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Reduced levels of cytochrome b_6f in transgenic tobacco increases the excitation pressure on Photosystem II without increasing sensitivity to photoinhibition *in vivo*

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Abstract

We have examined tobacco transformed with an antisense construct against the Rieske-FeS subunit of the cytochrome b_6f complex, containing only 15 to 20% of the wild-type level of cytochrome *f*. The anti-Rieske-FeS leaves had a comparable chlorophyll and Photosystem II reaction center stoichiometry and a comparable carotenoid profile to the wild-type, with differences of less than 10% on a leaf area basis. When exposed to high irradiance, the anti-Rieske-FeS leaves showed a greatly increased closure of Photosystem II and a much reduced capacity to develop non-photochemical quenching compared with wild-type. However, contrary to our expectations, the anti-Rieske-FeS leaves were not more susceptible to photoinhibition than were wild-type leaves. Further, when we regulated the irradiance so that the excitation pressure on photosystem II was equivalent in both the anti-Rieske-FeS and wild-type leaves, the anti-Rieske-FeS leaves experienced much less photoinhibition than wild-type. The evidence from the anti-Rieske-FeS tobacco suggests that rapid photoinactivation of Photosystem II *in vivo* only occurs when closure of Photosystem II coincides with lumen acidification. These results suggest that the model of photoinhibition *in vivo* occurring principally because of limitations to electron withdrawal from photosystem II does not explain photoinhibition in these transgenic tobacco leaves, and we need to re-evaluate the twinned concepts of photoinhibition and photoprotection.

Abbreviations: Chl – chlorophyll; DCMU – 3-(3′,4′-dichlophenyl)-1,-dimethylurea; Fo and Fo′ – minimal fluorescence when all PS II reaction centers are open in dark- and light-acclimated leaves, respectively; Fm and Fm′ – maximal fluorescence when all PS II reaction centers are closed in dark- and light-acclimated leaves, respectively; Fv – variable fluorescence (Fm − Fo) in dark acclimated leaves; Fv′ – variable fluorescence (Fm′ − Fo′) in light-acclimated leaves; NPQ – non-photochemical quenching of fluorescence; PS I and PS II – Photosystem I and II; P680 – primary electron donor of the reaction center of PS II; PFD – photosynthetic flux density; Q_A – primary acceptor quinone of PS II; q_P – photochemical quenching of fluorescence; V+A+Z – violaxanthin + antheraxanthin + zeaxanthin

Introduction

Photoinhibition is the loss of photosynthetic efficiency when the light-harvesting antennae absorb excitation energy in excess of that used for photosynthesis (Powles 1984; Osmond 1994). Of the four macromolecular complexes found in thylakoid membranes, Photosystem II (PS II) is the most vulnerable to dam-

age from excess irradiance, although damage to Photosystem I (PS I) has also been shown (Sonoike and Terashima 1994). In the field, the point where excitation capture exceeds the capacity for electron withdrawal from PS II varies, with effects of fluctuations in temperature, water and nutrient status superimposed on fluctuations in irradiance. Consequently, the responses of plants to variations in energy capture requirements are complex, and include both avoidance and tolerance mechanisms.

A range of avoidance mechanisms have been described for higher plants, including both long-term acclimative and fast dissipative mechanisms. Long-term responses include modulation of thylakoid composition (Anderson and Osmond 1987) and changes to leaf reflectance (Robinson et al. 1993). Fast avoidance mechanisms, estimated collectively by non-photochemical quenching of Chl fluorescence, dissipate absorbed photons as heat and rapidly down-regulate PS II energy capture efficiency during excess irradiance. Both reaction center and antenna-based mechanisms have been implicated in non-photochemical quenching of chlorophyll fluorescence, and the principal driving force behind these dissipative reactions is the development of the thylakoid ΔpH (Briantais et al. 1979; Krause et al. 1982). Low lumenal pH has been shown to slow electron donation from water to $P680^+$ and under these conditions excitation energy trapped by PS II may be dissipated as heat by fast internal charge recombination (Krieger and Weis 1990). The mechanistic basis for antenna-quenching, which is also driven by the ΔpH and is thought to be enhanced by the activity of the xanthophyll cycle (reviewed by Demmig-Adams and Adams 1992; Pfündel and Bilger 1994), remains unclear. Current models include both direct (Chow 1994; Frank et al. 1994; Owens 1994) and indirect (Ruban et al. 1992, 1993; Mohanty et al. 1995; Gilmore et al. 1996a, b) quenching roles for the xanthophyll zeaxanthin. However, the primary site(s) and mechanism(s) involved in antenna-quenching, and the degree to which such quenchers can divert energy from PS II, has not been established unequivocally. An important characteristic of these putative antennae-based photoprotective mechanisms is that they develop *coincidently* with the reduction of PS II electron acceptors, and as far as is known, they do not respond directly to the reduction state of the PS II electron acceptor pool.

