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Review

The importance of flavodoxin for environmental stress tolerance in photosynthetic microorganisms and transgenic plants. Mechanism, evolution and biotechnological potential

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ABSTRACT

Ferredoxins are electron shuttles harboring iron–sulfur clusters which participate in oxido-reductive pathways in organisms displaying very different lifestyles. Ferredoxin levels decline in plants and cyanobacteria exposed to environmental stress and iron starvation. Flavodoxin is an isofunctional flavoprotein present in cyanobacteria and algae (not plants) which is induced and replaces ferredoxin under stress. Expression of a chloroplast-targeted flavodoxin in plants confers tolerance to multiple stresses and iron deficit. We discuss herein the bases for functional equivalence between the two proteins, the reasons for ferredoxin conservation despite its susceptibility to aerobic stress and for the loss of flavodoxin as an adaptive trait in higher eukaryotes. We also propose a mechanism to explain the tolerance conferred by flavodoxin when expressed in plants.

1. Ferredoxins and flavodoxins

1.1. Electron shuttling: a common theme in oxido-reductive processes

Electron shuttling is a key feature of many redox pathways in all living organisms. This function is usually performed by diffusible electron carrier proteins which act as electronic switches between cellular sources of reducing power (i.e., light-driven reactions, pyridine nucleotides, sugars) and electron-consuming routes and processes. In organisms displaying oxygenic photosynthesis (plants, algae and cyanobacteria), ferredoxin (Fd) is a key player of electron shuttling [1]. There, Fds collect reducing equivalents generated in the photochemical reactions of the photosynthetic electron transport chain (PETC), and deliver them to a plethora of metabolic, regulatory, dissipative and developmental processes. A substantial fraction of photoreduced Fd is employed for the reduction of $NADP^+$ in an electron-hydride exchange reaction catalyzed by the flavoenzyme ferredoxin-$NADP^+$ reductase (FNR) [2,3]. The NADPH thus formed is subsequently employed for CO_2 fixation in the regenerative steps of the Calvin cycle and for other biosynthetic, regulatory and protective reactions. Reduced Fd molecules also act as electron donors for N and S assimilation, amino acid, fatty acid and secondary metabolism, reductive activation of enzymes, antioxidant regeneration, etc. (reviewed in [4]). A comprehensive list of known Fd partners is provided in Table 1.

Ferredoxins employ iron–sulfur clusters of different stoichiometry as prosthetic groups, with the photosynthetic Fd harboring a [2Fe–2S] center [1]. Canonical Fds have M_w of ~12 kDa and a midpoint redox potential of about −410 mV [5], which allows them to behave as low potential electron shuttles. Fd is found in a wide range of organisms pervading all kingdoms, aerobic and anaerobic, with plastid and mitochondrial variants in higher eukaryotes. Several isoforms are usually present in plants and cyanobacteria [6–9]. Expression of photosynthetic Fd is induced by light and declines under iron starvation [10–14]. Noteworthy, oxidative stress and adverse environmental situations (salt, extreme temperatures, water deficit) lead to down-regulation of Fd levels in both plants and cyanobacteria [14–18].

Many prokaryotes and some oceanic algae contain an isofunctional electron shuttle, flavodoxin (Fld), a small soluble protein (M_w = 15–22 kDa) which has a non-covalently bound FMN

Abbreviations: Fd, ferredoxin; Fld, flavodoxin; FNR, ferredoxin-$NADP^+$ reductase; Ga, billion years ago; GOGAT, glutamate–oxoglutarate amino transferase; PETC, photosynthetic electron transport chain; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; sFNR, soluble FNR; WT, wild-type

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Table 1
Identified partners of Fd and Fld in chloroplasts and cyanobacteria.

