl_{GG} of total Chl.

The similar Chl *a*/*b* ratios in the WT and all transgenic lines (Table 1) indicated that the assembly of the two photosystems and the photosystem stoichiometry is most likely not altered, provided that both Chl_{GG} and phytylated chlorophyll (Chl_{Phy}) are entirely proteinaceous constituents of the photosystems. The tocopherol content was significantly reduced in lines 6 and 10 and related to the relative proportions of Chl_{GG} in the transgenic lines. The reduction in tocopherol was in a similar range in leaves and isolated thylakoid membranes (Table 1).

The carotenoid composition underlined the differences between transformants and WT plants (Table 2). With the exception of the xanthophyll cycle pigments (VAZ pool), a reduction of all carotenoids was found per leaf area in the transgenic lines compared with the WT. However, an increase in the xanthophyll cycle

Table 1 Comparison of the chlorophyll and tocopherol contents of WT and *Chl P*-antisense plants of *Nicotiana tabacum*. The Chl compositions (Chl_{total}, Chl_{Phy}, Chl_{GG} and Chl *a*/*b*) and the tocopherol contents of WT and three different *Chl P*-antisense plants (lines 6, 10 and 20) were determined by HPLC. For Chl, all data are given per leaf area. The relative portion of pro Chl_{GG} is expressed in relation to the total Chl content of each line. For Chl, mean values of 10–12 different samples are shown ± SD. The absolute Chl content of WT plants was 14.8 ± 1.4 μmol Chl m^{-2} leaf area. The tocopherol content is either expressed in relation to leaf area (for leaves) or to total Chl (for thylakoids). The mean values of two measurements are shown. The absolute tocopherol content of the WT was 648 ng/100 mg fresh weight

	Chl_{total}	Chl_{Phy}	Chl_{GG}	Chl *a*/*b*	Tocopherol	
	(% WT)	(% WT)	(% Chl_{total})		(% WT)	
					Leaves	Thylakoids
WT	100	100	0	3.32 ± 0.10	100	100
6	62 ± 6	37 ± 4	41 ± 5	3.50 ± 0.17	41.5	21.4
10	70 ± 6	44 ± 5	37 ± 6	3.28 ± 0.09	37.5	29.6
20	61 ± 9	57 ± 8	7 ± 3	3.21 ± 0.09	95	66.7

Table 2 Comparison of the carotenoid compositions of WT and Chl P-antisense tobacco plants. The carotenoid contents of WT and *Chl P*-antisense plants (lines 6, 10 and 20) were determined by HPLC. Data are expressed on a leaf-area or Chl basis. Mean values of 30–40 different leaf samples are shown ± SD. The high SD for some values can be attributed to the heterogeneous pigmentation of the leaves, particularly for antisense plants. For the total Chl content see Table 1

	Chl/carotenoid	Pigment content (μmol)							
		Neoxanthin		Lutein		β-Carotene		Vx + Ax + Zx	
		m^{-2} leaf	$mmol^{-1}$ Chl	m^{-2} leaf	$mmol^{-1}$ Chl	m^{-2} leaf	$mmol^{-1}$ Chl	m^{-2} leaf	$mmol^{-1}$ Chl
WT	2.45 ± 0.17	7.8 ± 0.9	53 ± 2	25 ± 4	164 ± 14	20 ± 4	136 ± 8	0.83 ± 0.15	55 ± 8
6	2.30 ± 0.21	6.1 ± 1.3	65 ± 8	16 ± 3	176 ± 16	11 ± 1	116 ± 11	0.77 ± 0.17	83 ± 8
10	2.38 ± 0.29	7.5 ± 1.6	66 ± 10	19 ± 5	172 ± 24	12 ± 1	117 ± 5	0.88 ± 0.17	77 ± 8
20	2.54 ± 0.20	5.5 ± 1.0	54 ± 5	15 ± 1	159 ± 16	12 ± 2	124 ± 14	0.61 ± 0.09	62 ± 5

pigments could be observed relative to the Chl content in these transgenic plants, while all other carotenoids remained in the same proportions as WT contents. The similar stoichiometries of β-carotene, neoxanthin and lutein in relation to Chl indicate a similar antenna composition of both photosystems in all plants.

Pigment-binding proteins

The relative stoichiometries of the photosystems and the antenna composition were determined by Western blot analyses. Based on the same Chl content, all pigment-binding proteins of both photosystems were present in similar abundance in WT and transgenic plants (data not shown). This held true not only for the reaction-centre proteins, but also for the different antenna subcomplexes and indicates that even the presence of 40% Chl_{GG} did not disturb photosystem architecture and antenna protein assembly.

