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DARWIN REVIEW

The long goodbye: the rise and fall of flavodoxin during plant evolution

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Abstract

Ferredoxins are electron shuttles harbouring iron–sulfur clusters that connect multiple oxido-reductive pathways in organisms displaying different lifestyles. Some prokaryotes and algae express an isofunctional electron carrier, flavodoxin, which contains flavin mononucleotide as cofactor. Both proteins evolved in the anaerobic environment preceding the appearance of oxygenic photosynthesis. The advent of an oxygen-rich atmosphere proved detrimental to ferredoxin owing to iron limitation and oxidative damage to the iron–sulfur cluster, and many microorganisms induced flavodoxin expression to replace ferredoxin under stress conditions. Paradoxically, ferredoxin was maintained throughout the tree of life, whereas flavodoxin is absent from plants and animals. Of note is that flavodoxin expression in transgenic plants results in increased tolerance to multiple stresses and iron deficit, through mechanisms similar to those operating in microorganisms. Then, the question remains open as to why a trait that still confers plants such obvious adaptive benefits was not retained. We compare herein the properties of ferredoxin and flavodoxin, and their contrasting modes of expression in response to different environmental stimuli. Phylogenetic analyses suggest that the flavodoxin gene was already absent in the algal lineages immediately preceding land plants. Geographical distribution of phototrophs shows a bias against flavodoxin-containing organisms in iron-rich coastal/freshwater habitats. Based on these observations, we propose that plants evolved from freshwater macroalgae that already lacked flavodoxin because they thrived in an iron-rich habitat with no need to back up ferredoxin functions and therefore no selective pressure to keep the flavodoxin gene. Conversely, ferredoxin retention in the plant lineage is probably related to its higher efficiency as an electron carrier, compared with flavodoxin. Several lines of evidence supporting these contentions are presented and discussed.

Key words: Electron transfer, environmental stress, evolution, flavodoxin, ferredoxin, iron limitation.

Functional equivalence between ferredoxins and flavodoxins: how, why, and what for?

Electron shuttling as the currency for energy flow through cellular metabolism

Electron transfer reactions are the primary energy-transduction processes in all living organisms, and life on Earth most certainly began with the evolution of a limited number of metabolic routes coupled to oxido-reductive chemistry (Kim *et al.*, 2013). Reducing equivalents are ferried across networks of redox enzymes to the major metabolic sinks via a small set of diffusible electron carriers. They act as electronic switches between cellular sources of reducing power (i.e. light-driven reactions, pyridine nucleotides, sugars, etc)

Abbreviations: Fd, ferredoxin; Fld, flavodoxin; FMN, flavin mononucleotide; FNR, ferredoxin–NADP$^+$ reductase; HGT, horizontal gene transfer; PETC, photosynthetic electron transport chain; ROS, reactive oxygen species; WT, wild-type.

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and electron-consuming routes and processes. The most extensively used of these electron shuttles is ferredoxin (Fd), a small mobile metalloprotein containing a [2Fe–2S] cluster as prosthetic group (Hase *et al.*, 2006). Fds are found throughout the tree of life, with different isoforms located in mitochondria and chloroplasts of eukaryotes. In organisms displaying oxygenic photosynthesis (plants, algae, and cyanobacteria), Fd collects reducing equivalents generated during photosynthetic electron transport, and delivers them to a plethora of metabolic, regulatory, dissipative, and developmental processes. A substantial fraction of photoreduced Fd is employed for $NADP^+$ reduction in an electron-hydride exchange reaction catalysed by the flavoenzyme ferredoxin-$NADP^+$ reductase (FNR) (Carrillo and Ceccarelli, 2003; Ceccarelli *et al.*, 2004). The NADPH thus formed provides the reducing power required for the regenerative step of the Calvin cycle (i.e. conversion of 1,3-bisphosphoglycerate into glyceraldehyde 3-phosphate), and for other biosynthetic, regulatory, and protective pathways. Reduced Fd also delivers electrons for nitrogen and sulfur assimilation; amino acid, fatty acid, and secondary metabolism; antioxidant defence; reductive activation of enzymes, etc. A comprehensive list of Fd-dependent reactions and redox partners in photosynthetic organisms is shown in Table 1.

Many prokaryotes (including cyanobacteria) and some algae express an isofunctional electron carrier, flavodoxin (Fld), a soluble flavoprotein that contains flavin mononucleotide (FMN) as prosthetic group instead of an iron–sulfur centre (Sancho, 2006). Fld properties as redox shuttle largely match those of Fd, being able to replace the metalloprotein in many reactions (Table 1). The subcellular location of Fld also corresponds to that of Fd, namely, the bacterial cytosol and algal chloroplasts (La Roche *et al.*, 1996). When both carriers are present in the same genome, the Fld gene is typically induced as an adaptive resource under environmental or nutritional hardships that compromise Fd expression or activity (i.e. iron limitation). Given the key position occupied by Fd in the network of electron distribution, its decline affects vast portions of central metabolism, as well as defensive, regulatory, and developmental processes (reviewed in Zurbriggen *et al.*, 2008). Fld induction mitigates these effects, permitting survival and reproduction under the adverse condition, although the relevance of Fld replacement varies among species. In the cyanobacterium *Synechocystis* sp. PCC 6803, for instance, disruption of the *fld* gene has no fatal consequences, even under iron limitation (Kutzki *et al.*, 1998), whereas the gene encoding the main Fd isoform was found to be essential in spite of Fld induction (Poncelet *et al.*, 1998). However, several exceptions to this rule are documented in algae and cyanobacteria (see below), and a few Fld-specific pathways have been described in prokaryotes. Indeed, Fld is an essential gene in *Escherichia coli* and *Helycobacter pylori*, whereas Fd is not (Zheng *et al.*, 1999; Freigang *et al.*, 2002; Puan *et al.*, 2005).

Both Fd and Fld are able to mediate $NADP^+$ reduction as substrates of FNR (Nogués *et al.*, 2005). This reaction proceeds backwards, from NADPH to oxidized Fd/Fld, in non-photosynthetic cells and tissues (Onda *et al.*, 2000; Ceccarelli *et al.*, 2004). NADPH is the normal reductant in heterotrophic microorganisms, mitochondria, and non-photosynthetic plastids, but carbohydrates can also be used as electron donors to reduce Fd/Fld by enzymes such as the pyruvate–Fd reductase of *E. coli* (Blaschkowski *et al.*, 1982). Flds are not found in plants or animals, except as domains in larger enzymes, usually oxidoreductases, where they play specific roles different from electron shuttling (Sancho, 2006).

Comparative properties of ferredoxins and flavodoxins

Many aspects of Fd structure and function have been extensively reviewed by Hase *et al.* (2006) and by Hanke and Mulo (2013). Therefore, we will provide herein only a brief summary of Fd properties with reference to those of Fld. The reader is referred to the above-mentioned articles for a more comprehensive description of Fd biochemistry. These electron shuttles employ iron–sulfur clusters of different stoichiometry as prosthetic groups, with the photosynthetic Fds harbouring a [2Fe–2S] centre (Hase *et al.*, 2006). The irons are bridged by the two sulfur atoms and bind to the sulfide side-chains of four highly conserved cysteines (Fig. 1A). The FMN group of Fld is non-covalently bound to the apoprotein, with the isoalloxazine moiety sandwiched between the coplanar aromatic side chains of a tyrosine and a tryptophan (Fig. 1A). Fds have a Mw of ~12 kDa (Hase *et al.*, 2006), whereas Flds are slightly larger, with Mw=15–22 kDa (Sancho, 2006).