The principal mechanism that enables higher plants to tolerate excess irradiance is the D1 protein repair cycle. Light-dependent turn-over of the D1 protein subunit of the PS II core was early identified with photoinhibition (Mattoo et al. 1981; Kyle et al. 1984) and net photoinhibition has been shown to occur when the rate of D1 protein degradation exceeds the capacity for D1 protein synthesis (Greer et al. 1986; Aro et al. 1993a,b). However, D1 protein synthesis has been shown to saturate at low irradiances (Aro et al. 1993b, 1994; Park et al. 1996a), and pea leaves are known to have the same intrinsic capacity for D1 protein synthesis regardless of growth irradiance (Park et al. 1995a,b, 1996a,b). Furthermore, the efficacy of this tolerance mechanism will vary depending on the particular environmental conditions (e.g. low temperature) that prevail, and when combined with high irradiance, results in photoinhibition.

A model has begun to emerge from the many biochemical and biophysical studies that suggests photoinhibition of PS II can occur because of either limitations in electron delivery to oxidised PS II reaction centers (donor-side limitation) or because of a limited capacity for electron withdrawal from PS II (acceptor-side limitation). *In vitro* studies have shown that D1 degradation can occur because of cleavage on either the lumenal or stromal side of the thylakoid membrane (De Las Rivas et al. 1992; Aro et al. 1993b). However, the evidence for which of these limitations is primarily responsible for photoinhibition *in vivo* in higher plant leaves remains equivocal. Some reports suggest primarily acceptor-side related damage (De Las Rivas et al. 1993; Shipton and Barber 1994) while others suggest significant donor-side related degradation of the D1 protein (Russell et al. 1995; Kettunen et al. 1996).

In normal higher plant leaves the coincident development of PS II reaction center closure, leading to acceptor-side limitations, and lumen acidification, which may give rise to donor-side limitations by slowing electron donation from water to $P680^+$ (Krieger and Weis 1990), can only be broken by chemical means such as with the use of uncouplers (e.g. nigericin) or inhibitors of electron transport (e.g. DCMU). In this paper we describe experiments using tobacco transformed with an antisense mRNA construct against the transcript for the Rieske-FeS subunit of the cytochrome b_6/f complex (Price et al. 1995). Even at low irradiances, the electron carriers between P680 and the cytochrome b_6f complex are highly reduced in these leaves, and under these conditions little non-photochemical quenching develops. The principal aim of these experiments was to test whether photoinhibition *in vivo* occurs primarily as the result of the overreduction of electron carriers on the acceptor-side

of P680. A secondary aim was to assess the role of non-photochemical quenching in photoprotection.

Materials and methods

Plant material

Tobacco (*Nicotiana tabacum* L. var. Wisconsin 38) transformed to express antisense mRNA against the Rieske-FeS subunit of the cytochrome b_6/f complex was used (Price et al. 1995). Plants were raised from R2 seed from line B6F2.2-513 (see Figure 4 in Price et al. 1995). These plants are homozygous for the antisense insert but have the advantage that the Rieske-FeS antisense phenotype appears and stabilises approximately 5 weeks post-germination (GD Price unpublished data). Both wild-type and anti-Rieske-FeS plants were grown in soil and maintained on a diurnal cycle of 20 h light (120 μmol m^{-2} s^{-1} PFD) and 4 h dark, with a constant temperature (25 °C). The most recent fully-expanded leaf was used for all experiments.

Determination of cytochromes f *and* b_6 *and Rieske-FeS protein*

The level of antisense suppression was determined by Western blot analysis using antibodies raised against both the Rieske-FeS (Price et al. 1995) and cytochrome *f* proteins (Barkan et al. 1986). The Western blot bands were developed and quantified using ImageQuant software (Molecular Dynamics, CA, USA) as described previously (Price et al. 1995). Cytochromes *f* and b_6 were also determined by reduced minus oxidised absorbance difference spectra (Bendall et al. 1971).

Chlorophyll fluorescence

Chlorophyll *a* fluorescence was measured using a modulated fluorometer (PAM Chlorophyll Fluorometer; H. Walz AG, Effeltrich, Germany) with the PAM 103 accessory and two Schott lamps (model KL 1500; Schott Glaswerke AG, Mainz, Germany), providing saturating flashes and actinic illumination. Light response curves were measured on three different attached leaves in air at 23 °C. Fluorescence induction kinetics were monitored at different PFD's ranging from 10 to 2000 μmol m^{-2} s^{-1}. Fluorescence characteristics were evaluated when the steady-state Fs level was reached which, depending on PFD, occurred 10 to 30 min after switching to the next higher light

