An *in vivo* system involving co-expression of cyanobacterial flavodoxin and ferredoxin–NADP+ reductase confers increased tolerance to oxidative stress in plants

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A B S T R A C T

Oxidative stress in plants causes ferredoxin down-regulation and NADP+ shortage, over-reduction of the photosynthetic electron transport chain, electron leakage to oxygen and generation of reactive oxygen species (ROS). Expression of cyanobacterial flavodoxin in tobacco chloroplasts compensates for ferredoxin decline and restores electron delivery to productive routes, resulting in enhanced stress tolerance. We have designed an *in vivo* system to optimize flavodoxin reduction and NADP+ regeneration under stress using a version of cyanobacterial ferredoxin–NADP+ reductase without the thylakoid-binding domain. Co-expression of the two soluble flavoproteins in the chloroplast stroma resulted in lines displaying maximal tolerance to redox-cycling oxidants, lower damage and decreased ROS accumulation. The results underscore the importance of chloroplast redox homeostasis in plants exposed to adverse conditions, and provide a tool to improve crop tolerance toward environmental hardships.

1. Introduction

The photosynthetic electron transport chain (PETC) of plant chloroplasts provides reduced ferredoxin (Fd), NADPH and ATP for oxygenic photosynthesis. Electrons abstracted by light-dependent reactions at the photosystems promote water splitting at the oxidative end of the PETC, and are conveyed through soluble and thylakoid-bound electron carriers to Fd at the reducing side [1]. Ferredoxins are small, soluble [2Fe–2S] proteins that play a key role in electron distribution in all types of plastids [2]. In chloroplasts, photoreduced Fd can interact with Fd–NADP+ reductase (FNR) to generate the NADPH required for carbon assimilation and other biosynthetic and protective pathways. It can also divert reducing equivalents back to the PETC via cyclic electron flow, or to a plethora of soluble electron acceptor enzymes involved in various metabolic, regulatory and dissipative routes of the stroma [3]. In cyanobacteria and some marine algae, Fd can be replaced by flavodoxin (Fld), an isofunctional electron shuttle not present in plants which contains flavin mononucleotide as the prosthetic group [4–6]. When present, Fld usually acts as a backup for Fd when the levels of the iron–sulfur protein decline due to environmental hardships such as iron deficiency [4]. Under these conditions, Fld expression is induced and the flavoprotein takes over Fd functions in photosynthesis and other metabolic pathways [4].

Dioxygen may act as an adventitious electron acceptor of the PETC, yielding excited or partially reduced forms such as singlet oxygen and the superoxide radical, collectively known as reactive oxygen species (ROS). They react with different biomolecules, inactivating them and causing severe damage [7]. Under normal growth conditions, electron leakage to oxygen is minimal (<10%), and the small amounts of ROS generated are readily scavenged by plastidic antioxidants [8]. However, adverse environmental situations increase ROS production several-fold. Two non-excluding mechanisms have been invoked to explain this phenomenon. First, stress conditions inhibit CO_2 assimilation and hence, NADPH oxidation in the regenerative stages of the Calvin cycle [7]. Second, many environmental insults cause down-regulation of Fd expression in plants and cyanobacteria [9–11]. Both effects contribute to decrease the levels of the major physiological PETC acceptors (oxidized Fd and NADP+). Under these conditions, the PETC should become over-reduced and the ability of O_2 to subtract electrons from the chain is expected to increase, leading to runaway ROS propagation [7].

Therefore, depletion of the electron sink at the reducing side of the PETC is proposed to be a major determinant for the establishment of oxidative stress in plants [12], although the evidence for such a mechanism is circumstantial. For instance, knocked-down plants in which the levels of either FNR or Fd had been decreased by RNA antisense or RNA interference approaches display enhanced susceptibility to several sources of stress, in addition to growth and reproductive penalties [13–17]. This line of reasoning predicts that replenishment of the electron acceptor pool and reconstruction of electron delivery to productive routes might alleviate this situation. However, transformation of tobacco plants with a plastid-targeted Fd failed to improve stress tolerance, due to down-regulation of the transgene under oxidative conditions [18]. Instead, expression of a cyanobacterial Fld in tobacco chloroplasts led to transgenic lines with enhanced tolerance to multiple sources of stress, including drought, chilling and redox-cycling herbicides [11]. Consistent with the previous arguments, the effect of Fld was dose-dependent, required plastid targeting and resulted in preservation of photosynthesis, lower ROS accumulation and decreased cellular damage [3,11]. The ability of the foreign Fld to be reduced by Fd-dependent chloroplast systems (*e.g.*, the PETC) appeared essential to its protective effect [11].