From sequence alignments and structural considerations, Flds can be divided into short-chain and long-chain classes, which differ by the presence of a 20-amino acid loop of a so far unknown function (López-Llano *et al.*, 2004). Phylogenetic analyses indicate that the two lineages have diverged once (Sancho, 2006). Only short-chain Flds have been found in Gram-positive bacteria (firmicutes), whereas cyanobacteria and algae harbour members of the long-chain class exclusively (Pérez-Dorado *et al.*, 2013). Other Gram-negative bacteria may contain Flds from both families, occasionally in the same genome. *E. coli*, for instance, has at least four genes predicted to encode Flds. Two of them (*fldA* and *fldB*) belong to the long-chain class, whereas the other two (*mioC* and *yqcA*) encode short-chain Flds (Birch *et al.*, 2000). Only members of the long-chain Fld class have been associated with stress protection (Zheng *et al.*, 1999; Sancho, 2006; Moyano *et al.*, 2014).

Both the [2Fe–2S] cluster of Fd and the flavin group of Fld can in principle exchange one or two electrons. However, empirical evidence indicates that they behave as obligatory one-electron carriers, switching between the $Fe^{+3}.Fe^{+3}/Fe^{+3}.Fe^{+2}$ (Bott, 1999) and the semiquinone/hydroquinone states, respectively (Nogués *et al.*, 2005). These transitions have similar redox potentials (–400 to –430 mV for the photosynthetic isoforms), which allow the two proteins to behave as low potential electron shuttles.

Photosynthetic Fd has been shown to be more efficient than Fld in most reactions assayed *in vitro*, including $NADP^+$ photoreduction by isolated thylakoids and thioredoxin reduction by Fd-thioredoxin reductase in reconstituted systems (Tognetti *et al.*, 2006), although exceptions to this tendency have been reported. Electron donation to the NiFe-hydrogenase from *Synechocystis* sp. PCC 6803 proceeds at

Table 1. *List of Fd- and Fld-dependent reactions and redox partners in plastids and cyanobacteria*

Protein partner	Function	Metabolic pathway	Organisms	References
Ferredoxin				
Photosystem I (PSI)	Photosynthetic electron transport	Photosynthesis	Cyanobacteria	Meimberg and Mühlenhoff (1999); Sétif (2001)
			Algae	Sétif (2001)
			Plants	Sétif (2001)
PGR5, FNR, and PSI	Cyclic electron flow	Photosynthesis	Cyanobacteria	Yeremenko *et al.* (2005)
			Algae	Hanke and Mulo (2013)
			Plants	Hanke and Mulo (2013)
Ferredoxin–$NADP^+$ reductase (FNR)	$NADP^+$ reduction	Photosynthesis	Cyanobacteria	Hase *et al.* (2006); Hanke and Mulo (2013)
			Algae	Peden *et al.* (2013)
			Plants	Hase *et al.* (2006); Hanke and Mulo (2013)
	NADPH oxidation	Heterotrophic pathways	Apicomplexa	Ceccarelli *et al.* (2004)
			Plants	Onda *et al.* (2000)
Nitrogenase	N_2 fixation	Nitrogen assimilation	Cyanobacteria	Masepohl *et al.* (1997)
Nitrate reductase	Reduction of NO_3^- to NO_2^-	Nitrogen assimilation	Cyanobacteria	Hase *et al.* (2006); Hanke and Mulo (2013)
Nitrite reductase	Reduction of NO_2^- to NH_4^+	Nitrogen assimilation	Cyanobacteria	Hase *et al.* (2006); Hanke and Mulo (2013)
			Algae	Vigara *et al.* (1998); Terauchi *et al.* (2009)
			Plants	Hanke and Mulo (2013)
Glutamine synthetase	Combination of NH_4^+ with glutamate to form glutamine	Nitrogen assimilation	Algae	Vigara *et al.* (1998)
Glutamate-oxoglutarate amino transferase (GOGAT)	Glutamate synthesis	Amino acid synthesis	Cyanobacteria	Hase *et al.* (2006)
			Algae	Hase *et al.* (2006)
			Plants	Hase *et al.* (2006)
Hydrogenase	H_2 formation/H_2 oxidation	Hydrogen metabolism	Cyanobacteria	Gutekunst *et al.* (2014)
			Algae	Peden *et al.* (2013)
Sulfite reductase (SiR)	Reduction of SO_3^{2-} to H_2S	Sulfur assimilation	Plants	Hase *et al.* (2006); Hanke and Mulo (2013)
Ferredoxin–thioredoxin reductase (FTR)	Thioredoxin (Trx) reduction	Redox regulation$^+$	Cyanobacteria	Hase *et al.* (2006); Hanke and Mulo (2013)
			Algae	Hase *et al.* (2006)
			Plants	Hase *et al.* (2006); Hanke and Mulo (2013)
Fatty acid desaturase (FAD)	Double bond formation in fatty acids	Lipid metabolism	Cyanobacteria	Hanke and Mulo (2013)
			Algae	Peden *et al.* (2013)
			Plants	Hanke and Mulo (2013)
Monodehydroascorbate reductase (MDHAR)	Ascorbate regeneration	Antioxidant defence	Plants	Hanke and Mulo (2013)
Heme oxygenase and phytochromobilin synthase	Phytochromobilin* synthesis	Development	Plants	Hanke and Mulo (2013)
Heme oxygenase and ferredoxin-dependent bilin reductases	Bilin pigment synthesis	Development	Cyanobacteria	Hanke and Mulo (2013)
Flavodoxin				
PSI	Photosynthetic electron transport	Photosynthesis	Cyanobacteria	Meimberg and Mühlenhoff (1999); Sétif (2001)
			Algae	Peleato *et al.* (1994)
FNR	$NADP^+$ reduction	Photosynthesis	Cyanobacteria	Sancho (2006)
			Algae	Peleato *et al.* (1994)
FNR and PSI	Cyclic electron flow	Photosynthesis	Cyanobacteria	Hagemann *et al.* (1999)
Nitrite reductase	Reduction of NO_2^- to NH_4^+	Nitrogen assimilation	Algae	Vigara *et al.* (1998)
Glutamine synthetase	Combination of NH_4^+ with glutamate to form glutamine	Nitrogen assimilation	Algae	Vigara *et al.* (1998)
Hydrogenase	H_2 formation/H_2 oxidation	Hydrogen metabolism	Cyanobacteria	Gutekunst *et al.* (2014)

$^+$Reduced Trx activates a number of chloroplast enzymes of the Calvin cycle, the malate valve, lipid and starch metabolism, translation, and antioxidant defence.
*Plant chromophore of the light sensor phytochrome.

a higher rate with the endogenous Fld, compared with one of the minor *Synechocystis* Fd isoforms or the main leaf Fd from spinach (Gutekunst *et al.*, 2014). In most studies, however, Fd proved to be the preferred redox partner. For instance, Fd photoreduction by PSI from *Synechococcus* sp. PCC 7002 showed a strong preference over Fld (Meimberg and Mühlenhoff, 1999), and the Fd from the green alga *Chlorella fusca* was more efficient than Fld as electron donor to nitrite reductase and glutamine synthetase, two enzymes involved in nitrogen assimilation (Vigara *et al.*, 1998).