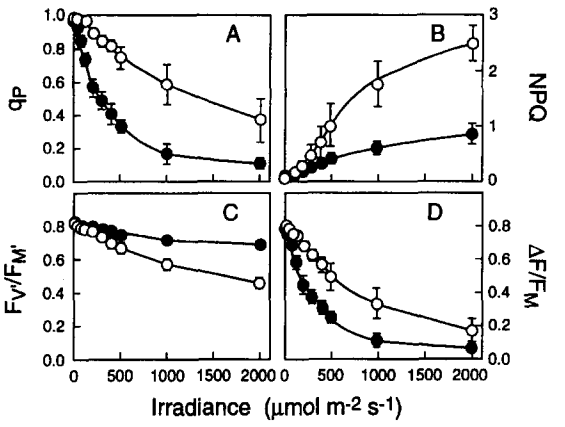

Figure 1. Irradiance response curves of Chl *a* fluorescence parameters measured on attached leaves in air at 23 °C. ○, wild-type; ●, anti-Rieske-FeS tobacco. Each point represents the mean ± SD of three different leaves.

level. Light response curves were also measured on leaf discs at 23 °C in humidified air. While leaf discs showed a more rapid reduction of the PS II acceptor pool, and more rapid development of NPQ, the differences between the anti-Rieske-FeS and wild-type leaves (Figure 1) were maintained (data not shown).

Fluorescence quenching characteristics were also monitored during subsequent high irradiance treatments. Leaf discs (1.0 cm^2) were cut from the leaves used to measure the light response curves and floated on distilled H_2O, adaxial face up. These were then dark-adapted for 30 min, and Fo and Fm determined with the PAM fluorometer. Trays containing the leaf discs were then placed on a gently shaking platform in a temperature-controlled water bath (20 °C) under a Phillips HPLR 1000 W mercury vapour lamp, with the light path interrupted by a glass heat and UV filter (Schott 115 Tempax). At different intervals, three replicate leaf discs were removed from the trays and immediately transferred to a leafdisc cuvette (model LD2; Hansatech Instruments Ltd, King's Lynn, Norfolk, UK) with the top modified to fit the optic fibre of the PAM fluorometer. The cuvette was pre-set to the irradiance of the treatment lamp (measured at the position of the leaf disc) so that the fluorescence characteristics could be monitored at the treatment irradiance as the experiment progressed. The proportion of PS II centers that were open (q_P), non-photochemical quenching (NPQ), the excitation capture efficiency of open reaction centers (Fv'/Fm'), and the yield of electron transport (ΔF/Fm), were calculated as described previously (Genty et al. 1989; Schreiber et al. 1994).

The leaf discs from the fluorescence quenching analysis, and those from the functional PS II measurements (see below), were combined and dark-adapted for 30 min. These leaf discs were used to quantify photoinhibition from fluorescence with a portable fluorometer (Plant Efficiency Analyser; Hansatech Instruments Ltd), using the parameter Fv/Fm.

Photon exposure, an estimate of the sum of photons captured per unit area (Park et al. 1996b) was calculated for the different experimental conditions using the following equation:

$$\text{Photon exposure} = \text{Irradiance} \times \text{Initial absorbance} \times \text{Time}$$

Functional PS II – oxygen flash yield

Photoinhibition was also quantified from the number of functional PS II reaction centers capable of oxygen evolution. The number of functional PS II reaction centers was determined from the oxygen yield per single-turnover flash during repetitive (10 Hz) saturating flash illumination and expressed on a Chl basis (Chow et al. 1991). Four leaf discs (4 cm^2) were enclosed in a modified Hansatech leaf disc cuvette (model LD2; Hansatech Lt., King's Lynn, Norfolk, UK) attached to a temperature controlled water bath set to 23 °C. Oxygen evolution was measured during a sequence of 4 min dark followed by 4 min of repetitive flash illumination. This sequence was repeated twice and the rate of gross O_2 evolution in the light was calculated from the average of these two measurement intervals. The duration of the flash, measured as the full width at half-peak height was approximately 2.5 μs. Background far-red light was used to avoid any limitation of electron transport by PS I. A small heating artifact resulting from the repetitive flash illumination was measured and subtracted from these calculations.

Pigments

Chl and carotenoids were measured from three replicate leaf discs (1.0 cm^2) collected at each time point during the high irradiance treatments, and frozen in liquid N_2. The frozen samples were ground in a mortar and pestle and extracted in ice cold 100% acetone (acetone-water mixtures did not improve carotenoid extraction). Pigments were measured by HPLC and separated on a Spherisorb ODS1 column (Alltech Associates, Sydney, Australia) at a flow rate of 1 ml min^{-1} with solvents A and B as described previously (Gilmore and Yamarnoto 1991). The injection volume was 20 μl. The column either was calibrated with standards prepared by TLC (Demmig et al. 1987) (neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, Chl *a*, β-carotene) or using commercial preparations (Chl *b*; Sigma, St. Louis, MO, USA). The concentration of Chl was checked spectrophotometrically in 80% buffered acetone (Porra et al. 1989).