Following this rationale, our working hypothesis is that even higher degrees of tolerance could be achieved by improving the capacity for Fld reduction *in planta* under stress conditions, and by relieving electron acceptor limitation of the PETC. Since NADPH build-up is another unwanted consequence of stress episodes, overexpression of FNR seems to be the logical choice in order to use the excess of NADPH as electron source for Fld reduction. Soluble FNR catalyzes Fld reduction by NADPH with high efficiency [19], but FNR normally binds to the thylakoid membrane, and when membrane-bound the preferred direction of the reaction is still under debate [20]. In this report we describe the generation of a soluble cyanobacterial FNR (sFNR) obtained by removing a membrane-binding N-terminal domain, and the design of an *in vivo* system for Fld reduction and NADPH oxidation, involving co-expression of Fld and sFNR in tobacco chloroplasts. The resulting transgenic lines displayed higher tolerance to oxidative stress and lower ROS build-up as compared to wild-type (*wt*) plants and single transformants.

2. Materials and methods

2.1. Design and characterization of transgenic tobacco plants expressing Fld and sFNR

A DNA fragment encoding a C-terminal region of FNR (sFNR) from *Anabaena* PCC7119 (without the phycobilisome binding domain) was obtained by PCR amplification of the whole gene cloned into plasmid pTrc99a [21], using primers 5′-CCGAGCTCACACCAT-GACTCAAGCGAA-3′ and 5′-ACGTCGACCAACTTAGTATGTTTCTAC-3′. A PCR product of the predicted length (940 bp) was digested with *Sac*I and *Sal*I and cloned into compatible sites of a pUC9-derived recombinant plasmid harboring the entire pea FNR precursor gene between *Bam*HI and *Sal*I restriction sites, and from which the DNA fragment encoding the mature region of pea FNR had been removed by digestion with *Sac*I and *Sal*I. This generated an in-frame fusion of the chloroplast transit peptide (TP) derived from pea FNR with the mature region of *Anabaena* FNR. The chimeric gene was excised from the corresponding plasmid by digestion with *Bam*HI and *Sal*I, cloned between the CaMV 35S promoter and polyadenylation regions of pDH51 [22]. The entire cassette was further isolated as an *Eco*RI fragment and inserted into the corresponding *Eco*RI site of the binary vector pCAMBIA2200 [23]. The resulting plasmid was introduced into the genome of tobacco (*Nicotiana*

tabacum cv. Petit Havana) through *Agrobacterium tumefaciens*-mediated leaf disc transformation [24].

Primary transformants expressing high levels of sFNR, as evaluated by SDS–PAGE and immunoblotting, were self-pollinated and all subsequent experiments were carried out with the homozygous progeny.

The preparation of double expressing plants was performed by cross-pollination. Transgenic plants expressing sFNR from *Anabaena* (*psfnr*) and a stable homozygous line expressing high levels of *Anabaena* Fld in chloroplasts (*pfld*) [11] were used as parentals. Primary double heterozygous transgenic plants expressing sFNR and Fld (*pfld/psfnr*) were self-pollinated and double homozygous plants selected by SDS–PAGE and immunoblotting.

Seeds were germinated on Murashige–Skoog agar supplemented with 3% (w/v) sucrose and, in the case of transformants, 100 μg mL^{-1} kanamycin. Three-week-old seedlings were transferred to soil or grown hydroponically in nutrient medium [25], and illuminated at 200 μmol quanta m^{-2} s^{-1} and 25 °C to provide a 16-h photoperiod. Intact chloroplasts were isolated and osmotically shocked as described [26]. Levels of sFNR and Fld in cleared leaf and chloroplast extracts were analyzed by 12% SDS–PAGE, followed by immunoblot analysis with antisera raised against *Anabaena* FNR and Fld.