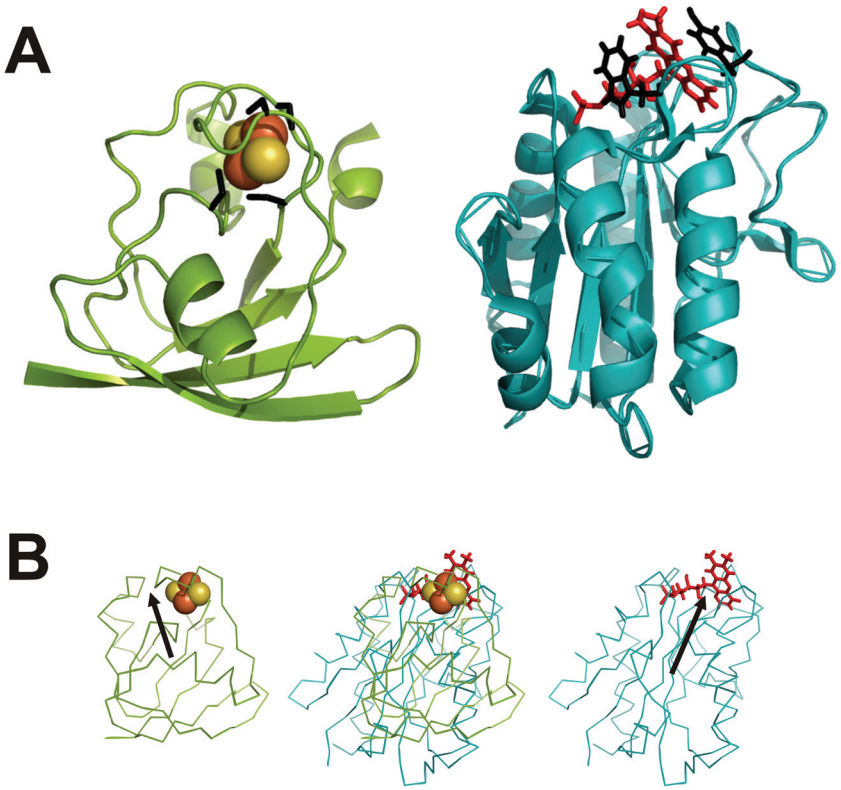

Fig. 1. Comparative structures of *Anabaena* ferredoxin and flavodoxin. (A) Ribbon diagrams of Fd (left) and Fld (right) showing interactions of the prosthetic groups at the active sites. The Fe–S cluster of Fd is represented as red-orange balls, and the FMN of Fld as red sticks. The side chains of the binding cysteines of Fd and of the stacking aromatic amino acids of Fld are coloured as black sticks. (B) Fd and Fld can be compared on the basis of their surface electrostatic potentials. The left and right panels show the *Anabaena* Fd and Fld, respectively, as Cα-traces. Prosthetic groups are highlighted. The two proteins are aligned according to the procedure derived by Ullmann *et al.* (2000). In the central picture, the structures are superimposed, showing co-localization of the redox centres. For clarity reasons, the vectors corresponding to the dipolar moments (black arrows) are drawn from positive to negative, opposite to conventional representations.

FNR-mediated reactions are the best characterized at the kinetic level, revealing some interesting features. The k_{cat}/K_M value of *Anabaena* Fd was reported to be ~25-fold higher than that of Fld (Medina *et al.*, 1998). The strength of the interaction with *Anabaena* FNR, as reflected by the Michaelis constants, was similar for both carriers, indicating that the gain in efficiency was at the expense of electron transfer rates (Medina *et al.*, 1998). It is likely that the complex electronic configuration of the flavin is not flexible enough to attain the high electron transfer rates typical of transition metals. Iron ions contain incompletely filled *d* orbitals which can readily accept electrons from different partners with various geometries, making them particularly versatile in oxido-reductive processes. It is indeed remarkable that some of these electron transfer reactions can be mimicked by the particular arrangement of π-orbitals found in the isoalloxazine ring system.

Functional equivalence without sequence and structural conservation

Fd and Fld do not share any structural similarity, and yet they can engage in essentially the same oxido-reductive reactions. The key to this apparent paradox resides in the very function of these proteins. One of the most desirable features of an electron shuttle is promiscuity, namely, the ability to exchange reducing equivalents with different redox partners. Accordingly, Fd and Fld have been tailored by evolution to be loosely selective in their interactions. Analysis of plant Fd binding sites in various Fd-dependent enzymes revealed no obvious homology (Hase *et al.*, 2006; Hanke and Mulo, 2013). Then, docking of Fd (and Fld) must be determined by general features of the active sites rather than contacts with specific conserved amino acids. The prosthetic groups of both proteins are eccentric and surrounded by patches of negatively charged residues, whereas their enzyme partners harbour a crown of positively charged amino acids around their exposed cofactors (Hase *et al.*, 2006; Hanke and Mulo, 2013). Initial interactions are steered by electrostatic attractions that help to stabilize the binary complexes, and serve to position the corresponding prosthetic groups at the proper distance and geometry to allow direct outer-sphere electron transfer between them (Kurisu *et al.*, 2001). Water exclusion from the hydrophobic area between the two proteins further strengthens binding (Kurisu *et al.*, 2001; Hanke and Mulo, 2013). The charged regions in Fd and Fld are remarkably insensitive to mutations, and different spatial arrangements of the two proteins at the FNR binding site (i.e. rotations) are allowed without losing the ability for electron transfer (Gómez-Moreno *et al.*, 1994; Goñi *et al.*, 2009).

Although Fd and Fld differ in virtually all structural features, they can be aligned on the basis of their Coulomb electrostatic potentials. Applying the Hodgkin index to evaluate their similarity in this sense, Ullmann *et al.* (2000) obtained a significant overlapping. The cofactors, rather than their centres of mass, coincided in the alignments (Fig. 1B). Both proteins have strong dipolar moments (380–700 Debyes), with the vectors of the negative dipoles pointing toward the active site regions (Fig. 1B). These considerations provide a rationale to understand why Fd and Fld are able to interact with so many different enzymes, and why they can be exchanged as substrates of a given partner without major loss in efficiency.

Ferredoxins and flavodoxins in plants, algae, and cyanobacteria

Ferredoxin accumulation in response to different environmental stimuli

The paralogy of Fd-coding genes varies widely among species (Table 2). Most cyanobacteria contain three or more isoforms, including highly divergent Fds of unknown function such as FdC1 (Poncelet *et al.*, 1998; Voss *et al.*, 2011). Among eukaryotes, glaucophytes and algae from the red-plastid lineage usually have a single Fd, whereas green algae and land plants contain numerous isoforms (Table 2). Six different Fd variants have been described in the unicellular green alga *Chlamydomonas reinhardtii*, a freshwater chlorophyte (Peden *et al.*, 2013), in the C3 plant *Arabidopsis thaliana*, with isoforms located in leaves and roots (Hanke *et al.*, 2004; Voss *et al.*, 2011), and in the C_4 plant maize, present in root plastids and in mesophyll and bundle sheath chloroplasts (Matsumura *et al.*, 1997; Sakakibara, 2003; Cheng *et al.*, 2008). Additional putative Fds have been retrieved from some sequenced genomes (i.e. maize), but they have not been functionally characterized. In general, the main Fd isoform engaged in photosynthetic electron transport accounts for 80–90% of the total Fd pool in leaves.

Tissue specificity broadly correlates with labour division and expression patterns. In the case of leaf Fd isoforms, *cis*-acting elements located upstream of the transcription initiation site provide for moderate light responsiveness (Vorst *et al.*, 1993). However, replacement of these regions by a constitutive promoter does not abolish light induction (Elliott *et al.*, 1989),

Table 2. *Phylogenetic distribution and copy number variation of long-chain Flds and cyanobacterial-/plastidic-type Fds*

Domain of life				Long-chain Fld	Cyanobacterial-/plastidic-like Fd*
Eukarya	Archaeplastida	Glaucophytes		0	1
		Red algae		0–1	1
		Chlorophytes		0–2	3–6
		Streptophytes (charophytes + land plants)		0	5–8
	Protists	Alveolates	Photosynthetic	1	1
			Non-photosynthetic	0	0–1
		Rhizaria	Photosynthetic	1	1–3
			Non-photosynthetic	0	0
		Stramenopiles	Photosynthetic	0–2	1–4
			Non-photosynthetic	0	0
		Haptophytes		1	4
		Cryptomonads		0–1	1
		Euglenids		NA	1
	Animals			0	0
	Fungi			0	0
Archaea				0–1#	0–6
Bacteria	Cyanobacteria			0–5	2–8
	Proteobacteria			0–2	0–2
	Firmicutes			0	0
	Actinobacteria			0	0–2
	Bacteroidetes			0–4	0
	Chlorobi			0–2	0
	Chlamydiae			0	0
	Spirochaetes			0–1	0
	Tenericutes			0–1	0
	Fusobacteria			0–3	0
	Chloroflexi			0–1	0
	Acidobacteria			0–1	0