Protein partners	Function	Metabolic pathway	Organisms	References
Ferredoxin				
Photosystem I (PSI)	Photosynthetic electron transport	Photosynthesis	Cyanobacteria, algae, plants	[82]
FNR	$NADP^+$ reduction	Photosynthesis	Cyanobacteria, plants	[1,83]
Nitrite reductase	Reduction of NO_2^- to $NH4^+$	Nitrogen assimilation	Cyanobacteria, algae, plants	[1,83]
Nitrate reductase	Reduction of NO_3^- to NO_2^-	Nitrogen assimilation	Cyanobacteria	[1,83]
Nitrogenase and pyruvate:Fd oxidoreductase or FNR	N_2 fixation	Nitrogen assimilation	Cyanobacteria	[84]
Hydrogenase	H_2 formation	Hydrogen metabolism	Cyanobacteria	[84]
Glutamate–oxoglutarate amino transferase (GOGAT)	Glutamate synthesis	Amino acid synthesis	Cyanobacteria, algae, plants	[1,83]
Sulfite reductase	Reduction of SO_3^{2-} to H_2S	Sulfur assimilation	Plants	[1]
Ferredoxin-thioredoxin reductase	Thioredoxin reduction	Redox regulation[a]	Cyanobacteria, algae, plants	[1,83]
Fatty acid desaturase	Double bond formation in fatty acids	Lipid metabolism	Cyanobacteria, plants	[83,85]
Monodehydroascorbate reductase	Ascorbate regeneration	Antioxidant defense	Plants	[86]
Heme oxigenase and phytochromobilin synthase	Phytochromobilin[b] synthesis	Development	Plants	[87,88]
Heme oxigenase and phycocyanobilin:Fd oxidoreductase	Phycocyanobilin[c] synthesis	Development	Cyanobacteria	[83,89,90]
PGRL1, PGR5, FNR and PSI	Cyclic electron flow	Photosynthesis	Algae, plants	[91,92]
Flavodoxin				
PSI	Photosynthetic electron transport	Photosynthesis	Cyanobacteria, algae	[82]
FNR	$NADP^+$ reduction	Photosynthesis	Cyanobacteria, algae	[22,93]
FNR and PSI	Cyclic electron flow	Photosynthesis	Cyanobacteria	[94]
Nitrogenase	N_2 fixation	Nitrogen assimilation	Cyanobacteria	[95]
Hydrogenase	H_2 formation	Hydrogen metabolism	Cyanobacteria	[84]

[a] Reduced thioredoxin activates key chloroplast enzymes of the Calvin cycle, the malate valve, etc.
[b] Plant chromophore of the light sensor phytochrome and intermediate in the synthesis of chlorophyll.
[c] Chromophore of the light sensor phytochrome in cyanobacteria and green algae, and precursor of the chromophores of the light-harvesting phycobiliproteins.

molecule as prosthetic group instead of an iron–sulfur cluster [19]. Unlike Fd, which is an obligatory one-electron carrier, the flavin group of Fld can in principle exchange one or two electrons, oscillating between the oxidized, the semiquinone and the hydroquinone states [20]. However, empirical evidence indicates that Fld behaves as a one-electron carrier under all circumstances, switching between the semiquinone/hydroquinone states [21]. This transition has a redox potential close to that of the Fe^{+3}/Fe^{+2} reaction of Fd, whereas the conversion of the oxidized form into the semiquinone is usually 200 mV less negative.

Fld properties as redox shuttle largely match those of Fd (Table 1), and the flavoprotein can replace the metalloprotein in most reactions [22]. In organisms in which both electron carriers are present, Fld is typically induced as an adaptive resource under environmental or nutritional hardships that compromise Fd expression or activity (i.e., iron limitation), allowing survival and reproduction under conditions that would be otherwise deleterious. However, a few Fld-specific metabolic routes have been described. Indeed, Fld is an essential gene in *Escherichia coli* and *Helicobacter pylori*, whereas Fd is not [23–25]. Both Fd and Fld are able to mediate $NADP^+$ photoreduction via FNR [26]. This reaction can proceed backwards, from NADPH to oxidized Fd/Fld, for instance in non-photosynthetic plant tissues (i.e., roots) and cyanobacterial heterocysts [3]. NADPH is the normal reductant in heterotrophic microorganisms and mitochondria, although carbohydrates can also be used as electron source to reduce Fd/Fld by committed enzymes such as the pyruvate-Fd reductase of *E. coli* [27].

Fld is present in prokaryotes (including cyanobacteria) and some algae, but has not been found in the genomes of plants (or animals), indicating that this adaptive resource was irreversibly lost in the long evolutionary history that led to current day streptophytes and metazoans [28].

1.2. The basis for functional equivalence: promiscuity as a virtue

Fld and Fd do not share any significant similarity in primary, secondary or tertiary structures, and yet they can interact productively with the same redox partners with comparable efficiency. The key to this apparent paradox resides in the very function of these proteins. The most desirable property of an electron shuttle is the ability to exchange reducing equivalents with the highest possible number of different redox partners. Accordingly, Fd and Fld have been tailored by evolution to be promiscuous in their interactions. Analysis of plant Fd binding sites in various Fd-dependent enzymes revealed no obvious homology [1]. Then, docking of Fd (and Fld) must be determined by general features of the proteins rather than contacts with specific conserved amino acids. The prosthetic groups of both proteins (flavin and [2Fe–2S]) are eccentric and surrounded by patches of negatively charged residues, while their enzyme partners harbor a crown of positively charged amino acids around their exposed cofactors [1]. Initial interactions are therefore steered by electrostatic attractions that help to stabilize the binary complexes, and serve to position the corresponding prosthetic groups at the proper distance to allow direct outer-sphere electron transfer between them. These charged regions are remarkably insensitive to mutations, and different accommodations of the two proteins (i.e., rotations) are allowed without losing the ability for efficient electron transfer [29–31].

