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Feedback regulation of photosynthetic electron transport by NADP(H) redox poise

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ABSTRACT

When plants experience an imbalance between the absorption of light energy and the use of that energy to drive metabolism, they are liable to suffer from oxidative stress. Such imbalances arise due to environmental conditions (e.g. heat, chilling or drought), and can result in the production of reactive oxygen species (ROS). Here, we present evidence for a novel protective process — feedback redox regulation via the redox poise of the NADP(H) pool. Photosynthetic electron transport was studied in two transgenic tobacco (*Nicotiana tabacum*) lines — one having reduced levels of ferredoxin $NADP^+$-reductase (FNR), the enzyme responsible for reducing $NADP^+$, and the other reduced levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the principal consumer of NADPH. Both had a similar degree of inhibition of carbon fixation and impaired electron transport. However, whilst FNR antisense plants were obviously stressed, with extensive bleaching of leaves, GAPDH antisense plants showed no visible signs of stress, beyond having a slowed growth rate. Examination of electron transport in these plants indicated that this difference is due to feedback regulation occurring in the GAPDH but not the FNR antisense plants. We propose that this reflects the occurrence of a previously undescribed regulatory pathway responding to the redox poise of the NADP(H) pool.

1. Introduction

In plant thylakoid membranes, light drives the flow of electrons from water, via photosystem (PS) II, cytochrome (cyt) b_6f and PSI to ferredoxin. Reduced ferredoxin is oxidised by ferredoxin $NADP^+$-reductase (FNR), producing NADPH. The primary use of this NADPH is in the Benson–Calvin cycle, where it reduces 1,3-diphospho glycerate, forming glyceraldehyde-3-phosphate, a reaction catalysed by the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This latter reaction is regarded as being crucial in controlling electron flow to the Benson–Calvin cycle, as it is the first step regulated by light, with GAPDH being activated via thioredoxin [1–4].

If the flow of electrons through the electron transport chain exceeds the capacity of metabolism to consume the reductant produced, then potentially harmful side reactions are liable to occur. Notably, electron flow to oxygen, the Mehler reaction [5], results in the production of superoxide, $O_2^{\cdot -}$, at the acceptor side of PSI (for reviews, see [6–8] and references therein). Superoxide can dismutate to form hydrogen peroxide, which, if not scavenged, can give rise to highly damaging hydroxyl radicals. Additionally, over reduction of PSII leads to charge recombination reactions, producing singlet excited oxygen (see [9–11]).

To avoid such reactions, plants possess an array of regulatory mechanisms that limit damage. The best characterised is non photochemical quenching (NPQ) [12–14]. Under excess light, a high pH gradient across the thylakoid membrane builds up, probably due to the occurrence of cyclic electron flow around PSI (see discussion in [15]). This stimulates the synthesis of the carotenoid zeaxanthin, which, together with a high ΔpH, causes an increase in the efficiency with which absorbed light energy is dissipated as heat.

A further regulatory mechanism is suggested to specifically limit the reduction of oxygen by PSI [16,17]. Under conditions where the capacity of the Benson–Calvin cycle is limited, e.g. when drought stress causes stomatal closure, limiting the entry of CO_2 into the leaf, electron flow through the electron transport chain is limited by regulation of the cyt b_6f complex [16–20]. Based on *in vitro* evidence, this down regulation was initially suggested to be mediated by the pH of the thylakoid lumen, with low pH inhibiting the oxidation of plastoquinol (see discussion in [21]). Under some conditions, however, regulation cannot be explained in this way, as non-correlations between NPQ and down regulation of electron transport indicate that the latter can vary under conditions where ΔpH is constant and visa versa [16,17]. Instead, Johnson [22] provided evidence that the down regulation of electron transport might proceed in response to the redox poise of the chloroplast stroma. It is recognised that the chloroplast stromal redox poise is highly regulated, such that the ratio of $NADP^+$ to NADPH and oxidised to reduced ferredoxin are maintained in a narrow range [23]. It is well known that the activity of the Benson–Calvin cycle is regulated by feed-forward mechanisms (see e.g. [24,25] for reviews). In particular, the disulphide

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Abbreviations: as, antisense; cyt, cytochrome; ΔpH, pH gradient across thylakoid membrane; FNR, ferredoxin $NADP^+$-reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $NADP^+$, oxidised nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP(H), total pool of $NADP^+$ and NADPH; NPQ, non photochemical quenching; NPQ_f, fast relaxing NPQ; NPQ_s, slow-relaxing NPQ; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species

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reducing enzyme thioredoxin alters the activity of a range of enzymes, itself being reduced by ferredoxin, via the enzyme ferredoxin thioredoxin reductase. Johnson [22] proposed that the redox poise of thioredoxin might also play a feedback role, regulating the flow of electrons through the cyt b_6f complex.

To test the redox hypothesis for regulation of electron transport, we have examined two lines of plants in which the overall capacity for photosynthesis is impaired by antisense suppression of either FNR [26] or of GAPDH [27]. We compared plants in which the degree of inhibition of overall photosynthesis was similar and show that whilst GAPDH antisense (as-GAPDH) plants are able to regulate the electron transport chain such that superoxide production is suppressed, this is not the case for FNR antisense (as-FNR) plants. This suggests that the signal regulating electron transport comes not from ferredoxin but rather from the NADP(H) pool.

2. Materials and methods

Seeds from wild type tobacco (*Nicotiana tabacum*) and from tobacco plants with down regulated FNR by antisense techniques [26] were supplied by Prof. U. Sonnewald (Gatersleben, Germany). Seeds from tobacco plants with down regulated GAPDH by antisense techniques [27] were supplied by Prof. D. Price (Canberra, Australia) and Prof. C. Raines (University of Essex, UK). Plants were grown in peat based compost in a controlled-environment growth chamber at an irradiance of 80 μmol m^{-2} s^{-1} with a 16 h day. Day/night temperatures were 20/16 °C.