Sources: Integrated Microbial Genomes (IMG) dababase (http://img.jgi.doe.gov; Markowitz *et al.* 2012) and National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).
*The presence of Fd versions with sequence similarity to proteobacterial-/mitochondrial-like Fds is not indicated.
#Long-chain Fld forms found in 3 out of 456 sequenced genomes.
NA, no genomic sequence available

indicating that Fd expression depends on sequences located in the transcribed portion of the gene and is therefore subject to post-transcriptional regulation. Using chimeric constructs of transcribed and flanking sequences of the Fd gene fused to various reporter genes, Dickey *et al.* (1992) could identify a region of light responsiveness within the transcription unit itself, extending both up- and downstream of the translation initiation codon. This mode of regulation seems to be an evolutionary novelty, as the vegetative Fd gene of cyanobacteria is light-modulated exclusively through sequences located in the 5′-untranscribed region (Mazouni *et al.*, 2003).

Light also stimulates expression of some Fd isoforms in algae (Lemaire *et al.*, 1999; Whitney *et al.*, 2011). In the diatom *Thalassiosira pseudonana*, Fd expression is coordinated with a diel cycle, reaching its maximum transcription levels during the light period. However, this oscillating expression pattern is eliminated under continuous light, indicating that it is not under circadian control (Whitney *et al.*, 2011). In contrast, the expression of the photosynthetic Fd in *C. reinhartii* is regulated by the circadian cycle, as well as by the redox state of the photosynthetic electron transport chain (PETC) (Lemaire *et al.*, 1999).

Non-photosynthetic Fds participate in the assimilation of nitrate as electron donors for nitrite reductase (Terauchi *et al.*, 2009; Peden *et al.*, 2013). Several components of this metabolic pathway are induced in roots when plants are grown on oxidized nitrogen sources (i.e. nitrate), and they are repressed by ammonia (Matsumura *et al.*, 1997; Patterson *et al.*, 2010). Two Fd isoforms are present in maize root plastids; one of them is expressed constitutively (FdIII), whereas the other (FdVI) responds to nitrate (Matsumura *et al.*, 1997). FdVI is also nitrate-induced in mesophyll chloroplasts (Sakakibara, 2003). It is unclear whether this Fd is co-expressed with the photosynthetic isoform in the same cells and chloroplasts, or if it is present in heterotrophic cells of the leaf (Hanke and Mulo, 2013). Some of the non-photosynthetic isoforms have less negative midpoint redox potentials compared with photosynthetic Fds (Hanke *et al.*, 2004; Gou *et al.*, 2006), indicating that they may have a narrower range of suitable electron partners (Peden *et al.*, 2013).

Each of the six Fds of *C. reinhardtii* harbours a chloroplast-targeting sequence. Expression of the various isoforms responds to different environmental inputs, correlating with their functions. The most abundant among them, the photosynthetic Fd encoded by the *petF* gene, is induced by light (Terauchi *et al.*, 2009). Its expression is not affected by nitrate, oxidative stress, anoxia, or copper deprivation. One of the minor non-photosynthetic isoforms, Fdx2, is induced by nitrate and H_2O_2 and is a preferred substrate for nitrite reductase, whereas PetF Fd preferentially interacts with FNR and Fd-thioredoxin reductase (Terauchi *et al.*, 2009; Peden *et al.*, 2013). The results suggest that labour division and differential expression of Fd variants preceded tissue differentiation and the advent of land plants. Interestingly enough, *C. reinhardtii* lacks Fld (Merchant *et al.*, 2007).

Dinitrogen fixation in microorganisms is an anaerobic process because nitrogenase is very sensitive to oxygen inactivation (Schrautemeier *et al.*, 1995). Nitrogen-fixing cyanobacteria usually express one or more Fd isoforms (generically named FdxH) as dedicated electron donors for the nitrogenase, in addition to the major Fd variants that mediate photosynthesis. Expression of these "diazotrophic" Fds is regulated by different mechanisms depending on the morphological and physiological strategy for dinitrogen fixation adopted by the host. In those cyanobacteria in which heterocysts provide the anaerobic environment, such as *Anabaena* sp. PCC 7120, an Fd isoform specific for these differentiated cells is expressed under dinitrogen-fixing conditions (Masepohl *et al.*, 1997). In contrast, in the non-heterocystous, filamentous cyanobacterium *Plectonema boryanum*, which fixes nitrogen only in low oxygen environments, the single FdxH variant accumulates under microaerobic conditions (Schrautemeier *et al.*, 1994). The two types of FdxH isoforms and regulatory mechanisms can be present in the same organism, as it occurs in heterocyst-forming *Anabaena variabilis* (Schrautemeier *et al.*, 1995). Some non-heterocystous cyanobacteria do not need a microaerobic environment because they temporally separate photosynthesis and dinitrogen fixation, so that the latter process is carried out during the night. Examples of this strategy are *Cyanothece* sp. ATCC 51142 and *Crocosphaera watsonii* WH 8501, where an Fd isoform is expressed during the dark period (Stöckel *et al.*, 2008; Shi *et al.*, 2010). All these "diazotrophic" Fds are coded by genes located in *nif* operons (Schrautemeier *et al.*, 1994, 1995; Welsh *et al.*, 2008; Shi *et al.*, 2010).

Down-regulation of ferredoxin levels by iron deficit and environmental stress

Fd expression is universally repressed under conditions of iron limitation, as revealed by various experimental approaches, including genome-wide microarray analyses in both plants (Thimm *et al.*, 2001) and cyanobacteria (Singh *et al.*, 2003; Thompson *et al.*, 2011). The six Fd isoforms of *C. reinhardtii* also decline under iron starvation, even though transcription of at least two of the non-photosynthetic isoforms (Fdx3 and Fdx6) was stimulated under these conditions (Terauchi *et al.*, 2009). The transcriptional control of genes related to iron metabolism is carried out by Fur (ferric uptake regulator) in most prokaryotes, but Fd repression in iron-starved cyanobacteria is independent of this mechanism (Ghassemian and Straus, 1996). Instead, it has been reported that iron levels control the stability of Fd mRNA in *Synechococcus* sp. PCC 7942 and *Anabaena* sp. PCC 7937 (Bovy *et al.*, 1993a). *Cis*-acting elements located at the 5′ extreme of the mRNA are involved in this regulation (Bovy *et al.*, 1993b).

Although the main value of Fld under iron limitation is certainly the taking over of Fd functions as the major electron-distributing hub in chloroplasts and cyanobacteria (Table 1), it is conceivable that this substitution might also contribute to plant welfare by permitting reallocation of the scarce available iron to other metal-dependent routes. The question is then how substantial this contribution could be, and the response differs depending on the organism and/or tissue. In Fe-replete *Thalassiosira weissflogii*, 30–40% of the cellular iron was found to be associated with Fd (Erdner and Anderson, 1999). As a complete PETC has 22 Fe atoms, this would represent

a stoichiometry of 4–5 Fd molecules per chain unit, in fairly good agreement with experimental determinations (Böhme, 1978). The iron share of Fd seems to be lower but still significant in plants (Terry and Abadía, 1986; Shikanai *et al.*, 2013). Stromal iron represents 20–30% of total leaf Fe, with Fd and ferritin as the most abundant metalloproteins (Terry and Abadía, 1986). Interestingly, when iron is withheld from plants, the stromal fraction declines much faster than the thylakoid or extrachloroplastic fractions (Terry and Low, 1982), confirming the high sensitivity of Fd to iron limitation. On the other hand, heterotrophic Fd isoforms account for less than 10% of their leaf counterparts (Voss *et al.*, 2008), indicating that they play a marginal role in cell iron homeostasis.