Although Fd and Fld differ in nearly all structural features, they could be aligned on the basis of their Coulomb electrostatic potentials. Applying the Hodgkin index to evaluate their similarity in this sense, Ullmann et al. [32] obtained a significant overlapping. The cofactors, rather than their centers of mass coincided in the alignments. Both proteins have strong dipole moments (380–700 Debyes), with the vectors of the negative dipole pointing toward the flavin ring in Fld and the iron–sulfur cluster in Fd [21,33]. These considerations provided a conceptual framework to understand why these electron carriers are able to interact with so many different enzymes, and why they can be swapped without major loss in efficiency.

1.3. Ferredoxin: the Achilles' heel of aerobic life?

Iron–sulfur clusters are sensitive to oxidation and low iron availability, which are the hallmarks of aerobic environments. And yet they are probably the cofactors more diversely employed by contemporary organisms. Why aerobes rely so heavily on chemical groups which appear ill-suited for an oxygen-rich habitat? Iron–sulfur centers in general, and Fds in particular, are very ancient biocatalysts that were already present in early organisms. Life in Earth originated about 3.5 billion years ago (Ga) in an anaerobic environment where oxygen was largely absent and Fe^{+2} and sulfide, plentiful (Fig. 1A).

Unlike organic prosthetic groups, iron–sulfur centers can be formed spontaneously from these simple compounds, and eventually assembled into extant polypeptide structures, since analogous clusters can be created in vitro by incubating ferrous and sulfide salts with organic thiolates. In addition, iron–sulfur centers exhibit great chemical versatility: they can accept and donate electrons in a range of oxido-reductive processes, act as Lewis acids during dehydration of carbonyl compounds in hydro-lyases, and mediate derivatisation of aliphatic metabolites by radical-based mechanisms [34]. These remarkable traits favored dispersion of organisms containing iron–sulfur proteins throughout the primordial anaerobic world, and placed these cofactors among the earliest catalysts. Indeed, one of the most provocative theories on the origin of life, developed by Wächtershäuser [35,36], proposes that they actually were the first catalysts and that life originated on the surface of pyrite (an iron–sulfur complex) deposits in the oceans.

The preceding discussion was intended to explain why iron–sulfur clusters were widespread when, approximately 2.75 Ga, cyanobacteria evolved photosystem II (PSII) and hence oxygenic photosynthesis, relieving these prokaryotes from the need of external electron donors. Oxygen concentrations remained low over the following two billion years or so, limited by both the paucity of oceanic phosphorous to support ATP synthesis, and by oxygen removal through reaction with dissolved ferrous and sulfide ions [37]. The rise of oxygen levels at the brink of the Precambrian (~0.8 Ga) led to one of the most catastrophic evolutionary stresses since early biotic history (Fig. 1A).

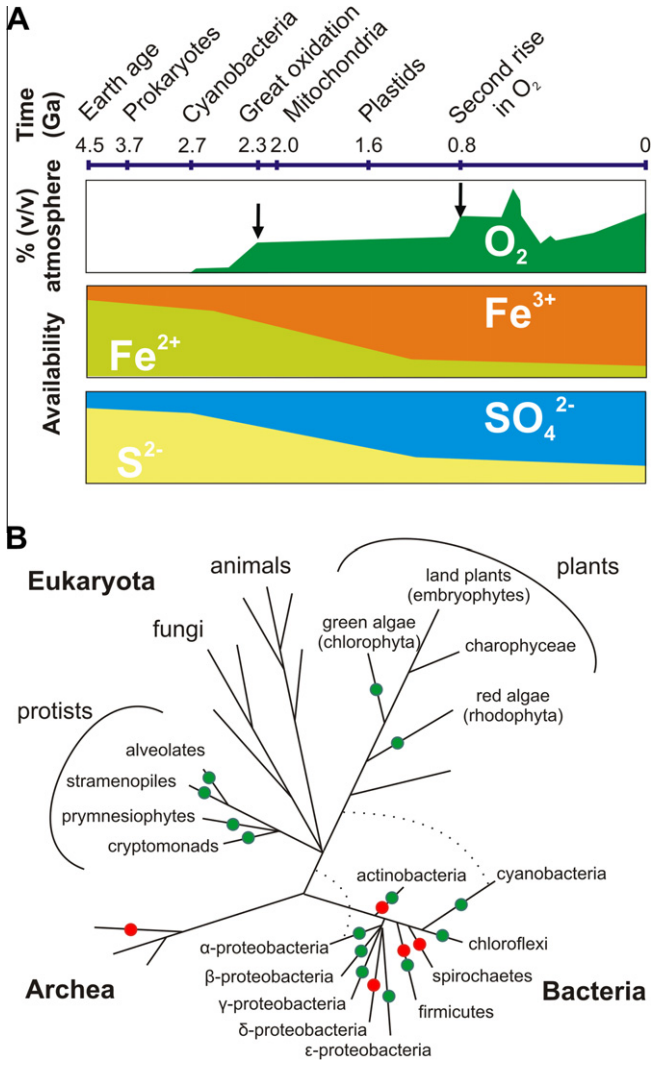

Fig. 1. Interplay between geochemical changes and biological evolution and its implications on the current Fld distribution. (A) Temporal relationships between atmospheric O_2 concentrations, availability of iron and sulfur and major evolutionary events along the Earth's life (adapted from [80,81]). (B) Fld distribution. The different colors indicate the two classes of Flds: red for the 'short chain' family (~140 amino acids) and green for the 'long chain' family (~170 residues) [22]. Dashed lines indicate primary endosymbiotic events that gave rise to plastids and mitochondria. Secondary endosymbioses were omitted for simplicity. Branch lengths are not in scale.