2.2. Determination of photosynthetic parameters

Chlorophyll fluorescence measurements were performed at 25 °C on dark-adapted leaves using a Qubit Systems pulse-modulated fluorometer. The F_v and F_m parameters were determined after 30 min in the dark, and the light-adapted values (F'_v and F'_m) were measured after 30 min of illumination at 200 μmol quanta m^{-2} s^{-1}. Photosynthetic parameters (F_v/F_m, Φ_{PSII}, NPQ, 1-qP) were calculated as described [27]. CO$_2$ assimilation rates were measured with an infra-red gas analyzer LI-6200 (LI-COR) at 500 μmol quanta m^{-2} s^{-1}. Induction/relaxation NPQ curves were determined using the program of the MINI-PAM 2000 fluorometer. Light induction measurements were performed on dark-adapted leaves by application
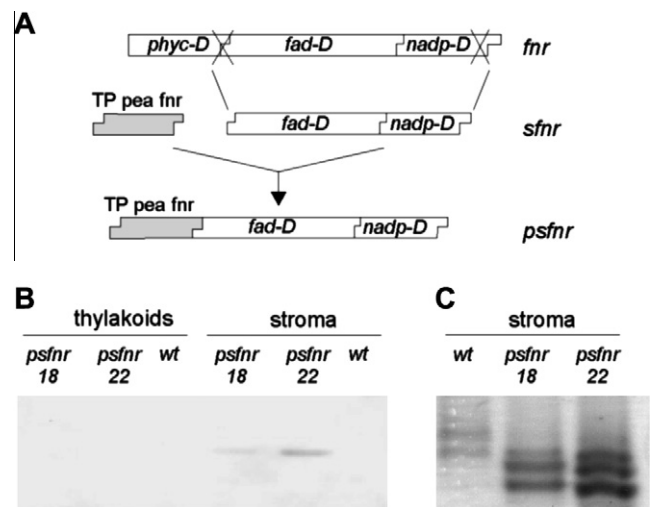
Fig. 1. Expression of soluble cyanobacterial FNR in chloroplasts. (A) Schematic representation of the chimeric gene used to generate transgenic plants expressing sFNR in chloroplasts. *Phyc-D*, *fad-D* and *nadp-D* indicate the phycobilisome-, FAD- and NADP(H)-binding domains, respectively. The coding sequence of sFNR was fused in-frame to the pea FNR transit peptide (TP) and cloned in pCAMBIA2200. (B) sFNR accumulation in chloroplasts. Stromal and membrane fractions from osmotically shocked chloroplasts (corresponding to 4 μg of chlorophyll) from *wt* and two independent transgenic lines (*psfnr18* and *psfnr22*) were fractionated by SDS–PAGE and blotted onto nitrocellulose membranes for immunodetection with antisera directed against *Anabaena* FNR. (C) NADPH-diaphorase activity in stromal fractions of *wt* and transgenic plants. Extracts corresponding to 30 μg of stromal proteins were resolved by native electrophoresis and stained as indicated in Section 2.

of a saturating pulse to obtain the F_m values, and a second pulse 5 s later. After 30 s, NPQ induction was followed over 6 min at 200, 600, 1000 and 2500 μmol quanta m^{-2} s^{-1} of actinic light, and saturating pulses were applied every 30 s to obtain F'_m. ETR values were obtained at 200 and 600 μmol quanta m^{-2} s^{-1} of actinic light using the equation: $\Phi_{PSII} \times$ actinic light intensity $\times 0.5 \times 0.84$. The actinic light was subsequently turned off, and the dark relaxation process was monitored at various times (30 s and 1, 2, 5 and 10 min) using saturating pulses [17].

2.3. In-gel diaphorase activity

For the identification of enzymes displaying NADPH-dependent diaphorase activity (largely FNR), stromal extracts corresponding to 30 μg of soluble protein were resolved by nondenaturing PAGE on 12% polyacrylamide gels. After electrophoresis, the gel was stained by incubation in 50 mM Tris–HCl, pH 8.5, 0.3 mM NADP$^+$, 3 mM Glc-6-P, 1 unit mL^{-1} Glc-6-P dehydrogenase, and 1 mg mL^{-1} nitroblue tetrazolium until the appearance of the purple formazan bands [26].