Plants were dark-adapted for 1 h prior to measurement. Leaves were clamped into an infra-red gas analyser chamber (Ciras-1; PP-Systems, Hitchin, UK), enabling CO_2 to be controlled. Nitrogen and oxygen were controlled by MKS mass flow controllers (MKS Instruments, Mass., USA). Measurements of chlorophyll fluorescence and PSI were carried out as described previously [18]. Briefly, chlorophyll fluorescence was measured with a Walz PAM-101 fluorimeter (Walz, Effeltrich, Germany) attached to a PAM101-ED emitter–detector unit. P700 redox state was monitored using a PAM-101 fluorimeter in combination with a ED-P700DW-E emitter–detector unit. Actinic and saturating lights were provided using Schott KL1500 halogen lamps, switched on and off using Uniblitz shutters (Vincent Associates, Rochester, NY, USA). Shutters were controlled and all data were recorded using a National Instruments PCI-6220 data acquisition card fitted to a PC running software written using Labview (National Instruments, Austin, Texas). Fluorescence parameters were calculated as described by Maxwell and Johnson [28]. Photosystem I parameters were calculated as described by Golding and Johnson [18]. The maximum P700 signal was calibrated by imposing a far red light on the leaf provided by a high powered LED chip peaking at 740 nm (LED740-66-60, Roithner Lasertechik, Vienna, Austria), filtered through a Schott RG710 glass filter. This was judged to be saturating as imposition of a saturating flash of white light on top of the far red did not give a transient increase in P700 oxidation. Measurements of the light response curve were obtained using a new leaf at each of the different irradiances. Measurements of the CO_2 response curve were obtained by first supplying external CO_2 at 2000, then 180, 45, 0, 90, and lastly 360 μl l^{-1}, and were performed on the same leaf at each CO_2 concentration. All measurements, except those where CO_2 was being used as variable, were performed at 2000 ppm CO_2. This was chosen to avoid possible variability due to differences in stomatal conductance amongst the three lines that might interfere with experimental analyses.

Measurements of cyt *f* were carried out using a laboratory built spectrophotometer, identical to that described by Joliot et al. [29].

For quantitation of FNR, protein extracts from 0.10 g of as-FNR and wild type leaves were separated by SDS-PAGE. Immunoblotting was performed using standard protocols, using an FNR antibody. GAPDH activity was determined as described by Ruuska et al. [30].

Thylakoids were prepared by harvesting leaves of at least five different dark-adapted wild type, as-FNR and as-GAPDH plants. Leaves were placed in an ice-cold blender. 250 ml of slushy grinding medium (330 mM sorbitol, 20 mM HEPES, 10 mM NaCl and 5 mM $MgCl_2$, pH 7.6) were added, and pulses were applied to grind the leaves. The extract was filtered through two layers of muslin, then through one layer of absorbent cotton wool. The filtrate was centrifuged at 3500 rpm for 5 min. The pellet was resuspended in shocking medium (5 mM HEPES, 5 mM $MgCl_2$, pH 7.6) and recentrifuged. The pellet was resuspended in grinding medium and recentrifuged, following by resuspension in a small volume of grinding medium. Samples of each thylakoid preparation were loaded onto SDS-PAGE on equivalent chlorophyll content. Immunoblotting was performed using antibodies raised against PsbA, PsaD and cyt *f* for quantitation of cyt b_6f, PSI and PSII complexes, respectively.

Chlorophyll content was assayed immediately after analysis of each leaf, by extraction in 80% acetone, and estimated using the equations of Porra et al. [31].

Extraction of pyridine nucleotides was carried out as described [32], and $NADP^+$ and NADPH levels were determined by the method developed by Zhang et al. [33]. NADP-MDH activation state was measured as described by Ruuska et al. [34]. Lipid peroxidation was estimated as malondialdehyde formation as described by Heath and Packer [35]. Data were analysed using SPSS. Two-sided *P* values <0.05 were considered statistically significant.

3. Results

Antisense plants lacking FNR or GAPDH were grown under identical conditions. Lines were selected in which there was a similar, severe, degree of down regulation of the enzyme concerned (see Fig. 1). Down regulation of either enzyme lead to plants with considerably slowed growth, even under low light conditions. Consistent with previous observations [36] as-FNR plants appeared to be substantially photobleached. In contrast, however, as-GAPDH plants appeared healthy with no obvious visual signs of stress (Fig. 1A). This difference in appearance was reflected in estimations of chlorophyll content — whilst both as-FNR and as-GAPDH had reduced chlorophyll content, this effect was more marked in the as-FNR plants (Fig. 1B).

For the plants used, maximum photosynthesis was inhibited by approximately 50% in each case (Fig. 1C). The impact of this on PSII photochemistry was monitored at a range of irradiances using chlorophyll fluorescence analysis (Fig. 2). The quantum efficiency of PSII (Φ_{PSII}) falls with increasing irradiance, reflecting the progressive saturation of PSII. In both as-FNR and as-GAPDH plants, Φ_{PSII} was lower at any given irradiance than in wild type plants (Fig. 2A) reflecting the overall inhibition of photosynthesis. The degree of inhibition was almost identical in the two lines.

With increasing irradiance, total NPQ rose in all plants (Fig. 2B). At irradiances up to 600 μmol m^{-2} s^{-1}, wild type and as-FNR plants had similar NPQ, whereas in as-GAPDH this rose more steeply with increasing irradiance. The maximum levels of NPQ observed in this experiment were, however, higher in as-FNR plants than in either as-