On the plus side, microbes were presented with the opportunity to use oxygen as a terminal oxidant, an adaptation that required surprisingly little molecular evolution [34]. Since aerobes kept many of the catabolic and biosynthetic pathways present in their anaerobic ancestors, maintenance of most iron–sulfur protein families was ensured. At the same time, oxygen build-up negatively affected the function of these metalloproteins in a number of ways. First, spin-pairing rules dictate that molecular oxygen accepts electrons one at a time rather than in pairs, discouraging reaction with most organic biomolecules but facilitating oxidation of transition metals, which are good univalent electron donors. As a consequence, oxygen oxidized ferrous iron in the environment to its ferric form, which rapidly precipitated as ferric hydroxide or formed insoluble complexes with anionic salts. The upshot was that as oxygen accumulated, iron decreased its bioavailability and became a limiting nutrient in many aerobic habitats (Fig. 1A) [34].

Second, partial reduction of oxygen generates oxidants with even higher reactivity, such as hydrogen or organic peroxides and the superoxide radical, collectively known as reactive oxygen species (ROS). Even under optimal growth conditions, ~5% of all electrons moving through the photosynthetic or respiratory chains are adventitiously delivered to oxygen with concomitant ROS generation. In photosynthetic organisms, this fraction increases dramatically under adverse environmental conditions [38]. Stress-dependent Fd down-regulation likely contributes to the establishment of the oxidative condition by self-propagating successive rounds of ROS synthesis. The decline in Fd amounts leads to over-reduction of the PETC due to shortage of electron acceptors. Under such circumstances the electron surplus could be passed straight to oxygen resulting in ROS generation [39–41].

Iron–sulfur centers are vulnerable to ROS attack to various extents, depending on solvent exposure and the polypeptide environment surrounding the cluster. Oxidation yields unstable forms that quickly decompose, resulting in protein inactivation and iron release. Elevated concentrations of free iron can wreak cellular havoc and lead to oxidative damage by engaging in Fenton-type reactions with hydrogen peroxide to generate the extremely toxic hydroxyl radical [34].

Thus, the high iron demand that modern aerobes inherited from their anaerobic ancestors does not suit well an oxygen-rich world. Air-thriving organisms tackled these problems at various levels by improving iron uptake, storage and mobilization, replacing ROS-sensitive targets by resistant ones (i.e., Fd by Fld), and developing more sophisticated antioxidant and repair systems. The many adjustments that have been made are expensive and bestow only a limited capacity to tolerate this threat. It is doubtful that iron–sulfur clusters could have emerged as central catalysts had life originally evolved in an aerobic environment. Largely because of their reliance on these cofactors, aerobes remain vulnerable to iron restriction and oxidative stress.

1.4. Flavodoxin as a backup for ferredoxin in different organisms

Flavodoxins are found in all major prokaryotic taxa including cyanobacteria and α-proteobacteria, the types of organisms that gave origin to modern day chloroplasts and mitochondria (Fig. 1B). As indicated above, the flavoprotein is absent from plants

and animals, except for the "enslaved" Fld-like domains of certain complex enzymes [22,42]. Fld absence in mitochondria seems to be universal, suggesting that the original endosymbiont already lacked the Fld gene, or that it was lost very early after integration. The situation in photosynthetic organisms is different. Following endosymbiosis, the gene was transferred to the nucleus of primitive green algae and the product redirected to plastids [43]. Phylogenetic analysis of available eukaryotic Fld sequences indicates that the gene is present in all major algal taxa [44,45], including those resulting from subsequent endosymbiotic events such as dinoflagellates, prymnesiophytes, diatoms and cryptomonads [45,46]. However, it has not been found in streptophytes, the phylogenetic branch composed of embryophytes (land plants) and charophytes [47–49]. This distribution suggests that the gene was lost somewhere in the transition between green algae and terrestrial plants.

Although Fld expression is induced under oxidative and environmental stress conditions, for instance during the *soxRS* response of *E. coli* [50], iron deficit appears to be the most critical imperative that determined its adaptive value [45]. The occurrence of this electron shuttle has been more extensively studied in marine organisms. Coastal waters typically contain high nutrient concentrations (including iron), due to inputs from land and sediments [51]. On the contrary, iron tends to be chronically deficient in the open oceans [51,52], where Fe levels could be 100- to 1000-fold lower than in the coast [53]. Comparisons between related organisms that have colonized the two habitats indicate that the minimal amount of iron required for growth of coastal phytoplankton is higher than that required by oceanic siblings [54].