Results

Characterisation of the anti-Rieske-FeS tobacco

Tobacco showing homozygous expression of an antisense mRNA insert against the Rieske-FeS subunit of the cytochrome b_6/f complex was studied to assess the role of reduced electron carriers on the acceptor side of PS II in photoinhibition. The anti-Rieske-FeS transformed leaves contained only 15 to 20% of the wild-type amount of the Rieske-FeS and cytochrome *f* apoproteins as determined by specific antibodies, and these apoproteins were found to have a 1:1 stoichiometry in both anti-Rieske-FeS and wild-type thylakoids. The concentration of cytochromes *f* and b_6 were also determined from reduced minus oxidised absorbance spectra of isolated thylakoids. Thus, only 15 to 20% of the assembled cytochrome b_6/f complexes were present in the anti-Rieske-FeS tobacco. This resulted in a constitutively reduced electron transport chain between P680 and the donor-side of the cytochrome b_6/f complex, such that at the growth irradiance 25% of the Q_A pool was reduced in the anti-Rieske-FeS leaves, compared with only 3% in the wild-type. Further, the anti-Rieske-FeS leaves experienced much greater excitation pressure on PS II (lower q_P) than did wild-type tobacco over the full range of irradiances measured (Figure 1A). Thus in transformed leaves, functional PS II reaction centers were much more prone to closure than in wild-type leaves.

The anti-Rieske-FeS leaves also developed lower levels of NPQ than did the wild-type (Figure 1B). Previously, we have reported that anti-Rieske-FeS leaves have equivalent levels of ATPase activity (Price et al. 1995). Thus, the lower levels of NPQ reflect the reduction in proton translocation and release into the lumen because of a reduced amount of linear electron transport. One consequence of the lower level of NPQ was that the trapping efficiency of open PSIIs (Fv'/Fm') remained high in the anti-Rieske-FeS leaves (Figure

Table 1. Chlorophyll and carotenoid content of wild-type and anti-Rieske-FeS leaves. Leaf discs were cut from fully-expanded leaves 3 h into the growth photoperiod. Pigments were analysed by HPLC as described in the 'Materials and methods'. Numbers represent the mean ± SD of three 1.0 cm^2 leaf discs

Pigment (μmol m^{-2})	Wild-type	Anti-Rieske-FeS
Total chlorophyll	505±47	482±66
V+A+Z	23±3	19±3
Neoxanthin	27±4	24±2
Lutein	67±6	60±8
β-carotene	59±7	52±7
Total carotenoids	177±16	155±19
Chl *a/b*	3.3±0.1	3.1±0.1

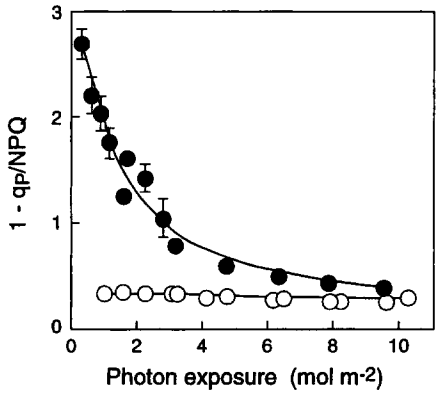

Figure 2. Relation between the ratio of 1 − q_P/NPQ and photon exposure. (○) wild-type; (●), anti-Rieske-FeS tobacco. Each point represents the mean ± SD of three replicate leaf discs. Fluorescence quenching parameters were measured using leaf discs in air at 23 °C.

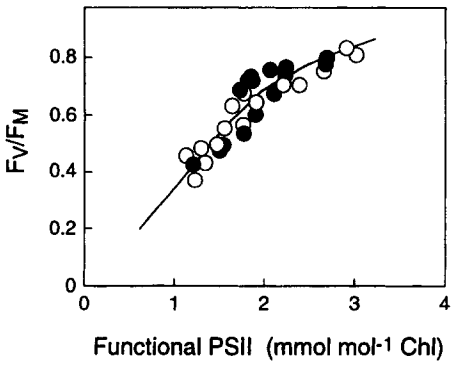

Figure 3. Relation between changes in functional PS II concentration and loss of dark-adapted Fv/Fm. (○), wild-type; (●), anti-Rieske-FeS tobacco.

1C). However, the quantum yield of electron transport (ΔF/Fm) was lower, at all but the lowest irradiances, due to the increased closure of PS II (Figures 1A and 1D).

The increase in excitation pressure on PS II in these leaves with a constitutivtely reduced electron transport chain did not greatly affect overall Chl or carotenoid content of the anti-Rieske-FeS leaves (Table 1). Nor was there a pronounced effect on the concentration of functional PS IIs, with the wild-type and anti-Rieske-FeS leaves having 3.0 and 2.7 mmol PS II mol^{-1} Chl, respectively. This was consistent with a slightly larger antenna size for PS II in the anti-Rieske-FeS leaves, and correlated with a decrease in the Chl *a/b* ratio (Table 1) (Price et al. 1995). Considering the proposed photoprotective role of the xanthophylls and the sustained closure of PS IIs experienced by these leaves under the growth irradiance, it was surprising to find that the anti-Rieske-FeS leaves had not increased the content of xanthophyll cycle pigments. If anything a small reduction from 45 to 39 mmol xanthophyll cycle pigments (V+A+Z) mol^{-1} Chl was found in the anti-Rieske-FeS leaves (Table 1).