We separated some of the photosynthetic pigment-binding complexes of the thylakoid membrane by isoelectric focussing (IEF), using a protocol established for spinach thylakoids (Färber et al. 1997). Three main fractions containing Lhcb1/2, Lhcb5/6 and PSI were separated and identified by their pigment composition and their position in comparison with the respective bands from spinach thylakoids. The Lhcb5/6 fraction obviously contained low amounts of PSII reaction-core proteins, as was apparent from its relatively high β-carotene content (Table 3). The PSI fraction could not be separated into antenna proteins and core complexes.

Analysis of the pigment composition of prepared thylakoid membrane (Table 3) showed a lower Chl_{GG} content in the transgenic lines than in extracts of intact leaves (cf. Table 1). This difference is apparently explained by the fact that thylakoids were prepared from the proximal part of leaves, which showed a significantly lower Chl_{GG} content than the distal part, which was used routinely for quenching and pigment analyses. The PSI fraction contained a lower proportion of Chl_{GG}, as found in the total pigment extracts from the respective

Table 3 Pigment contents of different IEF fractions separated from thylakoids of WT and *Chl P*-antisense plants of tobacco. The pigment contents of isolated thylakoid membranes and three different fractions obtained by IEF of detergent-solubilized membranes from WT and *Chl P*-antisense plants (lines 6, 10 and 20) were determined by HPLC. The assignment of each fraction to the different proteins was based on the comparison of both the pigment content and the focussing position on the gel with earlier data obtained with spinach and pea thylakoids. Chl_{GG} is given as a percentage of Chl_{total}. A typical single experiment is shown

		% Chl_{GG}	Chl *a*/*b*	Chl_{GG} *a*/*b*	Car/Chl	Pigment content (μmol $mmol^{-1}$ Chl)			
						Nx	Lu	Vx/Ax/Zx	Car
Thylakoids	WT	0	3.16	–	0.37	50	151	40	128
	6	29	3.04	3.20	0.35	51	147	47	107
	10	31	2.94	2.83	0.37	57	164	55	98
	20	3	3.35	3.24	0.35	47	154	36	118
Lhcb1/2	WT	0	1.37	–	0.41	90	262	38	22
	6	28	1.59	1.52	0.40	101	253	33	9
	10	31	1.58	1.36	0.42	104	255	43	19
	20	5	1.26	0.46	0.36	90	228	26	12
Lhcb5/6	WT	0	7.22	–	0.23	19	59	31	115
(PSII core)	6	25	3.34	2.62	0.32	56	127	76	57
	10	25	3.84	2.69	0.29	48	98	71	72
	20	2	4.96	7.05	0.28	37	108	44	88
PSI	WT	0	9.42	–	0.26	4	53	32	171
	6	17	7.17	8.69	0.28	0	53	39	176
	10	19	6.83	7.54	0.27	1	52	42	177
	20	2	11.45	7.46	0.22	3	43	24	151

thylakoid preparations (Table 3). The two PSII fractions, however, had a Chl_{GG} content similar to that of thylakoids. This could indicate a preferential incorporation of Chl_{Phy} into PSI in favour of Chl_{GG}. It cannot be deduced from our data whether the reaction-centre core or Chl *a*/*b*-binding antenna proteins contain different proportions of Chl_{GG}.

Photosynthetic activities

The photosynthetic activities of WT and transgenic plants were investigated in vivo and in vitro. The light saturation curve of CO_2 assimilation in intact leaves is shown in Fig. 1. Apparently similar curves were obtained for all plants. No significant differences were found for either the rate of dark respiration (around 0.6 μmol CO_2 $m^{-2}s^{-1}$) or the maximum rate of CO_2 assimilation (between 6 and 7 μmol CO_2 $m^{-2}s^{-1}$). Additionally, the quantum efficiency of CO_2 assimilation, estimated from the initial slope of the curves with about 20–30 photons per fixed CO_2, was similar in all plants. Obviously, neither the reduction in the total Chl content by 30–40% nor the presence of up to 40% Chl_{GG} in the transgenic plants led to a reduction in the photosynthetic activity under in vivo conditions.

The electron transport rates of both photosystems and the rates through the photosynthetic electron transport chain were analysed by measurements of oxygen evolution using isolated thylakoids. Figure 2 illustrates that significant differences could also not be detected in the photosynthetic activity of either PSI, PSII or the electron transport chain under these in vitro conditions. The similar ratio of coupled/uncoupled linear electron transport ($H_2O \rightarrow$ ferricyanide) in WT and transgenic plants indicates that membrane permeability is also not altered by the reduction in the tocopherol content of the two severely affected lines 6 and 10.