Oxidative stress and adverse environmental situations (salinity, extreme temperatures, water deficit) lead to down-regulation of Fd levels in both plants and cyanobacteria (Mazouni *et al.*, 2003; Singh *et al.*, 2003; Zimmermann *et al.*, 2004; Tognetti *et al.*, 2006; Terauchi *et al.*, 2009; Ceccoli *et al.*, 2011). Algal Fd isoforms also declined when *C. reinhardtii* cells were exposed to H_2O_2 (Terauchi *et al.*, 2009). Transcription of the gene encoding non-photosynthetic Fdx2 increased under oxidative conditions, but this induction was not reflected at the protein level. Moreover, when *C. reinhardtii* was grown in the presence of nitrate to obtain maximal Fdx2 accumulation, the contents of this isoform still declined after H_2O_2 treatment (Terauchi *et al.*, 2009).

Expression of *Anabaena* Fd in tobacco plants under control of a constitutive promoter resulted in the loss of light responsiveness. In contrast, when these transgenic plants were exposed to various stress conditions, the cyanobacterial Fd was down-regulated even faster than the endogenous counterparts (Ceccoli *et al.*, 2012). The results suggest that: (i) repression of the Fd gene by adverse environmental situations also has post-transcriptional components, and (ii) light- and stress-dependent regulation of Fd expression involve different responsive elements (Ceccoli *et al.*, 2012).

Then, data collected from a number of systems indicate that when plants are exposed to situations in which the Fd holoprotein cannot assemble (i.e. iron starvation) or is likely to be destroyed (i.e. oxidative stress), they respond by down-regulating accumulation of the corresponding transcripts. Such a response might seem odd, considering that Fd activity helps to relieve the electron pressure on the PETC and contributes to the antioxidant defence by providing reducing power for dissipative and scavenging processes. However, adoption of this pre-emptive strategy might respond to different imperatives; for instance, to save the energetic cost of synthesizing an apoprotein that cannot be used anyway. An additional advantage in the case of oxidative conditions is that formation of the Fe–S cluster would be prevented by lack of the Fd apoprotein, therefore eliminating a potential target for oxidants that upon destruction could lead to release of free iron and propagation of deadly hydroxyl radicals via Fenton-type reactions (see below). Besides these somehow obvious benefits, recent observations suggest that decline of Fd levels might play yet unknown roles in tolerance to certain environmental onslaughts. Mutant *Arabidopsis* lines deficient in the major photosynthetic Fd were, as expected, hypersensitive to a short-term treatment with high light (Liu *et al.*, 2013). However, these plants exhibited unpredicted tolerance to prolonged high light exposure compared with WT siblings, apparently profiting from a differential increase in PGR5-dependent cyclic electron flow (Liu *et al.*, 2013). Although it remains to be determined whether these effects could be extended to other stress situations, the results suggest that down-regulation of at least some Fd isoforms might contribute to long-term plant acclimation to adverse environments.

Flavodoxin and ferredoxin expressions display contrasting responses to environmental inputs

In most algal and cyanobacterial species, Fld is not expressed under iron-replete conditions. This restriction has limited studies on the light responsiveness of this protein, which is clearly involved in photosynthesis when present. The only exception is the *fld* gene from the cyanobacterium *Prochlorococcus marinus* MED4, which has been shown to be up-regulated under daylight as a typical photosynthetic gene (Zinser *et al.*, 2009). The mechanism of this diel mode of expression is unknown.

In general, the response of Fld expression to environmental stimuli follows a pattern opposite to that exhibited by Fd. In *E. coli* and other enterobacteria, the *fldA* and *fldB* genes are members of the *soxRS* regulon (Gaudu and Weiss, 2000), which orchestrates the defence of the bacterial cell against oxidants and redox-cyclic compounds (Giró *et al.*, 2006). The global trancriptional regulator Fur also belongs to this regulatory system (Zheng *et al.*, 1999), and its gene is located immediately downstream of that encoding FldA. The SoxS transcription factor binds to the promoter of the *fldA* gene, leading to the expression of a bicistronic mRNA that encompasses both *fldA* and *fur* (Zheng *et al.*, 1999). No SoxR/SoxS homologues have been identified in cyanobacteria and the regulator sustaining the superoxide response is still unclear in these phototrophs (Latifi *et al.*, 2009). On the other hand, the *fld* gene is part of the Fur regulon in cyanobacteria (González *et al.*, 2014), and FurA is also induced by oxidative stress (López-Gomollón *et al.*, 2009). In fact, Fld induction has been described in cyanobacteria under osmotic (Hagemann *et al.*, 1999; Rai *et al.*, 2014), heat (Kojima *et al.*, 2006), high light (Havaux *et al.*, 2005), and oxidants (Singh *et al.*, 2004), all conditions that lead to Fd down-regulation.

Iron deficit seems to be the most critical imperative that determines Fld adaptive value (Erdner *et al.*, 1999). However, iron responsiveness is not a universal feature of Fld genes. Although most cyanobacteria do respond to iron deficiency by inducing Fld (Singh *et al.*, 2003; Chappell and Webb, 2010), expression of certain Fld isoforms proved insensitive to this nutritional stress. Expression patterns correlate with phylogenetic positions, permitting allocation of cyanobacterial Flds to two different groups. One of them consists of those Flds whose expression is enhanced by iron deficiency (Singh *et al.*, 2003; Chappell and Webb, 2010). They form a highly supported clade in Fld phylogenetic trees (Lin *et al.*, 2009). The other group of Flds do not respond to iron deficiency and are only present in some diazotrophic cyanobacteria. They

cluster with the Flds of non-photosynthetic diazotrophic proteobacteria (Lin *et al.*, 2009), suggesting that they were transferred from proteobacteria to cyanobacteria by horizontal gene transfer (HGT).

As indicated previously, several diazotrophic cyanobacteria fix nitrogen in the dark, temporally separating photosynthesis and nitrogen fixation (Compaoré and Stal, 2010). In these organisms, the iron-unresponsive Flds have been observed to increase their abundance during the dark period, similar to the nitrogenase subunits and the "diazotrophic" Fd isoforms (Stöckel *et al.*, 2008; Shi *et al.*, 2010; Saito *et al.*, 2011), suggesting that these Flds might act as electron donors to nitrogenase. The use of Fld in nitrogen fixation has been proposed as part of a general metabolic strategy to spare iron for the iron-rich nitrogenase complex (Saito *et al.*, 2011).

Lack of iron responsiveness has also been observed in eukaryotes. In most algal species containing Fld, the gene is present as a single iron-responsive copy (Li *et al.*, 2004). Exceptions are some diatoms with two homologous genes, only one of them regulated by iron availability (Whitney *et al.*, 2011). A palindromic motif has been identified in the promoter region of low-iron regulated genes, including those coding for the iron-responsive Fld isoforms (Lommer *et al.*, 2012). The role of these *cis*-acting elements in iron-dependent gene regulation was confirmed by the promoter truncation technique in *Phaeodactylum tricornutum* (Yoshinaga *et al.*, 2014). On the other hand, iron-insensitive Fld isoforms display diel periodicities, reaching the highest transcript abundances during the dark period (Whitney *et al.*, 2011). The functions and redox partners of these "nocturnal" Flds are unknown.

The role of iron limitation in the spread and disappearance of Fld from the *Viridiplantae* kingdom will be discussed in a broader context in a forthcoming section.

The limits of flavodoxin distribution

Fds are found in a wide range of organisms pervading all kingdoms. They include cyanobacteria and α-proteobacteria, the types of organisms that gave origin to modern day chloroplasts and mitochondria (Table 2).