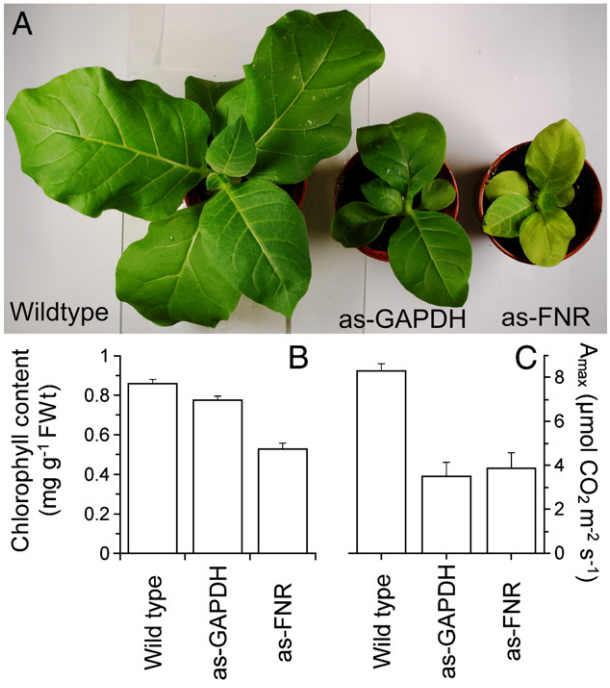

Fig. 1. Phenotype of wild type, as-GAPDH and as-FNR tobacco plants grown at an irradiance of 80 μmol m^{-2} s^{-1}. A. Appearance of as-GAPDH and as-FNR plants with 10–15% and 25–35%, respectively, of the relevant enzyme activity of wild type plants. Plants for physiological measurements were selected based on visual symptoms and the degree of suppression of the relevant protein confirmed using immunoblotting for FNR or by measuring GAPDH activity enzymatically. B. Chlorophyll content of plants used in physiological measurements. Both as-GAPDH and as-FNR are significantly lower than wild type ($P=0.008$ and $P=0.000$ respectively). C. Maximum assimilation rate in wild type, as-GAPDH and as-FNR plants, measured at irradiance 2000 μmol m^{-2} s^{-1} and 2000 μl l^{-1} CO_2. Both as-GAPDH and as-FNR plants had a maximum assimilation rate that was significantly lower than the control ($P=0.002$ and $P=0.004$ respectively). In panel B and C the mean±SE of at least three measurements on separate plants are shown.

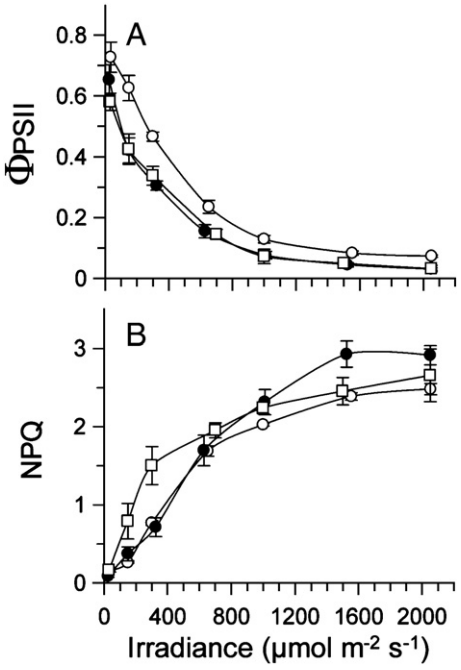

Fig. 2. Chlorophyll fluorescence quenching parameters in response to light in wild type and as-FNR and as-GAPDH plants. Quantum yield of PSII (Φ_{PSII}; A) and non photochemical quenching (B) in wild type (open circles), as-FNR (closed circles) and as-GAPDH (squares) tobacco plants subjected to a range of irradiances at 20% oxygen and 2000 μl l^{-1} CO_2. Plants were dark-adapted for 1 h prior to starting measurements. Leaves were pre-illuminated for 20 min, to allow photosynthesis to reach a steady state, before a saturating flash (8000 μmol m^{-2} s^{-1}) was applied to estimate fluorescence parameters. Each point represents the mean±SE of at least three measurements on separate plants.

GAPDH or wild type plants. NPQ is not only composed of pH-regulated protective processes, but also measures photoinhibition of PSII. To distinguish these processes at high light, we performed a quenching relaxation analysis (Table 1). In all plants, the majority of quenching relaxed rapidly in the dark (NPQ_f), indicating that it was pH dependent. Nevertheless, a proportion of the quenching was more persistent (NPQ_s), suggesting that there may be an increased contribution of photoinhibition. NPQ_s was greatest in as-FNR plants, suggesting that they had a greater vulnerability to photoinhibition. Photoinhibition cannot fully explain the elevated NPQ at high light in as-FNR plants, however, as NPQ_f was also greater (Table 1).

The redox state of the PSI primary electron donor, P700, can be monitored by measuring absorbance changes in the near infra-red [37]. In wild type, increasing irradiance resulted in increasing P700 oxidation (Fig. 3). This indicates that the effective slowest step in electron transport is prior to PSI. In as-GAPDH plants, P700 was more oxidised than in the wild type at any irradiance up to saturation. In as-FNR plants, in contrast, P700 was largely reduced across a wide range of irradiances. Upon a light–dark transition, oxidised P700 was re-reduced following a pseudo-first order decay. This decay is clearly not a simple first order process but has previously been observed to behave as such across a wide range of conditions and this feature can be used as a means of quantifying the decay [16,21,38]. Fitting this decay to an exponential equation yields an apparent first order rate constant (k). This was substantially lower across all irradiances in as-GAPDH plants than in wild type. In as-FNR plants, it was only at the highest irradiances that it was possible to measure any signal that could be ascribed to P700 oxidation. This signal decayed with a similar kinetic to wild type upon a light to dark transition, as indicated by the estimated values of k. At lower irradiances, there was evidence that no net oxidation of P700 was occurring, as the following P700 redox state continuously from the onset of illumination gave no net signal change and applying far red light immediately after actinic illumination gave rise to full oxidation of P700 (data not shown).