As the atmosphere became oxidant and the oceans iron-deficient, several mechanisms to survive iron limitation were developed by marine microorganisms, such as high surface-to-volume ratio to aid nutrient uptake [55], a more extensive machinery for metal storage [56], and a decrease of iron-rich PSI (12 iron atoms per complex) in favor of PSII (2–3 iron atoms per complex) [57]. Adaptation also included extensive replacement of iron-dependent proteins by isofunctional counterparts [51,56,58]. Known examples are the use of the copper protein plastocyanin instead of cytochrome c_6 (a hemoprotein), of cobalt-containing ribonucleotide reductase in place of the Fe-dependent isoenzyme, and of Mn-, Cu/Zn- and Ni-containing superoxide dismutases [43,59–61]. Within this context, expression of Fld as a backup of Fd is considered as the most crucial factor determining the colonization of iron-poor waters by phytoplankton [62]. The importance of Fld in the dynamics of sea ecology can be gauged by its use as a proxy for iron stress in the oceans [46]. Evaluation of metagenomic data showed that microorganisms lacking this flavoprotein are usually confined to coastal areas while Fld-containing marine microorganisms are preferably located in oceanic environments [56]. Moreover, closely related species may or may not contain Fld depending on their habitats [46,63,64].

Iron utilization also posed a serious challenge to plants after land colonization, but of a different nature. Iron is the fourth most common element (at least in the Earth's crust), and the problem of iron acquisition in soil is not of paucity but of availability. The main forms of iron in soils are ferric oxides, which are sparingly soluble at neutral pH and even less in alkaline medium [65]. It is worth noting that alkaline calcareous soils represent about one-third of the planet's cultivable land [65]. In this novel scenario, it is not clear why a trait with adaptive value such as Fld was not selected. One trivial explanation would be that the flavoprotein was unable to cope with the many changes in the metabolic networks that occurred during the radiation of terrestrial plants, and could no longer be used as a backup of Fd in these organisms. The following sections describe observations that contradict this contention.

2. Expression of cyanobacterial flavodoxin complements ferredoxin deficiency in transgenic plants

As discussed previously, the constraints for binding both Fd and Fld to cognate partners are less stringent than those of typical enzyme–substrate interactions. It was therefore not entirely unexpected that purified Fld could productively interact in vitro with plant enzymes whose prokaryotic ancestors used this flavoprotein as their common or occasional substrate. They include FNR, PSI and thioredoxin reductase for $NADP^+$ and thioredoxin reduction [15], as well as cyclic electron transport by isolated plant thylakoids [66]. *Anabaena* Fld is even able to engage in electron transfer reactions with the FNR from mammalian mitochondria [67], although this reductase is structurally unrelated to plant or cyanobacterial FNR and has a different evolutionary origin.

These observations strengthened the possibility that Fld could still function in planta, and that its introduction into the plant genome could improve stress tolerance in the same way as in microorganisms. The answers to these questions are not obvious. Even if Fld were able to interact with plant redox partners in vivo, replacement of Fd in stressed or iron-starved plants could be no longer critical for the survival of the host organism. Other proteins with higher sensitivity to these environmental hardships could have appeared during the evolution of terrestrial plants, and Fld might have become dispensable precisely because compensation of Fd decline ceased to be of selective advantage. Indeed, plants face the consequences of adverse environments (including Fd down-regulation) by alternative strategies. They deploy complex responses involving hundreds of genes whose products combat the stress situation at various levels, without resorting to the substitutive strategy found in prokaryotes and algae.

In spite of this uncertain prognosis, introduction of an engineered gene encoding a plastid-targeted Fld into the nuclear genome of various model and crop plants led to stable transformants that accumulated a properly assembled, active Fld [15]. Although the gene was placed under the control of constitutive promoters in all cases, the resulting plants contained various levels of the foreign flavoprotein due to position effects during integration of the T-DNA into the host genome [12,15,68]. When grown under normal conditions, these transformed plants did not differ significantly from their wild-type (WT) siblings with respect to growth rates, biomass accumulation, flower development and seed production [12,15,68]. The highest expressing lines displayed phenotypic traits similar to those of the wild type, except for a slight increase (~20%) in chlorophyll *a* and carotenoids levels [69]. In contrast, they were able to withstand a remarkable range of environmental adversities that proved detrimental to their WT counterparts, including drought, high light intensities, heat, chilling, ultraviolet radiation and poisoning with the contact herbicide paraquat [15,68]. Furthermore, Fld expression prevented ROS-triggered localized tissue death during inoculation with a non-host pathogen [70], and allowed growth and reproduction in iron-limited soils and media [12]. Interestingly, iron-starved Fld-expressing lines accumulated low levels of iron, similar to those of the wild type, and displayed a normal response to Fe deficit, indicating that the presence of Fld did not interfere with processes involved in iron status sensing, uptake or mobilization [12]. These plants simply lived and reproduced on lower iron quotas. Finally, transformation of either plant or rizhobia with a cyanobacterial Fld gene delayed legume nodule senescence [68,71], and protected nitrogen fixation activity of nodules exposed to salt or heavy metal toxicity [68,72].