In the case of Flds, as already indicated, they are absent from plants and animals, except for the "enslaved" Fld-like domains of complex enzymes (Sancho, 2006). Despite Fld presence in α-proteobacteria, they are not found in mitochondria, suggesting that the original endosymbiont already lacked this gene, or that it was lost very early after integration. The situation in chloroplasts is different. Fld is present in plastids from most major algal taxa (Table 2), including red and green algae, as well as those groups resulting from secondary endosymbiotic events such as dinoflagellates, haptophytes, diatoms, cryptophytes, and chlorarachniophytes (Fukuyama *et al.*, 1990; Erdner *et al.*, 1999; Inda and Peleato, 2002; Li *et al.*, 2004; Jaeckisch *et al.*, 2011; Whitney *et al.*, 2011; Curtis *et al.*, 2012; Read *et al.*, 2013). In contrast, it has not been found in streptophytes, which consist of land plants (embryophytes) and charophytes, a subgroup of freshwater green algae that represent the sister lineage of land plants (www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html; Timme *et al.*, 2012). This distribution suggests that the gene was lost somewhere in the transition between green algae and terrestrial plants.

Transgenic expression of flavodoxin in plants: and yet it works

In vitro experiments showed that cyanobacterial Flds were able to act as substrates for the plant descendants of many Fld-dependent prokaryotic enzymes (Scheller, 1996; Nogués *et al.*, 2004; Tognetti *et al.*, 2006). *Anabaena* Fld can even exchange electrons with the FNR from mammalian mitochondria (Zöllner *et al.*, 2004), although this reductase is structurally unrelated to plant or cyanobacterial FNRs and has a different evolutionary origin. Taking into account the loose requirements for productive binding of Fld and Fd to diverse redox partners, these results were not entirely unexpected. Then, both biochemical predictions and experimental observations suggested that Fld could function *in planta*. Confirmation of this hypothesis was obtained by introducing a cyanobacterial Fld gene into model and crop species (Tognetti *et al.*, 2006; Zurbriggen *et al.*, 2010). The transgenic product was targeted to chloroplasts and assembled there with FMN to yield a functional Fld holoprotein (Tognetti *et al.*, 2006).

Still, the possible effects of Fld presence in plant chloroplasts were not obvious. Plants have largely lost the substitutive strategies found in prokaryotes and algae, and respond to adverse environments by deploying complex defence systems involving many genes whose products combat the stress situation at various levels, such as increased repair and scavenging activities, metabolic reprogramming, and optimization of iron uptake (Zurbriggen *et al.*, 2008, 2010; Foyer and Shigeoka, 2011; Kobayashi and Nishizawa, 2012; Munné-Bosch *et al.*, 2013). The existence of these alternative strategies might simply indicate that replacement of Fd in stressed or iron-starved plants has no further adaptive value because other stress-sensitive proteins more critical to plant survival could have appeared along the evolutionary pathway that led to terrestrial plants.

Experimental evaluation of these possibilities revealed, however, that transgenic plants expressing a plastid-targeted Fld displayed increased tolerance, relative to their wild-type (WT) siblings, to multiple environmental adversities such as drought, high light intensities, heat, chilling, ultraviolet radiation, and poisoning with the contact herbicide paraquat (Tognetti *et al.*, 2006; Coba de la Peña *et al.*, 2010). Accumulation of reactive oxygen species (ROS) such as hydrogen or organic peroxides and the superoxide radical, which was prominent in stressed WT plants, was significantly mitigated in the Fld transformants (Tognetti *et al.*, 2006). 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 silencing (Blanco *et al.*, 2011). Fd deficiency caused growth arrest, leaf chlorosis, and photosynthetic impairment (Holtgrefe *et al.*, 2003; Hanke and Hase,

2008; Voss *et al.*, 2008; Blanco *et al.*, 2011). Expression of Fld resulted in partial recovery of all these parameters, with nearly WT phenotypes obtained in lines accumulating less than 15% of normal Fd levels (Blanco *et al.*, 2011). Photosynthesis and life were largely based on Fld in these plants.

Fld expression also prevented ROS-triggered localized cell death following inoculation of a non-host pathogen (Zurbriggen *et al.*, 2009), and allowed growth and reproduction in iron-limited soils and media (Tognetti *et al.*, 2007). Interestingly, iron-starved Fld-expressing lines accumulated WT (low) iron levels, 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 (Tognetti *et al.*, 2007). 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 (Redondo *et al.*, 2009; Coba de la Peña *et al.*, 2010), and protected nitrogen fixation activity of nodules exposed to salt or heavy metal toxicity (Coba de la Peña *et al.*, 2010; Shvaleva *et al.*, 2010).

For all types of stresses assayed, the effect of Fld was dose-dependent, saturated at a certain concentration threshold, and then became detrimental to plant fitness (Ceccoli *et al.*, 2012). Also, chloroplast location of the expressed product was mandatory. Transgenic plants that accumulated high Fld amounts in the cytosol displayed WT levels of stress tolerance (Tognetti *et al.*, 2006, 2007; Redondo *et al.*, 2009). 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 favoured delivery of reducing equivalents to productive metabolic, regulatory and dissipative pathways (Zurbriggen *et al.*, 2008). A model summarizing these findings is depicted in Fig. 2. The importance of Fld activity to bypass acceptor side limitation at the