To determine whether this difference in the rate of P700 reduction between as-GAPDH and wild type was due to an intrinsic difference in the capacity of electron transport (e.g. caused by a change in the ratio of the principal electron transport complexes) or due to a regulatory process, we examined the kinetics of $P700^+$ reduction 2 min after the end of actinic illumination (Fig. 3A, inset). Under such conditions, it is expected that the Benson–Calvin cycle will remain active, but that the pool of immediate acceptors from PSI will be oxidised. A 10 ms saturating flash of light was found to be sufficient to oxidise ~70% of P700 in both wild type and as-GAPDH plants. Longer flashes resulted in the P700 pool being re-reduced and exposing as-FNR leaves to such flashes did not give rise to any significant oxidation of P700. The kinetics with which $P700^+$ was re-reduced following a 10 ms flash in the case of wild type and as-GAPDH was very similar (k=0.19±0.005 and 0.21±0.025 ms^{-1} for wild type and as-GAPDH plants respectively). These values are similar to the maximum values seen at steady state light in wild type tobacco (Fig. 3C) and in other species (see e.g. [18]). Thus, we can conclude that as-GAPDH has a similar capacity for electron transport to wild type but that this is down regulated under steady state conditions. Immunoblot analysis supported the idea as there was only a subtle change in the stoichiometry of the major electron transport (Fig. 4). The amounts of cyt b_6f, PSI and PSII complexes were assessed by immunoblotting using antibodies raised against cyt f, PsaD and PsbA, respectively. This indicated an apparent modest reduction of both the cyt b_6f and PSI complex relative to PSII in the as-GAPDH lines. A similar stoichiometric change was observed in the as-FNR lines. Although they had similar degrees of inhibition of overall photosynthesis to the as-GAPDH plants, as-FNR plants maintained P700 in a largely reduced state across the entire light range (Fig. 3).

By lowering the CO_2 concentration, it should be possible, even in the antisense plants, to ensure that the slowest step in photosynthesis is at the CO_2 fixation step catalysed by Rubisco. With declining CO_2, Φ_{PSII} was found to progressively drop in wild type plants and in both antisense lines, however Φ_{PSII} was lower at any given CO_2 concentration in the antisense plants (Fig. 5A). The degree of inhibition was very similar in the two lines and the decline in Φ_{PSII} implies that CO_2 had become limiting in all cases. The drop in CO_2 also induced a rise in NPQ

Table 1
Quenching relaxation analysis of plants illuminated at high light

Measurement	Wild type	as-FNR	as-GAPDH
20% O_2 and 2000 μl l^{-1} CO_2			
NPQ_s	0.396±0.020	0.518±0.086	0.433±0.044
NPQ_f	2.104±0.018	2.512±0.386	2.177±0.153
NPQ_{total}	2.500±0.006	3.030±0.316	2.610±0.118
Φ_{PSII}	0.089±0.013	0.048±0.005	0.045±0.007
20% O_2 and 0 μl l^{-1} CO_2			
NPQ_s	0.539±0.054	0.665±0.076	0.504±0.015
NPQ_f	2.394±0.063	2.858±0.075	2.170±0.085
NPQ_{total}	2.753±0.063	3.523±0.133	2.674±0.096
Φ_{PSII}	0.018±0.006	0.011±0.003	0.011±0.002
2% O_2 and 2000 μl l^{-1} CO_2			
NPQ_s	0.344±0.058	0.344±0.045	0.330±0.053
NPQ_f	1.643±0.142	1.615±0.205	1.710±0.158
NPQ_{total}	1.986±0.122	1.959±0.170	2.040±0.192
Φ_{PSII}	0.075±0.009	0.031±0.007	0.038±0.003
Ratio Φ_{PSII} (2%/20% O_2) 2000 μl l^{-1} CO_2	0.84	0.65	0.84

Values of the fast- and slow-relaxing components of NPQ (NPQ_f and NPQ_s, respectively) and Φ_{PSII} measured at 2000 μmol m^{-2} s^{-1} light at varying CO_2 and oxygen concentrations. Mean values±SE of at least three measurements of wild type, as-FNR and as-GAPDH plants are shown.

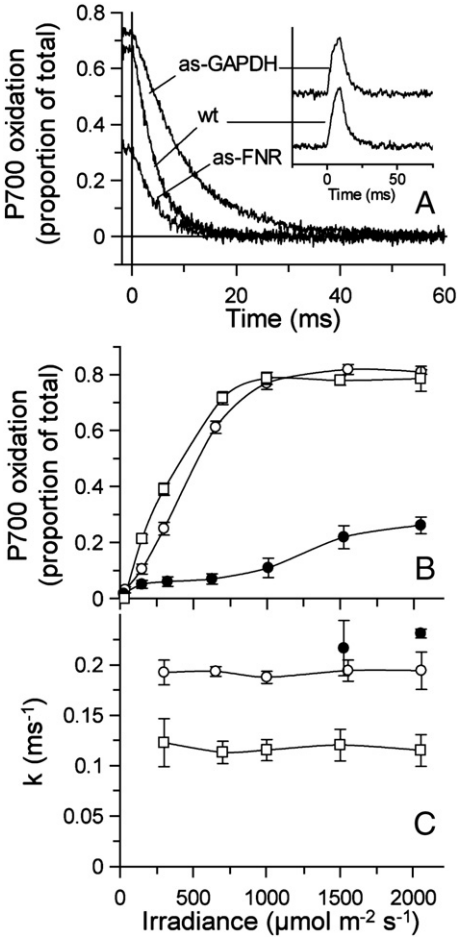

Fig. 3. Photosystem I photochemistry in response to light in wild type, and as-FNR and as-GAPDH plants. A. Representative curves showing the reduction of P700 following a light–dark transition following illumination at 2000 µmol $m^{-2}s^{-1}$, 2000 µl l^{-1} CO_2. Signals are normalised to the maximum signal obtained upon illumination of each leaf with a far red light (λ_{max}=730 nm), which provides an estimate of the total oxidisable pool of P700. Curves were fitted with a single exponential equation to allow estimation of a pseudo-first order rate constant (k) for the decay. Inset: Oxidation and re-reduction of $P700^+$ induced by a 10 ms flash of saturating white light 2 min after illumination of a leaf of as-GAPDH and wild type tobacco. For clarity, traces have been offset. B and C. Irradiance dependence of the degree of oxidation of P700 (B) and the rate constant for the re-reduction of PSI (C) in wild type (open circles), as-FNR (closed circles) and as-GAPDH (squares) tobacco plants. Measurements were made on leaves immediately after fluorescence measurements in Fig. 2. Values for k at lower irradiances are not shown for as-FNR plants as these could not be accurately measured. Each point represents the mean±SE of at least three measurements on separate plants.

in all cases, with as-FNR plants having a higher level of quenching across the entire CO_2 range (Fig 5B). This additional NPQ can be mostly ascribed to a rise in pH dependent high energy state quenching, as indicated by its reversibility when plants are transferred to darkness (Table 1).