ROS accumulation, which was prominent in stressed WT plants, was significantly mitigated in the transformants [15], especially in chloroplasts [70]. Complementation of Fd functions by Fld was

demonstrated by introducing a plastid-directed Fld into tobacco plants in which Fd expression had been knocked down using RNA antisense or RNA interference techniques [41]. Fd deficiency caused growth arrest, leaf chlorosis and photosynthetic impairment [6,39–41]. Expression of Fld resulted in partial recovery of all these parameters, with nearly WT phenotypes obtained in plants accumulating less than 15% of normal Fd levels [41]. Unexpectedly, expression of a plastid-directed Fd from cyanobacteria in WT tobacco failed to increase stress tolerance. The transgenic product declined even faster than the endogenous Fd when the transformed plants were exposed to various environmental hardships [18], revealing the existence of post-transcriptional control in the expression of this protein, as initially reported by Petracek et al. [11].

For all types of stresses assayed, the effect of Fld was dose-dependent, and targeting of the product to chloroplasts was mandatory. Transgenic plants that expressed Fld in high amounts in the cytosol displayed WT levels of stress tolerance [12,15,70]. The main conclusion drawn from these studies was that Fld contributed to the welfare of stressed plants by restoring chloroplast redox homeostasis compromised by stress-dependent Fd decline. The presence of the flavoprotein prevented electron misrouting and ROS formation, and favored delivery of reducing equivalents to productive metabolic, regulatory and dissipative pathways. The amount and redox state of Fld are obviously important to accomplish these tasks, suggesting that stress tolerance could be further manipulated by increasing Fld levels and reduction rates. In nuclear-transformed plants, accumulation of Fld in chloroplasts was limited by plastid import efficiency of the chimeric Fld precursor [15], with maximal leaf contents similar to those of endogenous Fd, ~3 μmol m^{-2} [18]. Likewise, Fld reduction by the PETC is limited by electron transfer efficiency, which was about 50% lower than that of plant Fd when assayed in vitro [15]. Attempts to overcome these limitations are described in the following section.

3. Improvement of tolerance by manipulation of Fld levels and redox state

3.1. Expression of Fld from the chloroplast genome. An overdose affair

For the analysis of dose dependency, Fld may be considered as a substrate. It is therefore expected that its effects saturate above a certain threshold (attained or not at the levels obtained in the transgenic plants), or even become detrimental to plant fitness (Fig. 2A). Introduction of the Fld gene directly into the tobacco chloroplast genome by homologous recombination circumvented the limits imposed by plastid import efficiency, resulting in transplastomic plants expressing the flavoprotein at levels that were ~4-fold above those of endogenous Fd [69]. Fld could be recovered as an active protein from leaves of the transformants, but even at these high concentrations it remained unnoticed in phenotypic terms in plants grown under normal conditions [69]. When these lines were compared with WT and nuclear-transformed plants expressing various levels of the flavoprotein, contents of photosynthetic pigments and photosynthetic performance displayed a moderate increase with Fld amounts up to 2.6 μmol m^{-2}, and then decreased to WT levels [69]. Tolerance to paraquat-mediated oxidative stress also exhibited a bell-shaped response, with a significant dose-dependent increase in tolerance followed by a drop in the high-expressing line [69]. The results indicated that optimal photosynthetic performance and higher stress tolerance were observed at Fld levels comparable to those of endogenous Fd. Further increases in Fld content become detrimental to plant welfare. Therefore, the dose-dependency response of Fld expressed in plant