Analysis of chlorophyll fluorescence quenching

The dependence of the quenching parameters of Chl fluorescence *q*P and NPQ on the light intensity during a 15-min illumination period were analysed in vivo (Fig. 3). No statistically significant differences were obtained between WT and transgenic plants. The similar values of *q*P indicate again a comparable photosynthetic utilization of absorbed light energy in all plants, in agreement with the data on CO_2 assimilation (cf. Fig. 1). Also, there were no dramatic differences between WT and transformants for the non-photochemical quenching, NPQ. While control plants and transgenic line 20 showed a saturation of NPQ above a PFD of 600 μmol m^{-2} s^{-1} (Fig. 3), a prolonged slow increase of NPQ at higher light intensities seemed to occur in the two strongly affected lines 6 and 10, which is consistent with a higher demand for heat dissipation of excitation

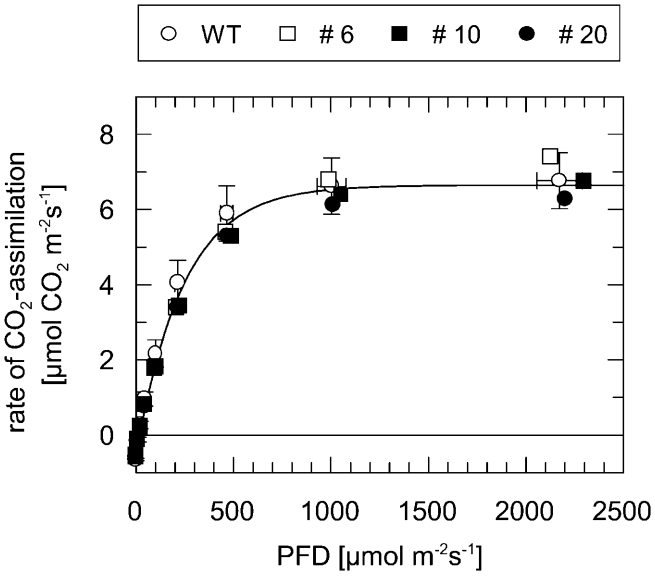

Fig. 1 Light saturation curve of CO_2 assimilation under in vivo conditions. The 3rd or 4th leaf of well-watered *Nicotiana tabacum* plants was put into the measuring cuvette and illuminated at different light intensities, starting with the highest intensity. Changes in the CO_2 concentration were recorded every minute during 20 min illumination at each light intensity. Single data points represent 12 to 15 different values obtained with each plant. Three different plants were analysed for each line (nos. *6*, *10*, *20*). For clarity, only the SD of the WT is indicated by bars. The SD was in a similar range for all transformants

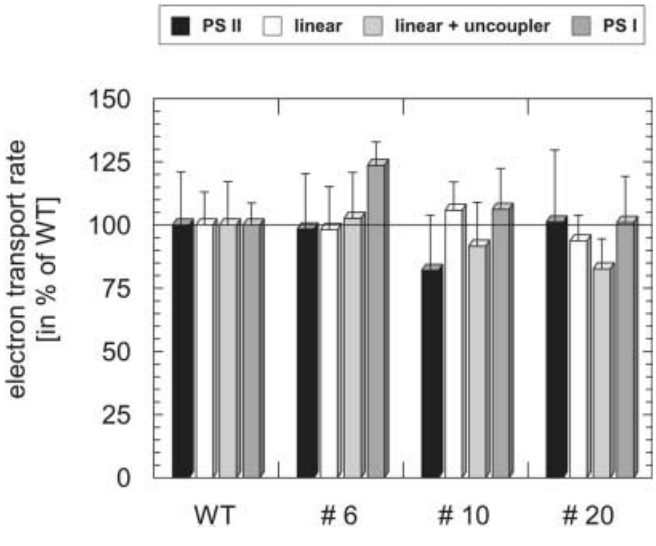

Fig. 2 Electron-transport rates of isolated thylakoid membranes of tobacco. Electron transport rates were calculated from measurements of oxygen evolution under saturating illumination using a Clark-type electrode. All electron transport rates are given as a percentage of the WT rates which were normalized to 100 for all measuring conditions. Absolute rates obtained with WT thylakoids were 780, 213, 450 and 1,100 μmol e^- $(mg\ Chl)^{-1}$ h^{-1} for PSII, linear electron transport, uncoupled linear electron transport and PSI, respectively. Data represent the mean of three different measurements performed with each of three different thylakoid preparations of each line. Bars indicate SD values

energy due to an apparent increase in photo-oxidative stress in both lines.

The relative contributions of the two major components of NPQ, namely *q*E, the energy-dependent quenching and *q*I, the photoinhibitory component to the total NPQ were determined. It is obvious from Fig. 3 that *q*I is increased by a factor of 2–3 in lines 6 and 10 compared with the WT and line 20. This increase in *q*I, on the other hand, was accompanied by a decrease in *q*E, resulting finally in a similar total value of NPQ. The general increase in *q*I in transgenic lines 6 and 10 indicates a higher susceptibility to photoinhibition, which should be more obvious under prolonged high light stress.