In wild type plants, lowering the CO_2 concentration resulted in an increased degree of oxidation of P700 (Fig 5C). This was also the case for as-GAPDH plants, except at the lowest CO_2 concentration used, where P700 started to become more reduced. This has been observed previously under conditions where low CO_2 is combined with low O_2 and has been taken to imply reduction of the PSI acceptor pool in the absence of any electron acceptor [21]. In as-FNR leaves, there is a marked increase in P700 oxidation with falling CO_2. Whereas at high CO_2 only about 10% of P700 was in the oxidised form under the conditions used, this rose to nearly 50% in the absence of CO_2. This oxidation was accompanied by a fall in the rate constant for $P700^+$ reduction (Fig. 5D). In as-FNR, k was close to or slightly higher than that in wild type across the entire CO_2 range, showing that electron transport could be efficiently down regulated in this plant. In as-GAPDH leaves, a similar effect was observed, however the effect was much less marked as the initial value of k was much lower. At the lowest CO_2 concentrations, the value of k converged in the three lines of plants.

The as-GAPDH and as-FNR plants used in this study had 10–15% and 25–35%, respectively, of the relevant enzyme activity of wild type plants. Measurements of PSII and PSI photochemistry were also performed on a range of antisense plants with a varying antisense suppression. Suppression of enzyme activity results in impairment of photosynthesis and thus a reduction of Φ_{PSII}, allowing this parameter to be indicative of the extent of enzyme suppression. Φ_{PSII} for all the plants measured was plotted against the extent of oxidation of P700 and the rate constant for $P700^+$ reduction (Fig. 6). Antisense plants with hardly any enzyme suppression had similar levels of Φ_{PSII}, P700 oxidation and rate constant of $P700^+$ reduction. On the other hand, with falling levels of Φ_{PSII}, the proportion of oxidised P700 rose in the as-GAPDH plants, whereas this proportion dropped markedly in the as-FNR plants. The decline in Φ_{PSII} was accompanied by decline in the rate constant for P700 reduction in the as-GAPDH plants, whereas values remained stable at wild type levels in the as-FNR plants.

The difference in the rate of P700 reduction comparing as-GAPDH and wild type plants shows that a limitation of the electron transport chain is occurring prior to PSI. The reduction of cyt f following a light–dark transition was measured as described by Joliot and Joliot [39], This was slower in as-GAPDH plants than in wild type, but was faster in the as-FNR plants (not shown). Chlorophyll fluorescence measurements indicated that the PSII acceptor pool is consistently more reduced in both antisense lines. This implies that the site of down regulation is at the step of plastoquinol oxidation by the cyt b_6f complex.

The effectiveness of the regulation of electron transport chains in the different plants can be assessed by assaying the redox poise of the

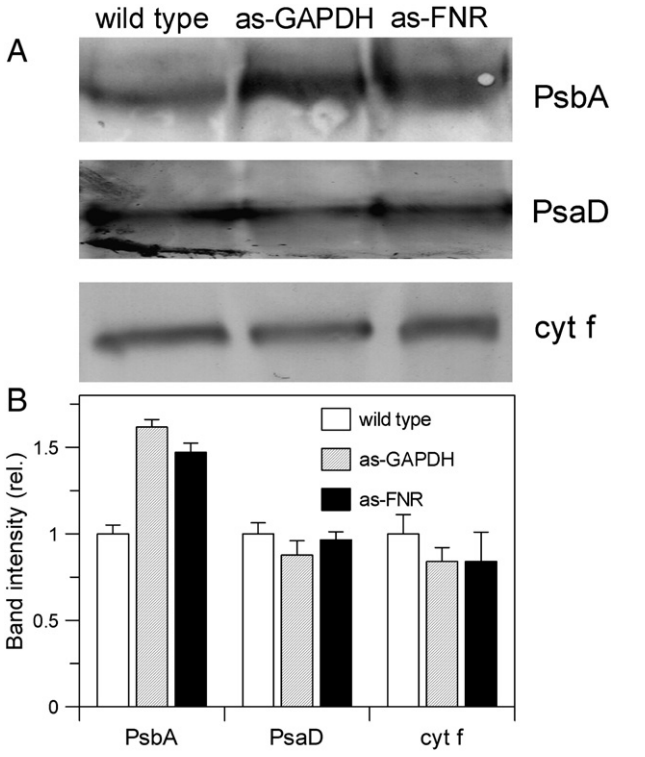

Fig. 4. Immunoblot analysis of thylakoid membranes of wild type, and as-FNR and as-GAPDH plants. A. Representative blots of PsbA, PsaD and cyt f. Thylakoid proteins were loaded on equivalent chlorophyll content basis. B. Protein bands were quantified with a GS-710 calibrated imaging densitometer (Bio-Rad). Results are means±SE of at least three blots.