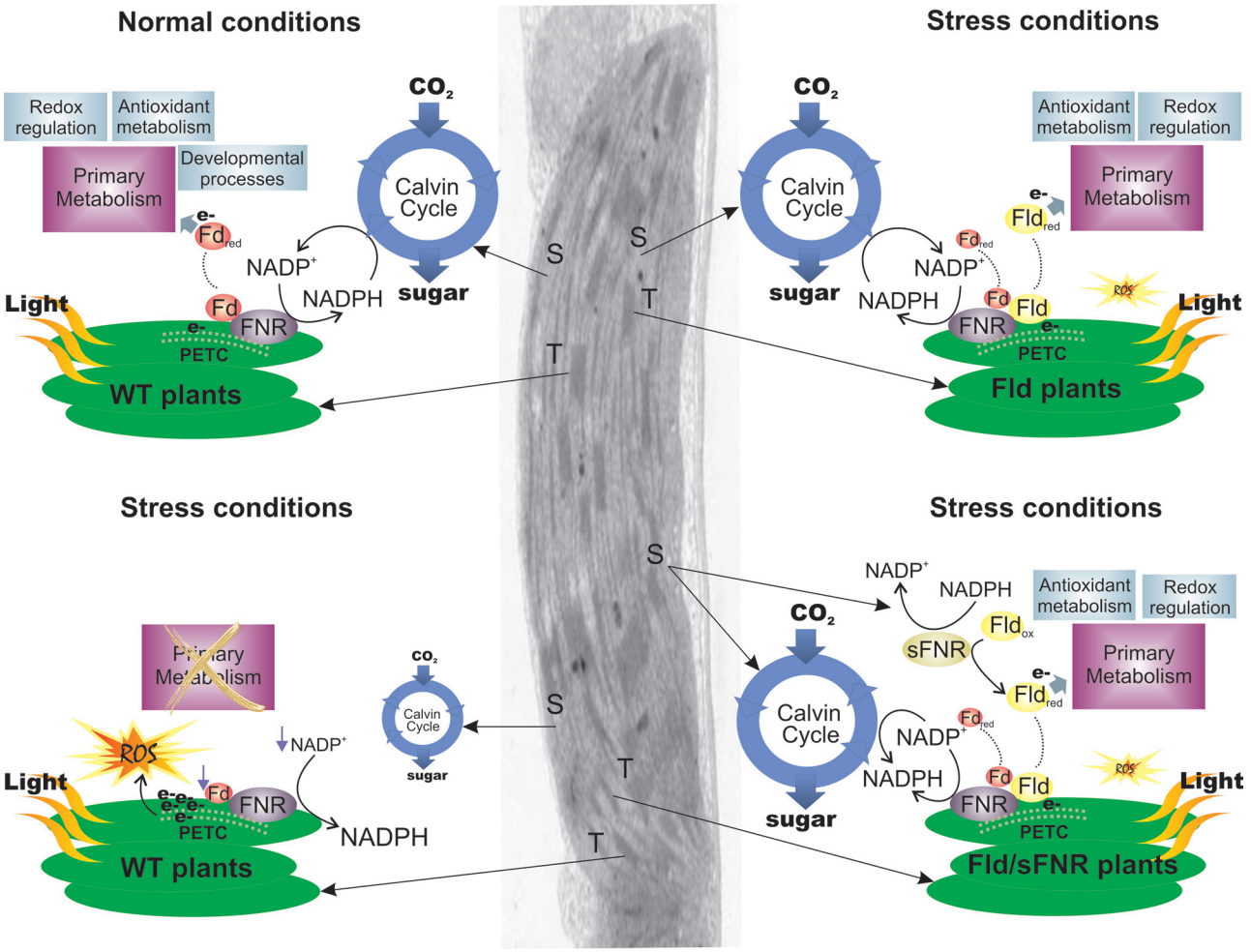

Fig. 2. Proposed model for the protective mechanism of Fld in plastids of transgenic plants. A transmission electron micrograph of a chloroplast is used to illustrate the places (thylakoids or stroma) where the various reactions are expected to occur. The situation in WT plants under normal growth conditions is shown in the top left panel, with Fd acting as the regular shuttle between the PETC and electron-accepting processes of the chloroplast. Stress conditions in WT lines (bottom left) lead to Fd down-regulation, $NADP^+$ exhaustion, and ROS build-up. Most central metabolic routes, including the Calvin cycle, are inhibited, whereas a few, such as respiration, are unaffected or even induced (not shown). Fld expression in stressed plants (top right) bypass Fd limitation, reactivating electron delivery to metabolic sinks. Simultaneous expression of Fld plus a soluble FNR (sFNR) in stressed plants (bottom right) further improves NADP(H) turnover and distribution of reducing equivalents to productive routes, resulting in increased tolerance to environmental hardships. T, thylakoids; S, stroma; ox, oxidized; red, reduced. Other abbreviations are given in the text.

reducing end of PSI (namely, Fd and $NADP^+$ shortage) was confirmed by introducing a second Fld reduction system. As NADPH build-up at the expense of $NADP^+$ is another unwanted consequence of stress episodes, overexpression of a soluble FNR seemed to be the logical choice to use the excess of NADPH as electron source for Fld reduction. Double-transgenic lines expressing cyanobacterial Fld and FNR had a higher $NADP^+$/NADPH ratio, and exhibited increased tolerance to paraquat-mediated oxidative stress relative to Fld-only siblings (Fig. 2) (Giró *et al.*, 2011).

The rise and fall of flavodoxin in photosynthetic organisms

Before flavodoxins, ferredoxins

The origin of life on Earth is placed at about 3,500 Mya in an anaerobic environment, and essential enzymatic mechanisms and biochemical pathways evolved in the absence of any selective pressure to avoid reactivity with oxygen or oxygen derivatives (Lane *et al.*, 2013). Whole genome analyses of phylogenetically diverse microorganisms suggest that the earliest proteins incorporated metals, and that metal usage evolved over time in accordance with its availability, which was in turn dependent on the redox state of the environment (Dupont *et al.*, 2006; Lane *et al.*, 2013). Iron is, by far, the most abundant transition metal on Earth (Taylor, 1964), and its bioavailability in the primitive Archaean and early Proterozoic oceans is reflected by its wide use as a catalyst for oxido-reductive proteins and enzymes. Indeed, iron is identified as a cofactor in two thirds of the oxidoreductases present in the PDB and Swiss-Prot datasets (Lane *et al.*, 2013). Fe-containing proteins can use the metal in a mineral form (as in superoxide dismutases), coordinated to imidazole nitrogens in porphyrins (haems), or complexed to sulfides in iron–sulfur clusters.

Several lines of evidence support the notion that Fds are among the oldest iron–sulfur proteins on Earth. First, Fe^{+2} and sulfide were thought to be plentiful in the anaerobic Archaean ocean, and unlike organic prosthetic groups, they can spontaneously form iron–sulfur centres and assemble into extant polypeptide structures. Indeed, analogous complexes can be created *in vitro* by incubating ferrous and sulfide salts with organic thiolates (Imlay, 2006). It has even been speculated that the earliest biologically relevant redox reactions occurred on the surface of iron–sulfur mineral deposits associated with hydrothermal vents (Wächtershäuser, 2007). Second, Fe–S groups 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 derivatization of aliphatic metabolites by radical-based mechanisms (Imlay, 2006). Third, phylogenomic analysis of protein architecture indicates that the Fd fold is very ancient. All Fds have a simple, conserved fold made up of ~60 amino acids that binds the Fe–S cluster and presumably evolved from an early gene duplication event of a 30-residue sequence, a "protoferredoxin" composed of a primeval subset of amino acids (Beinert, 1990; Darimont and Sterner, 1994; Eck and Dayhoff, 1966; Trifonov, 2000). Reconstruction of a phylogenomic tree of protein fold architecture using data from a domain census in almost 200 sequenced genomes of the three superkingdoms placed Fd as the fifth oldest domain on Earth (starting with the P-loop-containing NTP hydrolases), and second among redox-related domains, after the $NAD(P)^+$-binding Rossmann fold (Caetano-Anollés *et al.*, 2007).

These remarkable traits favoured dispersion of organisms containing iron–sulfur proteins throughout the anaerobic world, and placed these cofactors among the earliest catalysts. Other proteins containing identical [2Fe–2S] clusters, such as the Rieske proteins with their higher midpoint potentials, most probably evolved after Fd (Kim *et al.*, 2013).

If Fe–S clusters are indeed evolutionary relics of earlier biochemistries and prebiotic chemistries, it could be that the most ancient among extant organisms contained more Fe–S proteins. This contention has been put to test by Major *et al.* (2004) and Sousa *et al.* (2013), using 120 and 1606 bacterial genomes, respectively. They observed that methanogens, acetogens (clostridia), and sulphate reducers, which are considered the life forms that more closely resemble ancient organisms, contained the highest numbers of Fe–S proteins. These organisms thrive in the deep biosphere, where, as on the early Earth, there are limited ways to make a living.

The preceding discussion was intended to explain why iron–sulfur proteins were widespread when, approximately 2,700 Mya, cyanobacteria evolved 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 scarcity of oceanic phosphorous to support ATP synthesis, and by oxygen removal through reaction with dissolved ferrous and sulfide ions (Bjerrum and Canfield, 2002). The sharp rise of the oxygen levels at the brink of the Precambrian (~800 Mya) led to one of the most catastrophic evolutionary stresses of biotic history.

As 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 polyhydroxides or formed insoluble complexes with anionic salts. The upshot was that as oxygen accumulated, iron decreased its bioavailability and became a limiting nutrient in most aerobic habitats (Imlay, 2006).

Second, partial reduction of oxygen generates superoxide, H_2O_2, and other ROS, oxidants, which display still higher reactivity than oxygen. Even under optimal growth conditions, a fraction of the electrons moving through the photosynthetic or respiratory chains is adventitiously delivered to oxygen with concomitant ROS generation. This fraction

increases significantly under adverse environmental conditions (Mittler *et al.*, 2004).

Iron–sulfur centres are vulnerable to ROS attack to various extents, depending on solvent exposure and the polypeptide environment surrounding the cluster. Oxidation yields unstable intermediates 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 (Imlay, 2006). This process is generally self-propagating. In photosynthetic organisms, for instance, stress-dependent Fd down-regulation leads to over-reduction of the PETC owing to shortage of electron acceptors, and under such circumstances the electron surplus can be passed straight to oxygen resulting in runaway ROS generation (Holtgrefe *et al.*, 2003; Nogués *et al.*, 2004; Voss *et al.*, 2008; Blanco *et al.*, 2011).