We have used the ratio of (1 − q_P)/NPQ to show the balance between photochemical quenching and the capacity to develop antennae quenching. This relation has previously been proposed as an index of susceptibility of PS II to light stress (Osmond 1994). Wild-type leaves had a constant ratio over the range of photon exposures tested, a characteristic reported previously from studies using pea leaves (Park et al. 1995b, 1996b). In contrast, leaves of the anti-Rieske-FeS plants showed a poor capacity to maintain a balance between these two mechanisms and they were exposed to higher excitation pressures than wild-type leaves, particularly at low photon exposures (Figure 2). However, the anti-Rieske-FeS leaves did retain the capacity to develop NPQ, demonstrating that the quenching mechanisms themselves were not impaired but that NPQ develops more slowly in response to photon exposure in the transgenics. Thus, anti-Rieske-FeS leaves experience higher excitation pressures on PS II and this is prolonged by the slow rate at which NPQ is generated.

Response of low Rieske-FeS antisense plants to high irradiance

As the relative capacity to develop NPQ was different for the anti-Rieske-FeS and wild-type leaves, we have used both Chl *a* fluorescence and oxygen flash

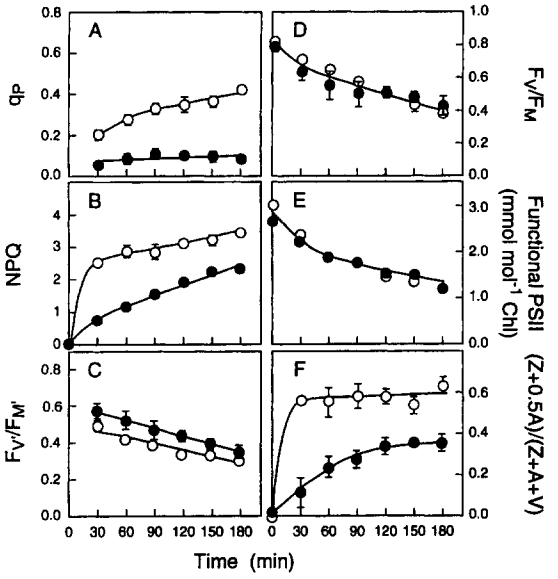

Figure 4. Response of Chl *a* fluorescence quenching, PS II function and violaxanthin de-epoxidation in wild-type (○) and anti-Rieske-FeS (●) tobacco to exposure to an irradiance of 1000 μmol m^{-2} s^{-1} PFD. Z = zeaxanthin, A = antheraxanthin, V = violaxanthin. Each point in the fluorescence quenching curves represents the mean ± SD of three replicate leaf discs. For Fv/Fm, $n = 7$ and for Functional PS II, $n = 4$.

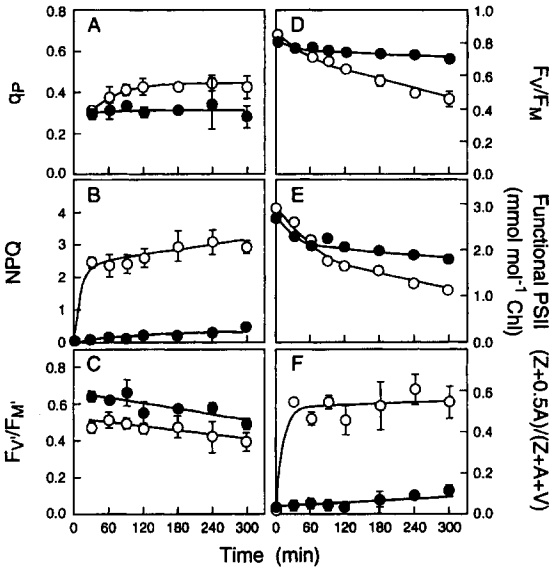

Figure 5. Experimental protocol similar to Figure 4 except that irradiance was varied to maintain equivalent excitation pressure on PS II ($q_P = 0.3$). For the wild-type (○) this required an irradiance of 650 μmol m^{-2} s^{-1} PFD and for the anti-Rieske-FeS (●) tobacco an irradiance of 175 μmol m^{-2} s^{-1} PFD. Z = zeaxanthin, A = antheraxanthin, V = violaxanthin. Each point in the fluorescence quenching curves represents the mean ± SD of three replicate leaf discs. For Fv/Fm, $n = 7$ and for Functional PS II, $n = 4$.

yield measurements to quantify photoinhibition during prolonged exposure to high irradiance. The relation between functionality of PS II as assessed by the fluorescence parameter Fv/Fm and by oxygen flash yield is shown in Figure 3. While the relation is curvilinear, it is not different for the anti-Rieske-FeS leaves. Thus, the changes in dark-adapted Fv/Fm and oxygen flash yield measured without a dark period reflect similar changes in PS II functionality in both the wild-type and anti-Rieske-FeS leaves.