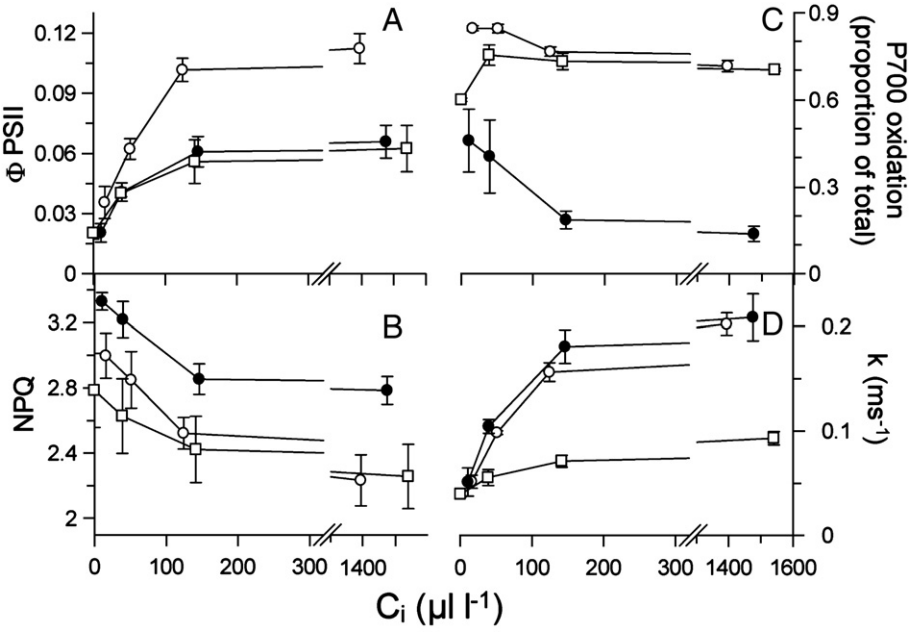

Fig. 5. Effect of altering CO_2 on chlorophyll fluorescence quenching parameters and photosystem I photochemistry in wild type and as-FNR and as-GAPDH plants. Quantum yield of PSII (Φ_{PSII}) (A) non photochemical quenching (B) P700 oxidation (C) and the rate constant for $P700^+$ reduction (D) in wild type (open circles), as-FNR (closed circles) and as-GAPDH (squares) tobacco plants subjected to a range of CO_2 concentrations at 20% oxygen and 1500 μmol m^{-2} s^{-1}. Plants were dark-adapted for 1 h prior to starting measurements. Leaves were illuminated for 20 min to allow photosynthesis to reach a steady state at 2000 μl l^{-1} CO_2 before a saturating flash (8000 μmol m^{-2} s^{-1}) was applied to estimate F_m. The CO_2 concentration was then altered and the leaf allowed to equilibrate for a further 10 min before the next measurement was made. Each curve represents the mean ± SE of at least three measurements on separate plants.

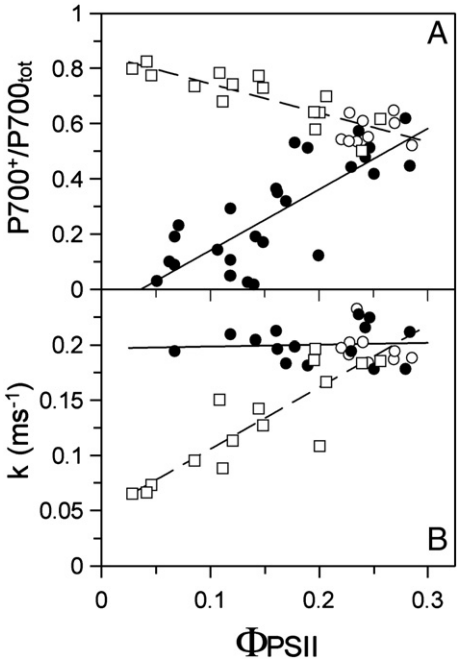

Fig. 6. Relation of the quantum yield of PSII to P700 oxidation and the rate constant for $P700^+$ reduction in response to high light in wild type and a range of as-FNR and as-GAPDH plants with different enzyme suppression. Quantum yield of PSII (Φ_{PSII}) was plotted against P700 oxidation (A) and the rate constant for $P700^+$ reduction (B) in wild type (open circles), as-FNR (closed circles) and as-GAPDH (squares) tobacco plants. Where P700 oxidation fell below ~0.2, no estimate of k was made due to the poor signal:noise ratio. Measurements were carried out in plants subjected to 640 μmol m^{-2} s^{-1} at 20% oxygen and 2000 μl l^{-1} CO_2. Plants were dark-adapted for 1 h prior to starting measurements. Leaves were illuminated for 20 min to allow photosynthesis to reach a steady state, before a saturating flash (8000 μmol m^{-2} s^{-1}) was applied to estimate fluorescence parameters.

PSI acceptor pool. The size of the total pool of $NADP^+$ and NADPH was similar in each plant (Table 2). However, this pool was significantly more oxidised in the as-FNR lines than in the wild type. In spite of the inhibition of assimilation seen in the as-GAPDH plants, no significant alteration in NADPH levels were observed in these plants. Ferredoxin redox state cannot readily be measured directly, however, an indication of this can be obtained by measuring the activation of malate dehydrogenase (Table 2). This was found to be significantly more active in as-FNR than in wild type plants, unlike as-GAPDH plants, which did not differ significantly from wild type. The importance of this is demonstrated by measurements of the extent of electron transport to oxygen in the Mehler reaction (Table 1). Whereas in both

Table 2
Redox poise of the PSI acceptor pool and lipid peroxidation

Measurement	Wild type	as-GAPDH	as-FNR
Total NADP(H) pool ($NADP^+$ + NADPH) (nmol/g fresh weight)	439 ± 73	447 ± 90	426 ± 71
$NADP^+$/NADPH at GL	1.353 ± 0.259	1.161 ± 0.130	2.803 ± 0.598
$NADP^+$/NADPH at HL	0.997 ± 0.140	1.047 ± 0.193	2.174 ± 0.364
NADP-MDH activation (%)	54.3 ± 2.3	56.6 ± 7.7	79.1 ± 5.6
Malondialdehyde (μmol/g fresh weight)	0.750 ± 0.046	0.720 ± 0.081	0.973 ± 0.058