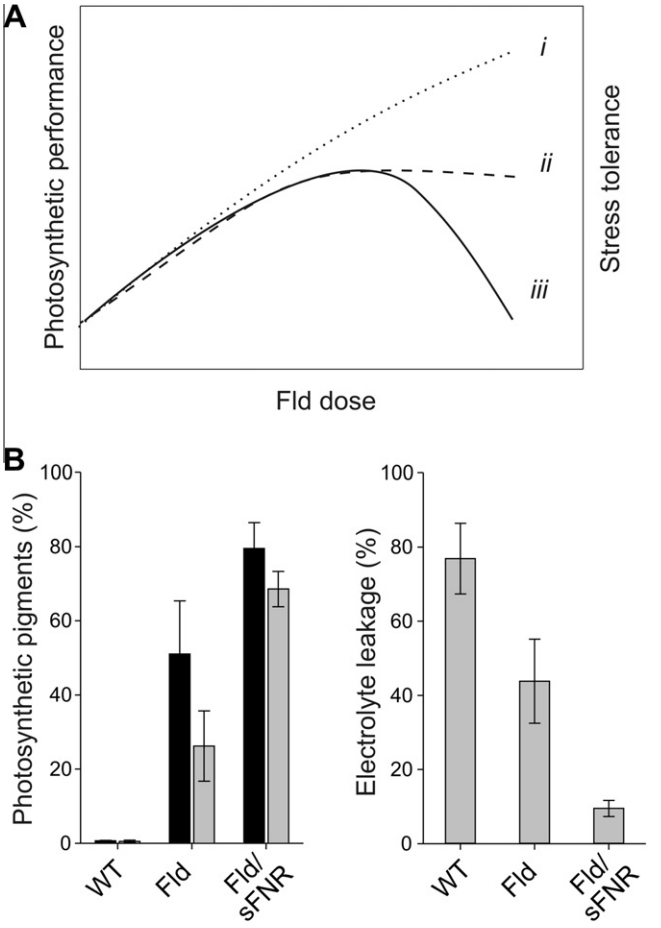

Fig. 2. Improvement of stress tolerance in transgenic tobacco plants. (A) Possible relationships between photosynthetic performance/stress tolerance and Fld doses of transgenic tobacco plants. Details of models *i* (*dotted line*), *ii* (*dashed line*) and *iii* (*solid line*) are given in the text. (B) Expression of Fld and sFNR in chloroplasts increase tolerance to paraquat toxicity. Total chlorophyll (black bars) and carotenoids (grey bars) remaining contents (left panel), and electrolyte leakage (right panel) were measured on leaf discs from WT and transgenic plants after incubation with 40 μM paraquat at 1000 μmol quanta m^{-2} s^{-1} for 7 h. Results are given as the mean ± SD of percentages of pigment content relative to discs incubated in water under the same condition, and of ion leakage relative to zero time.

chloroplasts conforms to the behavior described by curve *iii* in Fig. 2A.

It is possible that at the high levels obtained in the transplastomic lines, Fld competes with endogenous Fds for reducing equivalents generated at the PETC, and delivers them to stromal acceptors with lower efficiency. It cannot be ruled out, however, that an excess of electron acceptors at the reducing side of the PETC might introduce perturbations in the chloroplast redox homeostasis that the endogenous plastid systems are unable to compensate [73,74].

3.2. Optimization of Fld reduction in vivo. The rehab

Acceptor side limitation at the PETC (namely, Fd and $NADP^+$ shortage) is recognized as a major factor leading to runaway ROS propagation during environmental stress episodes [39–41]. The ability of Fld to bypass this blockade might be limited by the lower efficiency of this flavoprotein as electron acceptor of the PETC. Introduction of a second Fld reduction system could help to ameliorate this problem, and since NADPH build-up is another unwanted consequence of stress episodes, overexpression of FNR seems to be the logical choice to use the excess of NADPH as electron source for

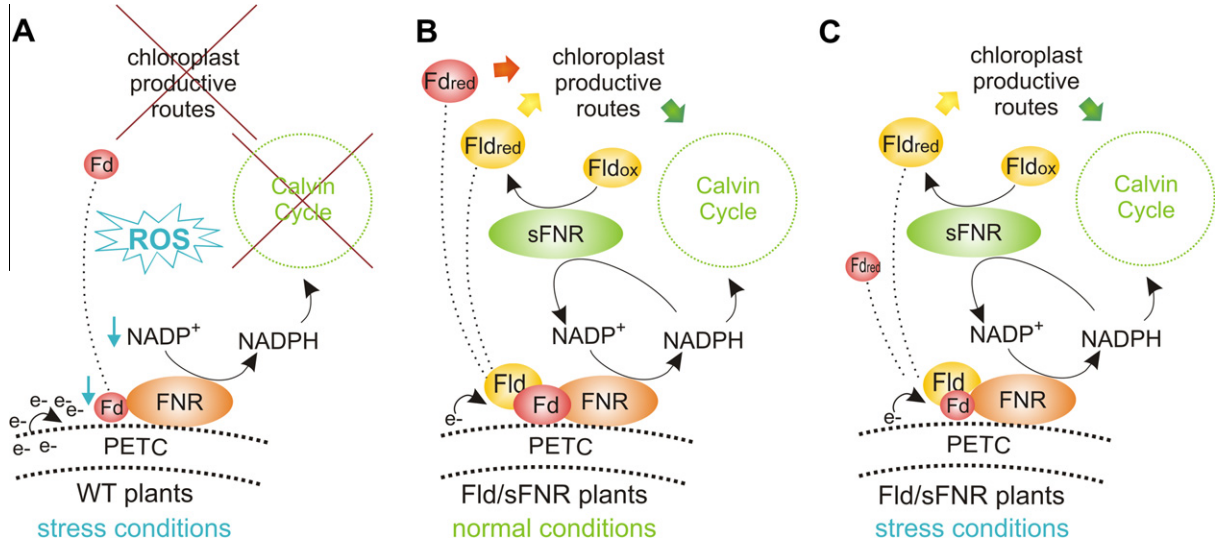

Fig. 3. Proposed model for the protective mechanism of Fld and sFNR in chloroplasts. (A) WT plants under stress conditions. (B) Fld/sFNR-expressing plants under normal growth conditions. (C) Fld/sFNR-expressing plants under stress situations. Details are given in the text; ox, oxidized; red, reduced.