Susceptibility to prolonged high-light stress

Inactivation of PSII (as determined by the slowly relaxing decrease in the ratio of variable to maximum fluorescence, F_V/F_M) after 1 h illumination at a PFD of 2,000 µmol $m^{-2}s^{-1}$ was used as indicator of the sensitivity of plants to high light stress. As illustrated in Fig. 4, the WT and line 20 again exhibited similar properties. For both plants, F_V/F_M was reduced during the high-light period to about 80% of the control value, and the restoration of the fluorescence signal was nearly complete during 2 h of recovery in low light. In contrast, lines 6 and 10 showed a much higher reduction in F_V/F_M to

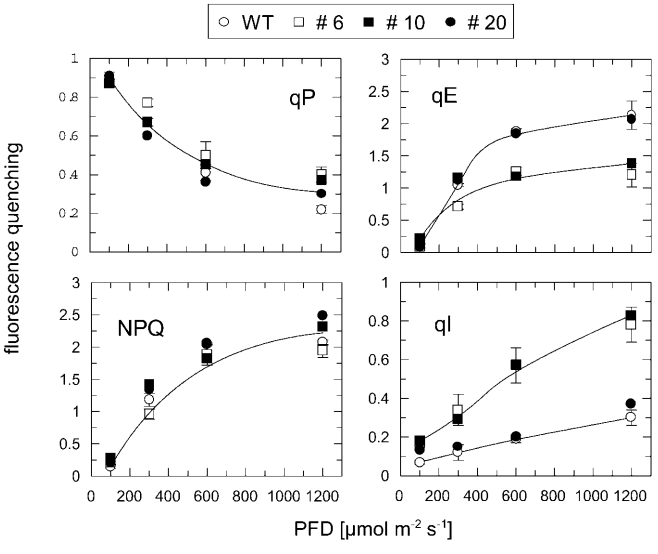

Fig. 3 Quenching of the variable Chl *a* fluorescence as a function of the actinic light (PFD). Tobacco leaf disks were illuminated for 16 min. Photochemical (*qP*) and non-photochemical (*NPQ*) fluorescence quenching parameters were determined from the fluorescence changes induced by a saturating flash at the end of actinic illumination. The two major components of non-photochemical quenching of variable fluorescence (*q*E, *q*I) and their respective Stern-Volmer coefficients were derived from the dark relaxation of F_V after 16 min illumination at different PFDs. The energy-dependent quenching (*qE*) was attributed to the portion that relaxes within the first 10 min of dark relaxation and photoinhibitory quenching (*qI*) to the remaining, slowly relaxing phase. The quenching by state transitions (*q*T) was found to be negligible under these measuring conditions. Data represent the mean of three different measurements performed with each of the four different lines (*WT*, nos. *6*, *10*, *20*). The SD is indicated by bars for the WT and line 6, and was in a similar range for the two other lines