The total pool of pyridine nucleotides ($NADP^+$ + NADPH) and the redox poise of that pool was measured in leaves of wild type, as-GAPDH and as-FNR tobacco plants as described in the Materials and methods. Total NADP(H) pool size did not vary significantly between plant lines. The ratio of $NADP^+$ to NADPH was measured in flash frozen leaf samples either at growth light (GL) or exposed to high light (HL, 550 μmol m^{-2} s^{-1} for 1 h). Results for as-FNR differed significantly from wild type (GL, P = 0.039; HL, P = 0.090), results for as-GAPDH did not (GL, P = 0.949; HL, P = 0.995). Lipid peroxidation was measured as malondialdehyde formation, with as-FNR differing significantly from wild type (P = 0.039), unlike as-GAPDH (P = 0.764). The activation state of NADP-MDH was measured as the ratio between initial and total activity. Result for as-FNR differed significantly from wild type (P = 0.018), and that for as-GAPDH did not (P = 0.896). Mean values ± SE of at least three measurements of wild type, as-FNR and as-GAPDH antisense plants are shown. For measurements of NADP-MDH activation and malondialdehyde formation, plants were dark-adapted for 1 h, and then exposed to 750 μmol m^{-2} s^{-1} for 1 h under ambient O_2 and CO_2 conditions. Leaf material was collected from plants and then flash frozen prior to subsequent analysis.

wild type and as-GAPDH plants lowering of oxygen concentration had a small effect on PSII efficiency, this inhibition was greater in as-FNR plants, demonstrating that oxygen is a more important sink for electrons in the latter than the former cases. This electron transport to O_2 can be shown to be important in generating the additional ΔpH seen at high light in as-FNR plants — in 20% O_2, as-FNR plants had the highest NPQ_f, at 2% O_2, they had the lowest. This did not, though, have a protective effect, since photoinhibition, measured as NPQ_s, was substantially lower when oxygen was removed.

The visual phenotype of as-FNR plants, compared to wild type and as-GAPDH (Fig 1), provides a strong indication that these plants differ in their vulnerability to oxidative stress. A more direct indication of this can be obtained from measurements of lipid peroxidation (Table 2). The extent of lipid peroxidation, measured as malondialdehyde formation, was significantly increased in as-FNR, but not as-GAPDH plants, compared to wild type.

4. Discussion

It has long been recognised that the redox poise of the chloroplast stroma provides a signal that is crucial in regulating the activity of enzymes of the Benson–Calvin cycle, this regulation being mediated by thioredoxin [24,25]. Results presented here provide strong evidence to support the idea that redox poise also has a feedback function, regulating the production of reducing equivalents to match the plant's capacity to consume them. Our results suggest that this form of regulation plays a vital role in limiting the production of reactive oxygen species in the chloroplast. As such, the efficiency of this regulation is likely to be an important determinant of stress tolerance in plants exposed to environmental stress. We propose that the primary signal for this regulation is the redox poise of the NADP(H) pool.

Photosynthetic electron transport has two main products — reducing equivalents (primarily as NADPH) and a ΔpH across the thylakoid membrane, which is used to drive the generation of ATP. Previous work has shown clearly that ΔpH is important in regulating the efficiency of light harvesting and so controls to some extent electron transport [40]. It has also been discussed that the ΔpH may directly control electron transport by limiting the rate of plastoquinone oxidation by the cyt b_6f complex (see [21] for discussion). As in previous studies [16,17] the direct involvement of ΔpH can be excluded in the present study. It is not possible to directly measure the ΔpH *in vivo*, however an indirect estimate can be obtained by measuring pH dependent quenching of chlorophyll fluorescence, NPQ_f. This does not vary linearly with ΔpH, but is thought to saturate *in vivo* at a pH below that required to affect cyt b_6f complex activity [41]. In our experiments, we examined a set of conditions in which NPQ varied, however the range of values observed were similar in all plants. Nevertheless, the degree of down regulation of electron transport differed substantially between the lines examined, even when comparing conditions with identical and non-saturated NPQ. For example, at low CO_2, there is clear indication from NPQ data that the ΔpH is higher in as-FNR leaves than in wild type or as-GAPDH, nevertheless the rate constant for reduction of $P700^+$ remains higher in as-FNR plants than in wild type (Fig. 5). Thus, we can conclude that ΔpH does not regulate electron transport under the conditions used here.

Previous studies have pointed to the possibility that the stromal redox poise might regulate the electron transport chain [16,17,22], however in none of these studies was it possible to identify precisely the redox signal involved. Johnson [22] suggested that this regulation might be achieved through a thioredoxin linked pathway. Isolated thylakoids were shown to be inhibited by the thiol reducing agent dithiothreitol, with a mid-point redox potential for this reduction being close to that of ferredoxin and NADP(H). Thioredoxin is well known to exert feed-forward activation of the Benson–Calvin cycle, however we can exclude the possibility that this same system also has a direct feedback role. Antisense suppression of FNR leads to increased reduction of thioredoxin, as evidenced by the increased activity of the thioredoxin regulated enzyme NADP-MDH (Table 2). This is not, however, accompanied by down regulation of the electron transport chain.

In contrast, in as-GAPDH plants, the limitation imposed on overall photosynthesis lies after the NADP(H) pool. In the absence of any regulation, this would tend to result in both the NADP(H) and the ferredoxin pools being more reduced at any given irradiance. However our results indicate that this is not the case — both the thioredoxin activation state and the $NADP^+$ to NADPH ratios are close to wild type, in spite of the downstream inhibition of photosynthesis. This implies that efficient regulation of electron transport must be occurring. Given the differences between the antisense lines examined, we can conclude that the signal that regulates electron transport is not the stromal thioredoxin redox state but rather must lie after the ferredoxin pool.

It is possible that the regulation we observe could be mediated by the FNR protein itself. FNR has previously been observed to bind to the cyt b_6f complex [42], however the significance of this binding is unclear [43]. It might be postulated for example that the binding of FNR results in an inhibition of the cyt b_6f complex. Such a mechanism seems to us unlikely, however. When the restriction in photosynthesis is moved away from FNR, as occurs when CO_2 is removed, as-FNR plants are capable of down regulating the electron transport chain as efficiently as wild type plants. No modulation of FNR binding to cyt b_6f in response to environmental conditions has been seen (data not shown; see also [43]). Hence, we can conclude that it is the redox poise of the NADP(H) pool itself that is likely to be providing the signal for the regulation.