Fld reduction. Plant FNRs are made up of two structural domains, bind extrinsically to the stromal side of thylakoids and are presumed to be membrane-bound when mediating $NADP^+$ photoreduction, whereas solubilized FNR catalyzes Fld reduction by NADPH with high efficiency [2,75]. Expression of a pea FNR in tobacco chloroplasts resulted in partition of the foreign flavoprotein between the thylakoids and the stroma [76]. The FNRs from *Anabaena* and related cyanobacteria contain an extra N-terminal domain, homologous to the CpcD phycobilisome linker polypeptide, which is responsible for membrane attachment through phycobilisome binding [77]. This region can be excised to abolish thylakoid interaction without affecting enzyme activity as an NADPH-dependent Fd/Fld reductase [78].

A gene encoding a plastid-directed soluble FNR (sFNR) was obtained by replacing the N-terminal domain of *Anabaena* FNR with a chloroplast-targeting transit peptide. Expression of sFNR in tobacco chloroplasts led to transgenic plants whose growth phenotypes and stress tolerance were similar to those of their WT siblings [79]. The foreign enzyme was quantitatively recovered in the leaf soluble fraction and exhibited NADPH-dependent catalytic activity. Double-transgenic lines expressing both cyanobacterial Fld and sFNR were generated by cross-fertilization of homozygous single transgenic lines. Under optimal growth conditions, they had a higher $NADP^+$/NADPH ratio, but failed to display phenotypic differences with respect to WT plants [79]. In contrast, the double transgenic lines exhibited higher tolerance to paraquat-mediated oxidative stress, as revealed by a 5-fold decrease (relative to " Fld only" siblings) in the amount of ion release due to membrane damage, and by higher levels of pigment preservation (Fig. 2B). ROS build-up was also differentially prevented in the Fld/sFNR-expressing lines [79].

These 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 stress tolerance. This system would function by recycling NADP(H) through the Fld/sFNR 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 to productive oxido-reductive pathways of the chloroplast will be favored by a continuous stream of reduced Fld. A model accounting for these observations is depicted in Fig. 3. In WT plants under stress conditions, Fd levels decline and NADPH accumulates, leading to over-reduction of the PETC and ROS build-up (Fig. 3A). In plants expressing Fld and sFNR, Fd and Fld can be reduced by both the PETC and 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 (Fig. 3B). Fld can replace declining Fd in stressed plants, restoring delivery of reducing equivalents to productive electron accepting routes in stressed double transgenic lines [4,15]. 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. 3C).

4. Conclusions and perspectives

Observations made in recent years have shed light on mechanistic and evolutionary aspects concerning the adaptive value of replacing Fd by Fld in stressed photosynthetic organisms and the structural bases for functional equivalence between the two electron shuttles. The results obtained have underscored the importance of maintaining redox homeostasis in hostile environments, and the deleterious effects resulting from its perturbation. Within this context, Fld emerged as a valuable tool to investigate the relationship between oxido-reductive processes and plant stress responses, and to probe the multiple roles played by ROS in stress-related sensing, signaling and regulation, as demonstrated in the case of biotic interactions [70]. However, a comprehensive understanding of the effects caused by Fd decline and Fld compensation is still due and will require genome-wide approaches involving extensive transcript and metabolite profiling. Introduction of a cyanobacterial Fld in plants led to transgenic lines with increased tolerance to a remarkable variety of stresses from biotic, abiotic and xenobiotic origin. These results indicate that stress-dependent Fd decline continues to be an important issue compromising plant survival, and that Fld still has adaptive value to correct this danger. Then, the reasons underlying Fld loss from the plant genome remain a mystery. One possibility is that the gene was already absent in the coastal macroalgae from which plants evolved. If iron limitation was indeed the major factor determining the value of Fld presence in the genome, selective pressure to retain this flavoprotein might have been low in the iron-rich coastal regions.

Finally, the use of the Fld gene to generate stress-tolerant crops has significant biotechnological potential and presents some unique advantages: multiple stress tolerance can be obtained with the introduction of a single gene, its prokaryotic origin precludes regulatory complications in planta, and it shows no developmental or reproductive penalties under normal growth conditions. A thorough understanding of Fld function in plants under different environmental regimes will help to design rational strategies to fully exploit this novel approach in the agricultural market.

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