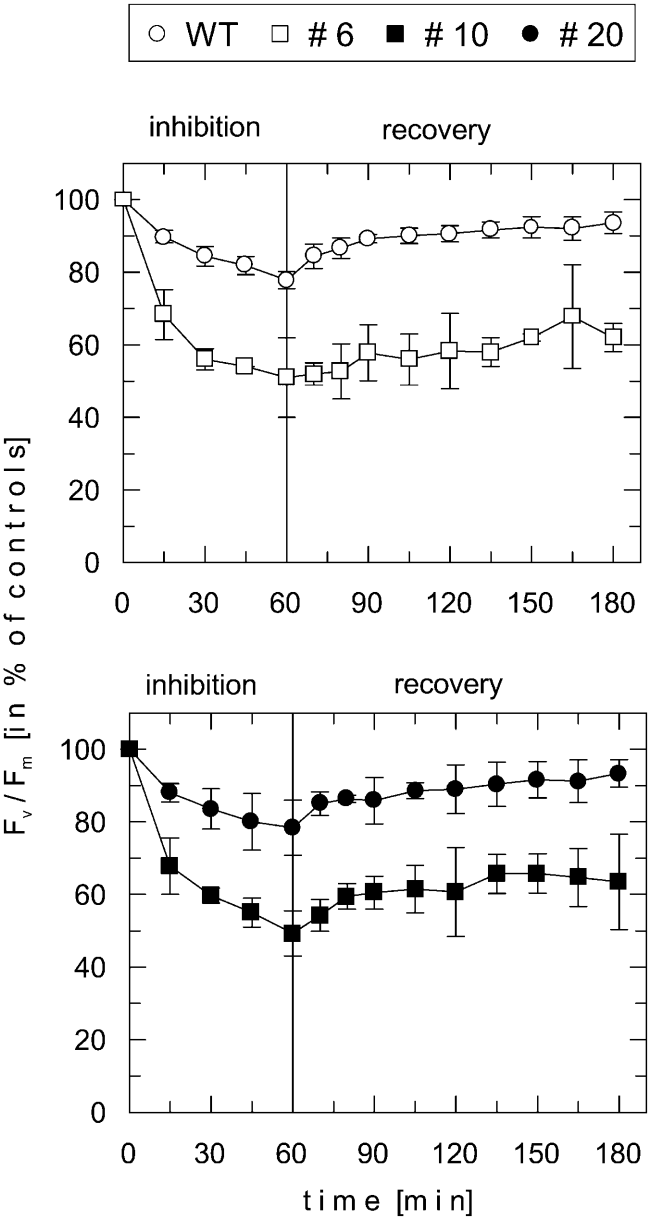

Fig. 4 Time course of photoinhibition and recovery. Tobacco leaf disks were illuminated for 1 h at a PFD of 2,000 µmol photons $m^{-2}s^{-1}$ and a temperature of 20 °C. Subsequently, leaf discs were transferred into low recovery light (PFD 20 µmol photons $m^{-2}s^{-1}$) for up to 2 h. At the given measuring points, leaf discs were dark-adapted for 10 min prior to measurement of instantaneous fluorescence (F_0) and maximum fluorescence (F_M) to allow for the relaxation of the pH-dependent fluorescence quenching (*q*E). Within one series each value was obtained with different leaf disks. Each value represents the mean of three different experiments performed with each of the different lines (*WT*, nos. *6*, *10*, *20*). For clarity, data are shown in two different diagrams; bars indicate SD values

about 50% after 1 h high light exposure. Additionally, only a small increase in the fluorescence could be detected after 2 h of subsequent low light exposure. These results indicate a dramatically increased sensitivity to high-light stress for the two strongly affected lines 6 and 10. The reduction in F_V/F_M was even more pronounced when leaves were illuminated at high PFDs for 2 h instead of 1 h. Under these conditions, the F_V was completely quenched and the recovery of the signal in low light was also abolished (not shown). Thus, the strong reduction of CHL P expression in lines 6 and 10 led to a strong increase in the susceptibility to high-light stress.

Xanthophyll cycle activity

The carotenoids zeaxanthin (Zx) and antheraxanthin (Ax), which are formed in the de-epoxidation reactions of the xanthophyll cycle, play a crucial role in the non-photochemical quenching of excitation energy. We investigated the activity of the cycle under the same illumination conditions as shown before (Fig. 4). The formation of Zx in high light was found to be similar in all plants (Fig. 5). The maximum de-epoxidation state was established within the first 15 min of illumination in all lines, indicating that the kinetics of Zx formation did not show remarkable differences. Only the increased de-epoxidation state after 1 h of high light in lines 6 and 10 might be indicative of a higher demand for energy dissipation in these plants.

Significant differences, however, were found with regard to the kinetics of epoxidation in the subsequent low-light phase. In both strongly affected lines (6 and 10) the conversion of Zx back to violaxanthin (Vx) was retarded. In agreement with earlier observations (Jahns 1995), these alterations most likely reflect the increased photo-oxidative stress in lines 6 and 10.