At 1000 μmol m^{-2} PFD, the anti-Rieske-FeS leaves experienced much higher excitation pressure on functional PS II reaction centers (lower q_P), and this remained constant over the 3 h period of the experiment (Figure 4A). However, the wild-type leaves showed a slight relaxation in closure of functional PS IIs over time. This opening of functional PS IIs correlated with the net loss of functional PS II centers (Figure 4E), suggesting that as functional centers were lost, those remaining were exposed to progressively less excitation energy. Therefore, non-functional PS II centers may act as effective quenching units in wild-type leaves. This was not so for the anti-Rieske-FeS leaves. However, the restriction in electron flow in these leaves may have been such that the remaining functional PS IIs were kept closed despite the formation of inactive centers. NPQ also developed more slowly in the anti-Rieske-FeS leaves (Figure 4B), due most likely to slower development of lumen acidification and the associated slower deepoxidation of violaxanthin to zeaxanthin (Figure 4F). Low lumenal acidification may also have contributed to the failure of inactivated PS IIs to form quenching centers in the anti-Rieske-FeS leaves. Similar conclusions were drawn from the limited capacity of low-light grown peas to develop quenching centers, despite the accumulation of inactive PS IIs (Park et al. 1996b). A higher trapping efficiency in the anti-Rieske-FeS leaves was associated with the lower NPQ (Figure 4C), but the difference was surprisingly small considering the marked differences in NPQ and in violaxanthin de-epoxidation.

In contrast to what we expected, the lower q_P and more slowly developing NPQ of the anti-Rieske-FeS leaves did not make these leaves more susceptible than wild-type to photoinhibition *in vivo* (Figure 4D and 4E). Overall susceptibility was not increased in the anti-Rieske-FeS leaves, despite the severe acceptor-side limitation for electron transfer and the reduced capacity to dissipate excitation energy, either in the

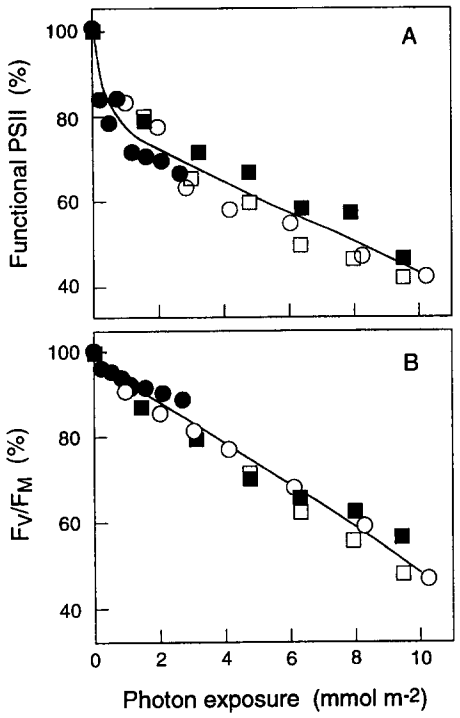

Figure 6. Changes in the proportion (% of initial) of functional PS II reaction centers and dark-adapted Fv/Fm expressed as a function of photon exposure. Wild-type leaves were exposed for different periods at irradiances of either 650 μmol m^{-2} s^{-1} PFD (○) or 1000 μmol m^{-2} s^{-1} PFD (□), and anti–Rieske-FeS leaves were exposed to either 175 μmol m^{-2} s^{-1} PFD (●) or 1000 μmol m^{-2} s^{-1} PFD (■). Photon exposure was calculated as Irradiance × Time × Initial absorbance. Data redrawn from experiments described in Figures 4 and 5.

antennae or via inactive PS IIs. Even more surprising, when the irradiance was regulated such that the excitation pressure on PS II was equivalent in both the anti-Rieske-FeS and wild-type leaves, the anti-Rieske-FeS leaves experienced much *less* photoinhibition than wild-type (Figure 5D and 5E). A further consequence of the different irradiances used to regulate q_P was that, under these conditions, only a very low amount of NPQ developed (Figure 5B) and almost no violaxanthin de-epoxidation occurred in the anti-Rieske-FeS leaves (Figure 5F). Consequently, trapping efficiency remained high in the anti-Rieske-FeS leaves (Figure 5C), but the combination of efficient energy trapping and the strong reduction of the PS II acceptor pool *did not* lead to increased photoinhibition. These data suggest that leaves are more, not less, prone to photoinhibition when PS II reduction occurs coincidently with the development of lumen acidification and NPQ.