2.4. Methyl viologen treatments

Leaf discs (12-mm diameter) were floated topside up in 1 mL water or 40 μM MV, and illuminated at 1000 μmol quanta m^{-2} s^{-1} for 7 h. Electrolyte leakage was measured as described previously [11]. For plants cultured in hydroponics, 100 μM MV was added to the nutrient solution.
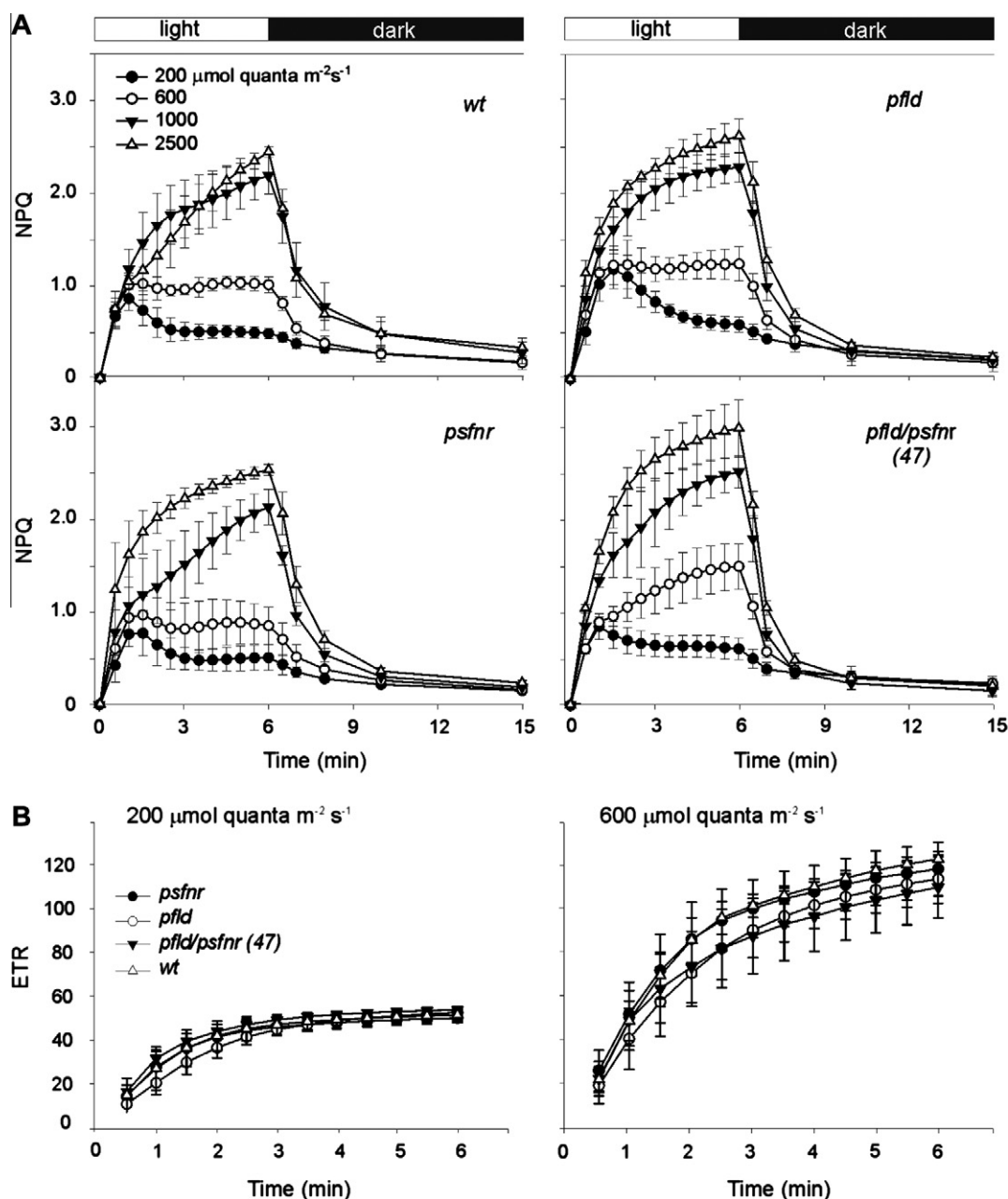
Fig. 2. Induction/relaxation curves of non-photochemical quenching. (A) NPQ measurements were carried out using induction (at the actinic light intensities indicated in *wt* panel) and relaxation periods of 6 and 9 min, respectively, as indicated by the boxes at the top. (B) Time courses of electron transport rates. ETR values were calculated as $\Phi_{PSII} \times 0.5 \times 0.84 \times$ actinic light intensity, at 200 and 600 μmol quanta m^{-2} s^{-1}. Plants grown at 200 μmol quanta m^{-2} s^{-1} were dark-adapted for 30 min prior to the 6-min measurements. Values are means ± SD of three assays on independent plants from each line. Results obtained with double homozygous line (47) are shown.
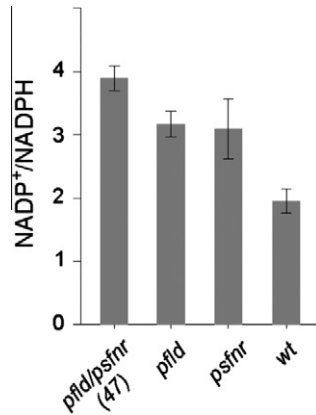
Fig. 3. Increased NADP$^+$/NADPH ratio in *pfld/psfnr* line (47). NADP(H) contents were measured in leaf extracts of 3-week-old plants as described in Section 2. Measurements were done in triplicate, and values are the mean ratios ± propagation of SE.