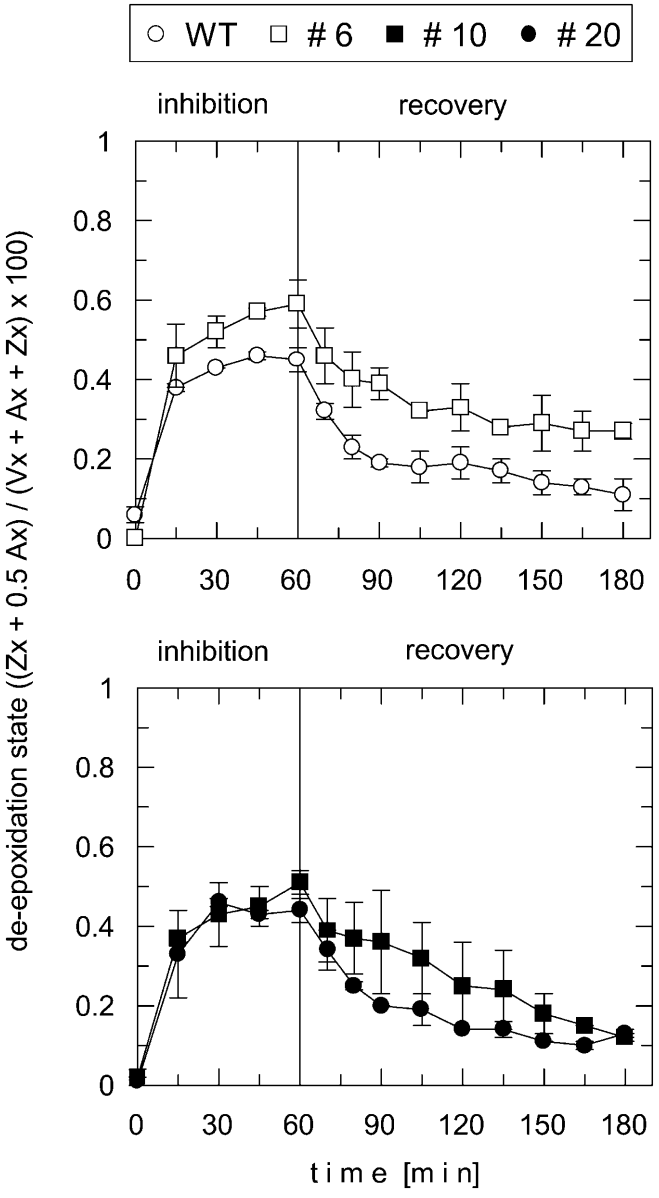

Fig. 5 Xanthophyll conversion during photoinhibition and recovery. Tobacco leaf disks from the experiment shown in Fig. 4 were used for analysis of the pigment content. Immediately after measuring the maximum fluorescence, leaf discs were frozen in liquid nitrogen. For pigment extraction, leaf discs were carefully disrupted in a mortar under addition of 1 ml acetone. After a short centrifugation of the samples, pigment extracts were filtered though a 0.2-µm filter and analysed by HPLC. The de-epoxidation state of the xanthophyll cycle pigments is calculated as $DEPS = (Zx + 0.5Ax)/(Vx + Ax + Zx) \times 100$. All other conditions were as in Fig. 4. Bars indicate SD values

Discussion

Our pigment and tocopherol data (Tables 1, 2) are in agreement with the former analysis of WT tobacco and the transgenic lines 6, 10 and 20 (Tanaka et al. 1999). A strong reduction in the tocopherol content always corresponds with a high proportion of Chl_{GG} in the transformants with reduced CHL P expression. The in vivo (Fig. 1) and in vitro (Fig. 2) measurements of the photosynthetic activities indicated that neither energy transfer from the antenna to the reaction centre nor the electron transport is influenced by the changes in the pigment composition and the tocopherol content. Thus, as has been previously shown for *bch P*-deficient *Rhodobacter* cells (Bollivar et al. 1994), the assembly and function of the antenna system in higher plants is also not disturbed when Chl_{GG} replaces Chl_{Phy}.

It is remarkable, however, that Chl_{GG} was not equally associated with all Chl-binding proteins (Table 3). Preferential binding of Chl_{Phy} instead of Chl_{GG} was particularly determined for PSI. Since the Chl_{GG} a/b ratio in the PSI fraction was in a similar range as the Chl_{Phy} a/b ratio, we conclude that core and peripheral antennae do not selectively distinguish between the geranylgeranylated and the phytylated Chl. The analysis of the Chl_{GG} content of the two photosystems in stroma and grana fractions of the membrane could clarify whether PSI has a preferential affinity for Chl_{Phy} or if assembly of Chl_{Phy} is favoured in distinct membrane regions.

The presence of Chl_{GG} had no influence on the activity of either photosystem. The same can be suggested for the putative presence of geranylgeranylated phylloquinone in the PSI reaction-centre core. If the phylloquinone precursor 1,4-dihydroxy-2-naphthoic acid cannot be specifically prenylated only with phytyl diphosphate, phylloquinone should be present to the same extent in the geranylgeranylated form as Chl.

Our fluorescence analyses indicated a strong increase in the susceptibility of PSII to high light intensities (reflected by the increase in *q*I) in the two severely affected lines 6 and 10 (Figs. 3, 4). Because of the unchanged photosynthetic capacity in these two lines, the changes in *q*I are predominantly attributed to the lower tocopherol content and not to the presence of Chl_{GG}. Chl_{GG} would probably impair energy transfer or antenna assembly in one or both photosystems, resulting in a lower photosynthetic capacity, but not in an increased susceptibility to photoinhibition. However, the almost identical rates of CO_2 assimilation in intact leaves, as well as the unchanged electron transport rates obtained with isolated thylakoids, argue against any restriction of the photosynthetic capacity due to a high Chl_{GG} content.

We can also exclude that the reduction of *q*E is responsible for the increase in *q*I. Firstly, a much more pronounced reduction of *q*E, e.g. in *chlorina f2* mutants of barley did not have a significant effect on the photoinhibitory fluorescence quenching *q*I in comparison with the respective WT plants (data not shown). Secondly, *q*E was found to be not reduced in lines 6 and 10 within the first 5 min of illumination in the quenching analysis (not shown), so that the reduction of *q*E seems to be rather a result of the increased *q*I after prolonged illumination than the other way round.