We recalculated photoinhibition as a function of photon exposure to account for the variation in irradiances used in these experiments (Figure 6). The loss of functional PS IIs assessed from oxygen flash-yield measurements shows an initial curvilinear relation, with a fraction of PS IIs (approx. 20%) being apparently very susceptible to inactivation at very low photon exposures (Figure 6A). However, no differences were found in the response of the anti-Rieske-FeS and wild-type leaves. After the initial fast drop, the loss of PS II function was linearly related to photon exposure in both the anti-Rieske-FeS and wild-type leaves regardless of photon fluence, extent of PS II closure or the level of NPQ. When we made a similar assessment by Fv/Fm, no rapid initial loss of functional PS IIs was observed (Figure 6B). This showed that a fraction of PS IIs could lose function without changing their fluorescence characteristics. These highly sensitive PS II reaction centers have been described and discussed in detail previously (Park et al. 1995a,b) and did not appear to vary between the anti-Rieske-FeS and wild-type leaves. Photoinhibition, assessed by Fv/Fm, was linearly correlated with photon exposure in both the wild-type and anti-Rieske-FeS leaves. This relation was not altered by the increase in excitation pressure or by the reduced capacity of the anti-Rieske-FeS leaves to develop NPQ.

Discussion

We have shown that a specific down-regulation of the amount and activity of the cytochrome b_6/f complex in the thylakoids of tobacco breaks the link between the reduction of PS II electron acceptors and the development of ΔpH-dependent NPQ (Figure 3) that is normally seen in higher plants (Osmond 1994; Park et al. 1996b). Consequently, photochemical quenching decreases with increasing irradiance much more rapidly in the transgenics than in wild-type leaves, and NPQ develops only slowly in the transgenic leaves (Figure 1 and 4). The lower NPQ and high antenna trapping efficiency of the transgenic plants was maintained even when the transgenic and wild-type leaves where exposed to irradiances resulting in a similar excitation pressure on PS II (similar q_p) (Figure 5). Significantly, the disruption of the relation between the reduction of PS II and the development of NPQ in the transgenics was achieved without greatly altering the light harvesting structures of PS II or PS I (Price et al. 1995). The anti-Rieske-FeS leaves had comparable Chl and

PS II reaction center stoichiometries and a comparable carotenoid profile to the wild-type, with differences of less than 10% an a leaf area basis (Table 1).

Due to lower NPQ and concomitantly higher antenna trapping efficiency in the anti-Rieske-FeS leaves, a photon absorbed by the light-harvesting antenna of PS II should have a higher probability of reaching the PS II reaction center. However, once at the PS II reaction center the photon should have a lower probability of entering the electron transport chain due to a more reduced Q_A pool. This should increase the probability of reactions such as charge recombination of the $P680^+Pheo^-$ radical pair and thus the risk of generating singlet oxygen thereby increasing photoinhibition (Barber 1995). Surprisingly, no increase in photoinhibition was observed. Strikingly, when photoinhibition in the wild-type and the anti-Rieske-FeS transformants was compared with respect to photon exposure (Irradiance × Time × Absorbance), no differences were found between the response of the wild-type and transgenic leaves (Figure 6). Despite the limited ability to generate NPQ, the high antenna trapping efficiency and increased reduction of Q_A for the transgenics, the dose response for PS II photoinactivation was similar to wild-type. This suggests that photoinactivation depends on the number of photons absorbed by PS II rather than the rate of photon absorption or the redox state of the Q_A pool, confirming earlier reports (Park et al. 1995a,b, 1996b). These results suggest that the model of photoinhibition *in vivo* occurring principally because of limitations to electron withdrawal from PS II (Barber 1995) does not explain photoinhibition in these transgenic tobacco leaves.

The main purpose of these experiments was to use antisense transgenics to perturb electron transport and the development of NPQ rather than use *in vitro* assays or chemical treatments, with their attendant problems of unknown concentration gradients between the solution and the site of activity in the chloroplast. As such, we assess the *net* level of photoinhibition of PS II function *in vivo*, where the repair phase is unable keep up with the rate of inactivation. We do not measure the rate or extent of total PS II inactivation. However, our previous studies have shown that peas have the same intrinsic capacity for D1 protein synthesis regardless of growth irradiance and light-harvesting antenna composition (Park et al. 1995a,b, 1996a,b). Further, D1 protein synthesis has been shown to saturate at low irradiances (Aro et al. 1993b, 1994; Park et al. 1996a). Thus, we conclude that the similar susceptibility of the anti-Rieske-FeS and wild-type leaves to net photoinhibition *in vivo* reflects similar rates of PS II inactivation.

If acceptor-side limitations in PS II are not the predominant triggers for photoinhibition *in vivo*, this raises questions regarding the role of NPQ and the xanthophyll cycle in photoprotection. The xanthophyll cycle is thought to enhance antennae-based ΔpH-dependent NPQ and in this way lower the probability of PS II becoming over-reduced (Demmig-Adams and Adams 1992; Owens 1994). It is clear from the body of work that has now accumulated that the de-epoxidation of some fraction of the violaxanthin pool to zeaxanthin in the presence of a *trans*-thylakoid pH gradient enhances the development of NPQ (see reviews by Demmig-Adams and Adams 1992; Pfündel and Bilger 1994; Horton et al. 1996). The results presented in this paper do not challenge this conclusion. However, while the evidence in support of a role for the xanthophyll cycle in the development of NPQ is convincing, the question of whether NPQ reflects primarily photoprotective mechanisms remains. Further, we have shown that the xanthophyll cycle is completely unresponsive to the prolonged reduction of the PS II acceptor pool (Table 1 and Figure 5 and 6). This is curious, considering that the over-reduction of PS II is the very condition the xanthophyll cycle is thought to have evolved to relieve.