2.5. Analytical procedures

Chlorophylls and carotenoids were determined spectrophotometrically after extraction with 96% (v/v) ethanol [28]. NADP(H) levels were estimated by a redox cycling assay, essentially as described by Slater and Sawyer [29], after extraction of the pyridine nucleotides from leaf tissue. Hydroperoxides were measured using a modified ferrous oxidation–xylenol orange assay [30].

3. Results and discussion

The preparation and phenotypic characterization of tobacco line *pfld*5–8, expressing Fld from *Anabaena* PCC7119, have been described elsewhere [11]. While plant FNRs are made up of two structural domains and bind extrinsically to membranes, *Anabaena* FNR is a 3-domain flavoenzyme, with the two C-terminal domains being homologous to their plant counterparts [19]. The N-terminal region in the cyanobacterial reductase is responsible for membrane attachment through phycobilisome binding [31]. This domain can be excised to abolish thylakoid interaction, without affecting enzyme activity [32]. Then, for the generation of sFNR-expressing plants, a truncated form of the FNR from *Anabaena* PCC7119 lacking the membrane-binding domain was fused in-frame to the transit peptide of pea FNR (Fig. 1A), and introduced into tobacco plants using *Agrobacterium*-mediated leaf disc transformation, to yield *psfnr* lines. The presence of sFNR was determined in cleared leaf extracts by SDS–PAGE and immunoblotting. Lines containing the highest amounts of the foreign flavoenzyme were made homozygous by self-pollination. Homozygosity of the transgene was confirmed in all cases by back-crosses to the wild type and by quantification of sFNR amounts (data not shown, but see below). Segregation analysis confirmed a single insertion *locus* per genome in selected lines. The sFNR product was targeted to chloroplasts and accumulated in the stroma, as revealed by immunoblots performed on different fractions of intact chloroplasts isolated from two different homozygous lines (Fig. 1B, lanes *psfnr18* and *psfnr22*). It is worth noting that antisera raised against *Anabaena* FNR did not cross-react with endogenous
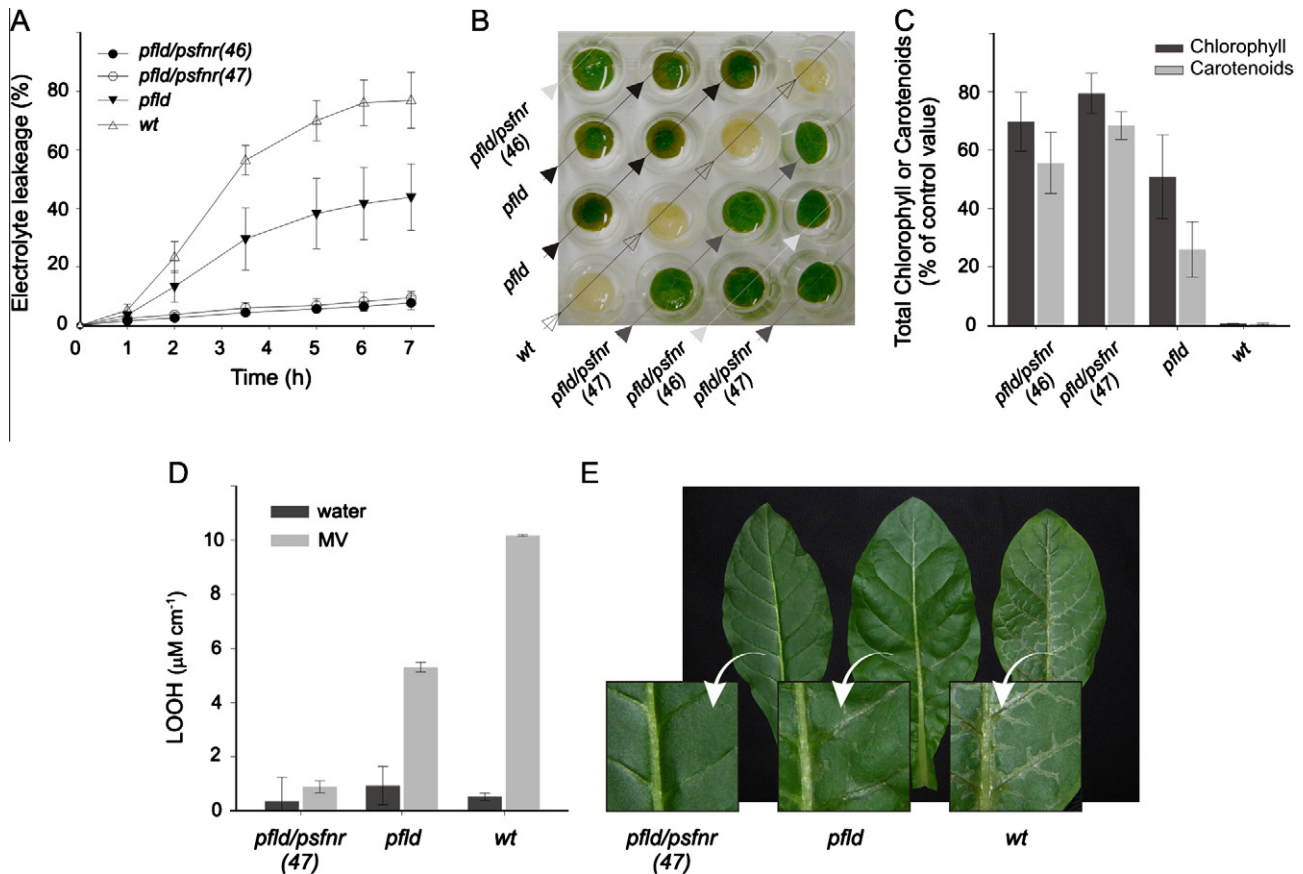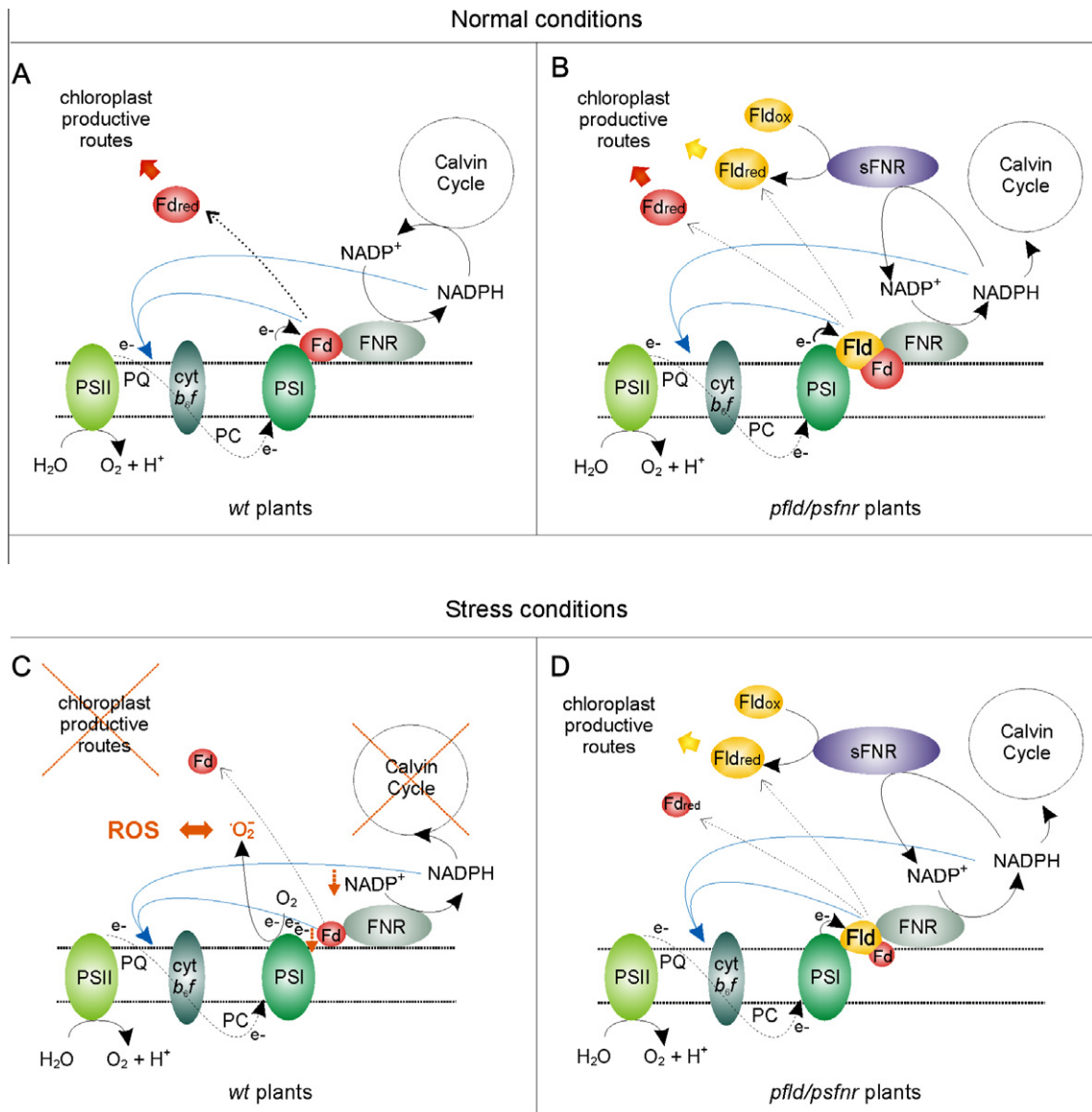