The concept that $NADP^+$ or NADPH provides signals regulating thylakoid processes is not without precedent in the literature. Rajagopal et al. [44] presented evidence for a direct effect of $NADP^+$ binding on the activity of PSI itself, with a change in the proportion of PSI centres that are photooxidisible being lowered in the presence of $NADP^+$. This phenomenon is consistent with the observation that low CO_2 increases the proportion of PSI centres measured as being active [18] however appears to be distinct from the type of regulation being measured here, which takes place prior to PSI. Over reduction of the NADP(H) pool has also been implicated in regulating the partioning of electrons between linear and cyclic electron flows, as indicated by far red induced oxidation of P700 [43,45–47]. Again, there is not clear mechanistic link between these findings and the phenomenon described here.

Measurements of chlorophyll fluorescence allow us to conclude that the plastoquinone pool is in a more reduced state in both as-FNR and as-GAPDH plants than in wild type (Fig. 2). At the same time, we were able to show that the reduction of cyt *f* is slowed in as-GAPDH, implying that the oxidation of plastoquinol by the cyt b_6f complex is the site of regulation of electron transport in these plants. The decrease in the rate constant for $P700^+$ reduction might be explained by a simple decrease in the pool of cyt b_6f complexes, such that this becomes overall the limiting step in photosynthesis. Such a change in complex stoichiometry would result in the pool of cyt *f* being maintained in a largely reduced state in the light. This is not the case, as we observe oxidation of cyt *f* under steady state light conditions (not shown). Also, based on the amplitude of the cyt *f* signal seen in far red light, as well as on immunoblot analysis of thylakoid membranes (Fig. 4), there is no indication that the stoichiometry of the major thylakoid membrane complexes is grossly altered in either antisense plant. Importantly, the changes in thylakoid complex ratios seen are similar in both as-FNR and as-GAPDH plants, so these cannot explain the differences between these plants. Finally, examination of $P700^+$ reduction following flash induced oxidation implies that the maximum rate of electron transport per PSI in as-GAPDH plants is similar to wild type (Fig 3A inset).

It is of interest to note that, although PSI centres in as-FNR plants were found to be in a largely reduced state, there is no indication that this results in increased fluorescence from these plants. In PSI centres, reduction of the FeS centres results in the production of a state $P700^+/A_1^-$ which is highly unstable and undergoes rapid charge recombination primarily via a short lived triplet state. The state $P700^+/A_o^-$ has an even shorter lifetime, approximately 30 ns, and decays either to ground state directly or via the triplet at a ratio of 7:3. Thus the PSI reaction centre remains an efficient quencher in all states (see discussion in [48] and references therein) as indicated by the higher rate constant for reduction of P700+ in as-FNR plants.

At present, we are not able to define in detail the regulatory pathway involved. The simplest model would be to invoke a direct inhibition of the cyt b_6f complex by NADPH, however in preliminary measurements in isolated thylakoid membranes, we were unable to find any evidence for a simple inhibition (data not shown). We therefore assume that the regulation needs to be mediated by some stromal component(s) lost in osmotically shocked thylakoids. Although a direct role of stromal thioredoxin can be excluded based on our results, the possible involvement of some thioredoxin-like protein cannot be excluded. For example, m-type thioredoxins are known to exist in the thylakoid lumen [49], the role of which remains to be elucidated. It has been suggested, by analogy with bacterial systems, that these might be reduced via an NADPH-thioredoxin reductase system operating across the membrane [50] and indeed an NADPH dependent thioredoxin reductase has been reported in chloroplasts [51,52], though it remains to be demonstrated whether this transfers reducing equivalents to the lumen. Regulation might then be achieved via reduction of a disulphide bridge on the Rieske FeS subunit of the cyt b_6f complex. Based on measurements in cyt b/c complexes, this is predicted to have a low potential and its reduction will inhibit the complex [53–55]. *In vitro*, this disulphide is known to interact with thioredoxin [56,57].

The importance of the regulatory pathway we have described is well illustrated by the visual symptoms observed. Whereas as-GAPDH plants showed only slowed growth, with no signs of damage, the leaves of the as-FNR plants, which had a very similar degree of inhibition of photosynthesis, were clearly stressed. This visual assessment is supported by measurements of chlorophyll loss (Fig. 1) and lipid peroxidation (Table 2), showing that the as-FNR plants are suffering from clear oxidative stress. The loss of PSI relative to PSII also does not explain the difference between the plants, since the PSI:PSII ratio (and also the content of cyt b_6f relative to the other two complexes) was similar in both antisense lines (Fig. 4).

Another notable feature of as-FNR plants is that they have increased vulnerability to photoinhibition (measured as NPQ_s) at high light, compared to the as-GAPDH plants (Table 1). This is in spite of the fact that Φ_{PSII} is equally suppressed in both plants and that, at high irradiances, NPQ_f is greatest in as-FNR plants. This difference is removed at low oxygen, where all three lines gave similar non photochemical quenching. The observation that NPQ_f was sensitive to oxygen levels in all plants, in contrast to previous observations in barley [20] suggests that electron flow to oxygen contributes significantly to the generation of ΔpH in these plants and also that the differences in NPQ_f between the three lines studied here can be explained, at least partially, by such a pathway. The increase in NPQ_s observed in the as-FNR plants is consistent with the notion that it ROS production at PSI, associated with the Mehler reaction, contributes to the photoinhibition seen in these leaves.

In conclusion, we have presented evidence that the capacity of the chloroplast electron transport chain is regulated in response to the concentration of its principal product, NADPH. Although the details of how this regulation is achieved remain to be elucidated, the importance of this process is immediately clear from the different phenotypes of the plants we have studied. Regulation by NADPH will play a vital role in the ability of plants to tolerate environmental stress and so a better understanding of the processes involved will contribute to our understanding of and our ability to enhance stress tolerance in plants.

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