Logically, if NPQ is primarily an antenna-based phenomenon (Ruban and Horton 1995; Gilmore et al. 1996a,b; Horton et al. 1996) that reflects a significant reduction in excitation flow to PS II it must be photoprotective regardless of whether the mechanism of photoinhibition is acceptor- or donor-side mediated or whether it is an intrinsic probability event associated with charge separation. In the current experiments, we compare the responses of wild-type and transgenic tobacco leaves, grown under the same irradiance and with comparable light-harvesting structures, to exposure to high irradiance. In these experiments both NPQ and violaxanthin de-epoxidation was 2-fold higher in wild-type compared with anti-Rieske-FeS leaves. This did not *increase* the resistance of the wild-type leaves to photoinhibition. Further, we have previously reported that tobacco expressing antisense transcripts against the δ-subunit of the chloroplastic ATP-synthase show increased NPQ and increased xanthophyll pool sizes compared with wild-type (Hurry 1995). Rather than being more tolerant of high irradiances, the anti-ATP-synthase leaves were more *sensitive* to photoinhibition than either the anti-Rieske-FeS or wild-type leaves (Hurry 1995). These data from the anti-Rieske-FeS

and the anti-ATP-synthase transgenic tobacco strongly suggest that closure of PS II, without a concomitant acidification of the thylakoid lumen, does not lead to rapid photoinactivation of PS IIs *in vivo*. However, when PS II closure is coupled to lumen acidification, PS IIs are inactivated at a faster rate measured either as dark-adapted Fv/Fm or as oxygen flash yield (Figure 5 and 6). This suggests that donor side limitations, perhaps developing because of lumen acidification (Krieger and Weis 1993; Krieger et al. 1993; van Wijk and van Hasselt 1993), may be important in the photoinactivation of PS II reaction centers *in vivo*. Recent studies of D1 protein turn-over and *in vivo* degradation patterns provide indirect evidence in support of donor side photoinhibition in intact leaves (Russell et al. 1995; Kettunen et al. 1996). Thus, rather than driving an exclusively photoprotective mechanism by promoting the development of NPQ, lumen acidification may contribute to photoinhibition *in vivo*.

Pfündel and Bilger (1994) point out that little is known of the signal that mediates between the environment and the xanthophyll cycle pigment pool. Several recent reports have suggested that the reduction state of PS II or the plastoquinone pool acts as the trigger for acclimation of the light-harvesting antennae (Allen 1993; Escoubas et al. 1995; Huner et al. 1995; Maxwell et al. 1995). In contrast to these reports, the data from the anti-Rieske-FeS tobacco showed that the marked alterations to the redox poise of intersystem electron transport had little effect on the PS II/PS I ratio and the size of the PS II light-harvesting antenna (Hurry 1995; Price et al. 1995) (Table 1). Further, the data presented in this paper, and our previous reports of the anti-ATP-synthase transformants (Hurry 1995; Price et al. 1995) clearly show that the reduction state of PS II or the plastoquinone pool is not the trigger for modifications to the size of the xanthophyll pool. Rather, this may be mediated by sustained energisation of the thylakoid membrane.

A final, and potentially important, difference between the anti-Rieske-FeS and wild-type leaves is that PS II closure in the wild-type leaves requires that the electron transport chain is reduced from the acceptor-side of PS I. This is not so for the anti-Rieske-FeS transgenics, where only electron carriers from the cytochrome b_6/f complex and up-stream to PS II become highly reduced. Consequently, photoinhibition in the anti-Rieske-FeS transformants does not involve the same generation of potentially reactive high-energy electrons at PS I. Therefore, the results presented in this paper may also point to an important role for electrons arriving at PS I without an acceptor in the photoinactivation of PS II *in vivo*, and this needs to be investigated further.

In summary, the antisense suppression of the Rieske-FeS-binding protein in tobacco leaves resulted in a constitutive marked increase in excitation pressure on PS II. This led to only a slight decrease in the Chl *a/b* ratio, to little change in the xanthophyll pigment content and, as previously shown (Price et al. 1995), to little change in the PS II to PS I ratio. When exposed to high irradiance, the antisense leaves showed a greatly increased closure of PS II and a much reduced capacity to develop non-photochemical quenching compared with wild-type. However, contrary to expectations, the anti-Rieske-FeS leaves were *not* more susceptible to photoinhibition than were wild-type leaves, strongly suggesting that photoinhibition *in vivo* is not due to the over reduction of electron carriers immediately on the acceptor-side of P680. Rather, the evidence from the anti-Rieske-FeS tobacco suggests that rapid photoinactivation of PS II *in vivo* only occurs when closure of PS II occurs coincident with lumen acidification.

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