Fig. 4. Expression of Fld and sFNR in chloroplasts increase tolerance to MV toxicity. Membrane damage (A), bleaching (B), and pigment degradation (C), were analyzed in five leaf discs from 6-week-old *wt* and transgenic plants exposed to 40 μM MV at 1000 μmol quanta m^{-2} s^{-1} for 7 h. Two *pfld/psfnr* lines were assayed. Values are expressed as the percentages ± SD of ion leakage relative to zero time of discs incubated under the same conditions (A), or as means ± SD of the percentages calculated against control discs incubated in water (C). (D) Leaf discs were challenged with 10 μM MV at 700 μmol quanta m^{-2} s^{-1} for 3 h, and hydroperoxides (LOOH) were determined by the xylenol orange method [30]. Bars are means of four independent determinations ± SD. (E) Four-week-old plants grown in soil were transferred to hydroponics solution for 3 days, and subsequently supplemented with new nutrient solution containing 100 μM MV. Pictures were taken 24 h after treatment.

tobacco FNR (Fig. 1B). Diaphorase activity staining of native gels revealed several reactive bands in the stroma of transformed plants (Fig. 1C, lanes *psfnr18* and *psfnr22*), migrating ahead of those corresponding to the tobacco flavoenzymes (Fig. 1C, lane *wt*). Micro-heterogeneity at the N-terminus during transit peptide processing might account for the various molecular species observed, as already reported [33]. Although in-gel determinations do not provide quantitative values, it is clear from Fig. 1C that the cyanobacterial reductase accounts for most FNR activity in the chloroplast stroma of the transgenic lines. Growth rates, biomass accumulation and photosynthetic activities of *psfnr* plants did not differ significantly from those displayed by *wt* siblings (Table S1).

Plants co-expressing Fld and sFNR were prepared by cross-fertilization of the respective individual transformants, and double homozygous lines were selected by a combination of segregation analysis and protein level determinations (Fig. S1). The general phenotypes of the double-transgenic lines (*pfld/psfnr*) were similar to those of *wt* plants (Table S1). Steady-state photosynthetic parameters, including photosystem (PS) II operating efficiency, maximum quantum yield of PSII and CO_2 assimilation activities, also failed to show significant differences (Table S1). The results indicate that the presence of the two interacting proteins remained largely unnoticed, in phenotypic terms, in unstressed plants. However, some subtle differences could be detected. First, the time course of NPQ build-up at high light intensities was faster and more extensive in the double transgenics, without significant changes in electron transfer rates through PSII (Fig. 2). NPQ is a dissipative response deployed by plants to relieve excess excitation energy on the PETC, and largely relies on Fd-dependent cyclic electron flow through PSI. Second, the $NADP^+/NADPH$ ratio was higher in the *pfld/psfnr* lines relative to *wt* and single transformants (Fig. 3).