The increased VAZ-pool size in lines 6 and 10 can be interpreted as an adaptation to an increased light sensitivity in these two lines. Similar changes in the VAZ-pool size were previously observed for sun leaves in comparison with shade leaves (Demmig-Adams 1998), young leaves (Krause et al. 1995), antenna-depleted plants (Jahns 1995) and plants grown under light or temperature stress (Król et al. 1999). In all cases, an increased demand for energy dissipation is required due to environmental stress conditions or a reduced capacity for non-photochemical quenching of excitation energy.

It is remarkable in this context that the absolute increase in the VAZ-pool size in lines 6 and 10 (about 15–20 per 1,000 Chl molecules, corresponding to roughly 1 per 40 Chl *a*) was in the same range as the tocopherol concentration in tobacco leaves (1 molecule tocopherol per 40–50 Chl was found by Tanaka et al. 1999). It has recently been suggested that the xanthophyll cycle pigments, in addition to their assumed dissipative function in the antenna, may play a crucial role in membrane fluidity, thermostability and, additionally, in the protection of the membrane against lipid peroxidation (Havaux 1998; Havaux and Niyogi 1999; Havaux et al. 2000). These proposed functions of the xanthophylls resemble those of α-tocopherol (Fryer 1992), so that a reduction in the α-tocopherol content could possibly be compensated by an increase in xanthophyll cycle pigments.

However, one would expect that such a pool of xanthophylls (at least in the de-epoxidized state) is not bound, or is only loosely bound, to antenna proteins. The relative increase in the xanthophyll cycle pigments in PSI and Lhcb5/6, as observed in the IEF experiment (Table 3), seems to be not consistent with this assumption. Moreover, the high susceptibility of lines 6 and 10 to photo-oxidative stress implies that carotenoids cannot entirely compensate for the deficient protective function of tocopherol against lipid peroxidation. But more careful quantitative analyses are required to determine the fractions of protein-bound and free xanthophylls, particularly in the de-epoxidized state.

The mechanism by which low tocopherol contents in the plastidic membranes caused increased inactivation of PSII in lines 6 and 10 remains elusive. Since changes in photosynthetic electron transfer in the mutants can be excluded from our study, it is most likely that an increased formation of reactive oxygen species in the tocopherol-deficient plants is involved. It is well known from the literature that active oxygen plays a central role in the high-light-induced inactivation of and damage to PSII (for a review, see Krause 1994). Thus, it is reasonable to assume that a reduced detoxification of reactive oxygen species in the membrane due to the reduced tocopherol content is responsible for the increased photoinactivation of PSII in lines 6 and 10.

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References

Asada K (1994) Production and action of active oxygen in photosynthetic tissues. In: Foyer CH, Mullineaux PM (eds) Causes of photo-oxidative stress and amelioration of defence systems in plants. CRC Press, Boca Raton, pp 77–104

Asada K (1999) The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 50:601–639

Asada K, Endo T, Mano J, Miyake C (1998) Molecular mechanism for relaxation of and protection from light stress. In: Satoh K, Murata N (eds) Stress responses of photosynthetic organsims: molecular mechanisms and molecular regulations. Elsevier V, Amsterdam, pp 37–52

Barber J, de las Rivas J (1993) A functional model for the role of cytochrome b_{559} in the protection against donor and acceptor side photoinhibition. Proc Natl Acad Sci USA 90:10942–10946

Bishop NI, Wong J (1974) Photochemical characteristics of a vitamin E deficient mutant of *Scenedesmus obliquus*. Ber Dtsch Bot Ges 87:359–371

Bollivar DW, Wang S, Allen JP, Bauer CE (1994) Molecular genetic analysis of terminal steps in bacteriochlorophyll *a* biosynthesis: Characterization of a *Rhodobacter capsulatus* strain that synthesizes geranyl-geraniol-esterified bacteriochlorophyll *a*. Biochemistry 33:12763–12768

Cobbett CS, May MJ, Howden R, Rolls B (1998) The glutathione-deficient, cadmium-sensitive mutant, cad2-1, of *Arabidopsis*

thaliana is deficient in gamma-glutamylcysteine synthetase. Plant J 16:73–78

Conklin PL, Pallanca JE, Last RL, Smirnoff N (1997) L-ascorbic acid metabolism in the ascorbate-deficient arabidopsis mutant vtc1. Plant Physiol 115:1277–1285

Demmig-Adams B (1998) Survey of thermal energy dissipation and pigment composition in sun and shade leaves. Plant Cell Physiol 39:474–482

Demmig-Adams B, Adams WW (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci 1:21–26

Fahrenholtz SR, Doleiden FH, Trozzolo AM, Lamola AA (1974) Quenching of singlet oxygen by α-tocopherol. Photochem Photobiol 20:505–509