Methyl viologen (MV) is a redox-cycling herbicide which propagates ROS by accepting electrons at PSI, one at a time, and transferring them to oxygen to yield superoxide radicals [34]. Previous

Fig. 5. Proposed model for the protective mechanism of Fld and sFNR in chloroplasts. Under normal growth conditions Fd/Fld tranfers electrons to different productive routes in chloroplasts. Under stress situations, Fd levels decline. In *pfld/psfnr* plants, sFNR oxidizes NADPH, regenerating $NADP^+$ and contributing to Fld reduction. Blue arrows indicate cyclic electron transport. Cytb_6f, cytochrome b_6f; PC, plastocyanin; PQ, plastoquinone; ox, oxidized; red, reduced.

results have shown that plants expressing Fld in chloroplasts displayed enhanced tolerance to several sources of stress including MV [11]. To probe the tolerance of the single and double transformants, membrane damage in the presence of MV was evaluated by measuring ion release from leaf discs. Initial rates of electrolyte leakage were ∼2-fold lower in *pfld* foliar tissue as compared to *wt* discs (Fig. 4A), whereas plants transformed with the gene encoding sFNR displayed *wt* levels of tolerance (Fig. S2). The combination of both flavoproteins in *pfld/psfnr* lines led to a further 10-fold decrease (relative to *pfld* siblings) in the rates of ion release, a dramatic improvement that was also evident at the end of the treatment (Fig. 4A). During that 7-h period of MV exposure, both *wt* (Fig. 4B) and *psfnr* discs (data not shown) were completely bleached, reflecting nearly quantitative destruction of leaf pigments (Fig. 4C). Plants expressing Fld alone retained about 50% and 25% of chlorophylls and carotenoids, respectively, in good agreement with previous reports [11]. Once again, the double transformants displayed the highest levels of pigment preservation: 70–80% for chlorophylls and 55–70% for carotenoids (Fig. 4C). In line with the stress protection conferred, ROS build-up was almost entirely prevented in lines co-expressing Fld and sFNR, about 5- and 10-fold below the levels accumulated in *pfld* and *wt* lines, respectively (Fig. 4D). When whole plants were exposed to MV toxicity in a hydroponic system, the extent of damage undergone by the leaves followed the same trend observed in disc experiments, with *pfld/psfnr* lines displaying the highest degree of tolerance (Fig. 4E).

The collected results indicate that replenishment of the acceptor sink at the PETC, and the presence of an additional electron source for Fld reduction in the double-transgenic plants significantly increased tolerance to oxidative stress. We propose that this system functions by recycling NADP(H) through the sFNR/Fld couple, thus relieving the electron pressure on the PETC and preventing excessive reduction of the NADP(H) pool under adverse situations. At the same time, proper delivery of reducing equivalents generated at the PETC to productive oxido-reductive pathways of the chloroplast will be favored by a continuous stream of reduced Fld. A model describing these findings and interpretation is provided in Fig. 5.

According to this rationale, under non-stressed conditions, Fd in *wt* plants will collect reducing equivalents from the PETC and distribute them to the Calvin cycle (via NADP⁺ photoreduction), to other stromal acceptors, and back to the PETC via cyclic electron flow (Fig. 5A). In the double transgenics (Fig. 5B), Fd and Fld can be reduced by both the PETC and NADPH (via sFNR). Fld can act in the same chloroplast oxido-reductive pathways as Fd, but the iron-sulfur protein will likely be the preferred electron shuttle [11]. The activity of sFNR has only limited effect on redox homeostasis, reflected by a moderate increase in the NADP⁺/NADPH ratio (Fig. 3) and faster NPQ build-up (Fig. 2). Under stress conditions, however, Fd levels decline and NADPH accumulates in *wt* plants, leading to over-reduction of the PETC and ROS build-up (Fig. 5C). Fld can replace declining Fd, restoring delivery of reducing equivalents to productive electron accepting routes [3,11]. The activity of sFNR will contribute to these Fld functions, and at the same time will consume the NADPH surplus, preventing over-reduction of the PETC and ROS propagation (Fig. 5D). Although the proposed mechanism is consistent with the observations reported here, some of its steps are speculative and require further experimentation to substantiate the contentions made. Accordingly, the model of Fig. 5 should be regarded as a working hypothesis.

Then, the combined action of Fld and sFNR can play a transient but crucial role in maintaining proper electron distribution during oxidative stress episodes. The results emphasize the paramount importance of chloroplast redox homeostasis in plants exposed to adverse conditions. Many environmental insults of agronomical relevance, such as drought, chilling and salinity, lead to the establishment of an oxidative stress situation that significantly contributes to the damage suffered by the plant [35]. Observations reported here indicate that the Fld/sFNR system could provide a promising biotechnological tool to improve crop tolerance towards these environmental hardships. Research is currently underway to evaluate this possibility.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fob.2011.10.004.

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