Färber A, Young AJ, Ruban AV, Horton P, Jahns P (1997) Dynamics of xanthophyll-cycle activity in different antenna subcomplexes in the photosynthetic membranes of higher plants: the relationship between zeaxanthin conversion and nonphotochemical fluorescence quenching. Plant Physiol 115:1609–1618

Foyer CH, Lelandais M, Kunert KJ (1994) Photo-oxidative stress in plants. Physiol Plant 92:696–717

Fryer MJ (1992) The antioxidant effects of thylakoid vitamin E (α-tocopherol). Plant Cell Environ 15:381–392

Halliwell B (1981) Chloroplast metabolism: the structure and function of chloroplasts in green leaf cells. Clarendon Press, Oxford

Havaux M (1998) Carotenoids as membrane stabilizers in chloroplasts. Trends Plant Sci 3:147–151

Havaux M, Niyogi KK (1999) The violaxanthin cycle protects plants from photo-oxidative damage by more than one mechanism. Proc Natl Acad Sci USA 96:8762–8767

Havaux M, Bonfils J-P, Lütz C, Niyogi KK (2000) Photodamage of the photosynthetic apparatus and its dependence on the leaf developmental stage in the *npq1 Arabidopsis* mutant deficient in the xanthophyll cycle enzyme violoxanthin de-epoxidase. Plant Physiol 124:273–284

Heber U, Walker D (1992) Concerning a dual function of coupled cyclic electron transport in leaves. Plant Physiol 100:1621–1626

Horton P, Ruban AV, Walters RG (1996) Regulation of light harvesting in green plants. Annu Rev Plant Physiol Plant Mol Biol 47:655–684

Hurry V, Anderson JM, Chow WS, Osmond CB (1997) Accumulation of zeaxanthin in abscisic acid-deficient mutants of *Arabidopsis* does not affect chlorophyll fluorescence quenching or sensitivity to photoinhibition in vivo. Plant Physiol 113:639–648

Jahns P (1995) The xanthophyll cycle in intermittent light grown pea plants: possible functions of chlorophyll *a*/*b* binding proteins. Plant Physiol 108:149–156

Kozaki A, Takeba G (1996) Photorespiration protects C3 plants from photooxidation. Nature 384:557–560

Krause GH (1994) The role of oxygen in photoinhibition of photosynthesis. In: Foyer CH, Mullineaux PM (eds) Causes of photo-oxidative stress and amelioration of defence systems in plants. CRC Press, Boca Raton, pp 43–76

Krause GH, Virgo A, Winter K (1995) High susceptibility to photoinhibition of young leaves of tropical forest trees. Planta 197:583–591

Król M, Ivanov AG, Jansson S, Kloppstech K, Huner NPA (1999) Greening under high light or cold temperature affects the level of xanthophyll-cycle pigments, early light-inducible proteins, and light-harvesting polypeptides in wild-type barley and the *chlorina f2* mutant. Plant Physiol 120:193–203

Munné-Bosch S, Alegre L (2000) Changes in carotenoids, tocopherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in *Rosmarinus officinalis* plants. Planta 210:925–931

Neely WC, Martin JM, Barker SA (1988) Products and relative reaction rates of the oxidation of tocopherols with singlet molecular oxygen. Photochem Photobiol 48:423–428

Niyogi KK (1999) Photoprotection revisited: genetic and molecular approaches. Annu Rev Plant Physiol Plant Mol Biol 50:333–359

Niyogi KK, Grossman AR, Björkman O (1998) *Arabidopsis* mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. Plant Cell 10:1121–1134

Quick WP, Stitt M (1989) An examination of factors contributing to non-photochemical quenching of chlorophyll fluorescence in barley leaves. Biochim Biophys Acta 977:287–296

Smirnoff N (1996) The function and metabolism of ascorbic acid in plants. Ann Bot 78:661–669

Tanaka R, Oster U, Kruse E, Rüdiger W, Grimm B (1999) Reduced activity of geranylgeranyl reductase leads to loss of chlorophyll and tocopherol and to partially geranylgeranylated chlorophyll in transgenic tobacco plants expressing antisense RNA for geranylgeranyl reductase. Plant Physiol 120:695–704

Thiele A, Schirwitz K, Winter K, Krause GH (1996) Increased xanthophyll cycle activity and reduced D1 protein inactivation related to photoinhibition in two plant systems acclimated to excess light. Plant Sci 115:237–250

Walters RG, Horton P (1991) Resolution of components of non-photochemical chlorophyll fluorescence quenching in barley leaves. Photosynth Res 27:121–133

Wise RR, Naylor AW (1987) Chilling-enhanced photooxidation: evidence for the role of singlet oxygen and superoxide in the breakdown of pigments and endogenous antioxidants. Plant Physiol 83:278–282