Then, the type of iron–sulfur chemistry 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 developing more sophisticated antioxidant and repair systems, and by reprogramming vast portions of metabolism from growth to defence (Foyer and Shigeoka, 2011; Munné-Bosch *et al.*, 2013). As the oceans became progressively iron-deficient, several mechanisms were also developed by marine microorganisms to overcome this limitation, such as high surface-to-volume ratio to aid nutrient uptake (Chisholm, 1992), a more extensive machinery for metal capture and storage (Toulza *et al.*, 2012), and a decrease of iron-rich PSI (12 iron atoms per complex) in favour of PSII (2–3 iron atoms per complex) (Strzepek and Harrison, 2004). The many adjustments made were expensive and bestowed 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.

As a consequence of these combined challenges, there was also intense selective pressure to replace oxidant-sensitive, iron-dependent proteins by oxidant-resistant, iron-free isofunctional counterparts (Palenik *et al.*, 2006; Allen *et al.*, 2008; Toulza *et al.*, 2012). 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 Cu/Zn-, Mn- and Ni-containing superoxide dismutases (De la Rosa *et al.*, 2006; Palenik *et al.*, 2007). Within this context, substitution of labile Fd acquired paramount importance.

Presumably a first step toward this direction was the development of more oxygen-tolerant Fd versions by improving protection of their Fe–S centres from oxidants. Some of the changes undergone by these proteins can be recognized by comparing extant aerobic and anaerobic isoforms. In typical anaerobic Fds, the bridging μ–sulfido atoms of the cluster are partially solvent-exposed. In contrast, the iron and sulfur atoms of the [2Fe–2S] centres of aerobic Fds are better shielded by the protein (Jagannathan and Golbeck, 2009). In spite of these improvements, oxygen tolerance was not complete and the question of iron shortage remained unsolved. Fld was the ultimate solution, as it is oxygen-insensitive and does not require iron.

The origin of flavodoxins

Expression of Fld to replace Fd is generally regarded as one of the most crucial factors determining the colonization of iron-poor waters by phytoplankton (Rocap *et al.*, 2003), and evaluation of metagenomic data confirmed that algae and cyanobacteria lacking this flavoprotein are usually confined to iron-rich coastal/freshwater habitats (Toulza *et al.*, 2012, see below). The importance of Fld in the dynamics of sea ecology is reflected by its use as a proxy for iron stress in the oceans (Erdner *et al.*, 1999). As the role of Fld as a backup of Fd seems to be a response to aerobiosis, it is natural to assume that this flavoprotein evolved well after Fd. Surprisingly, this does not seem to be the case.

Phylogenomic evaluation of protein-fold architecture actually placed the Fld structure among the nine most ancestral and widely shared folds, appearing immediately after Fd (Caetano-Anollés *et al.*, 2007). According to these data, the ancient folds represent architectures of fundamental importance encoded in a genetic core that can be tracked back to the universal ancestor of the three superkingdoms of life (Harris *et al.*, 2003). They appeared in a relatively short timeframe, long before diversification of prokaryotes (Caetano-Anollés *et al.*, 2007). Then, early Flds either played roles independent from Fd substitution or responded to environmental cues not related to aerobiosis. Most likely, they were recruited to replace iron- and oxygen-sensitive Fd at a later stage, after oxygen build-up. The widespread presence of Fld in anaerobes and the existence of Fld-specific metabolic routes (Freigang *et al.*, 2002; Puan *et al.*, 2005) provide circumstantial evidence to this tenet.

Flavodoxin entered the algal world and never came out; the phylogenetic patchwork

A simplified phylogenetic tree of photosynthetic eukaryotes is shown in Fig. 3. The initial endosymbiotic event that gave origin to all algal lineages is estimated to have occurred ~1,600 Mya (Hedges *et al.*, 2004; Yoon *et al.*, 2004). Glaucophytes were probably the first to diverge from the common ancestor, followed by the split between red and green algae, a few hundred million years after the primary endosymbiosis (Hedges *et al.*, 2004; Yoon *et al.*, 2004). Members of these latter lineages subsequently became the subjects of secondary and tertiary endosymbiosis (Fig. 3), spreading the algal heritage throughout all kinds of habitats. *Viridiplantae* separated ~1,000 Mya in two lineages: Chlorophyta and Streptophyta, which followed radically different evolutionary trajectories (Hedges *et al.*, 2004). Chlorophytes, which comprise the majority of green algal species, radiated in marine and coastal environments, whereas streptophytes, which include land plants (Embryophyta) and a paraphyletic assembly of green algae (charophytes), evolved largely in freshwater. Ancestral charophytes colonized the dry land ~450 Mya,

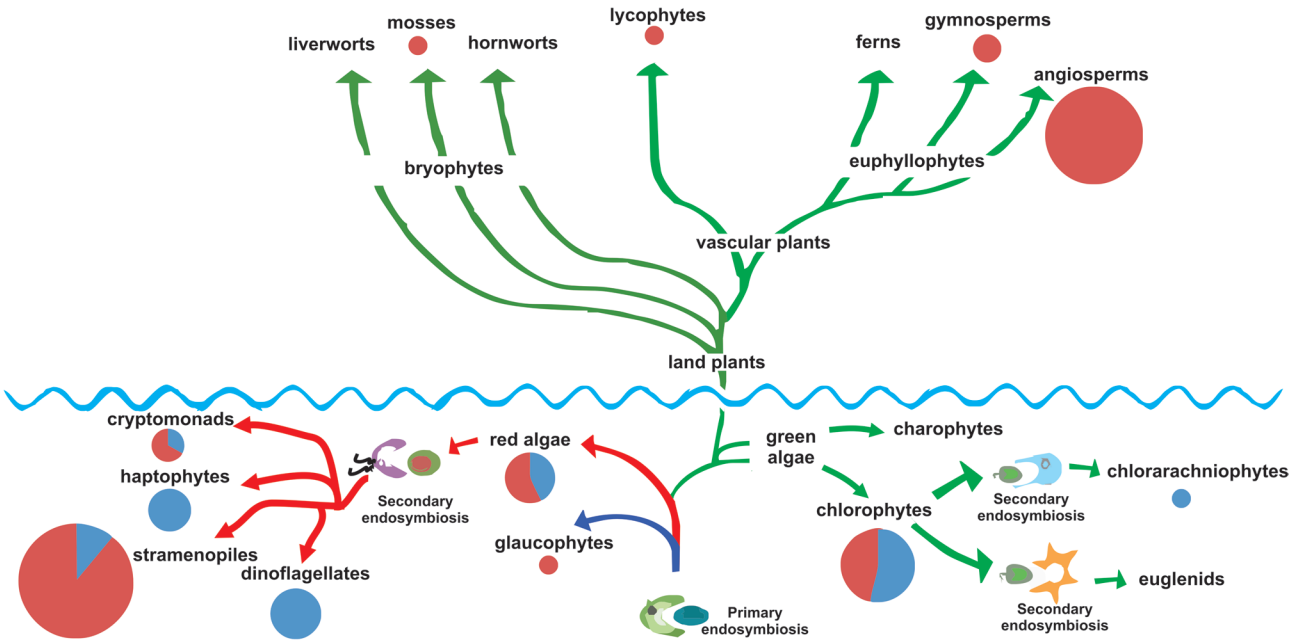

Fig. 3. An evolutionary framework of Fld distribution among photosynthetic eukaryotes. A simplified phylogenetic tree was constructed using data from Bowman (2013) and Keeling (2013). Circle sizes represent the absolute number of species analysed in each branch, whereas colours indicate the fraction of species containing (blue) or not (purple) the *fld* gene.

giving rise to land plants (Fig. 3), which have dominated the terrestrial environments since then, and some have even become secondarily adapted to freshwater or marine habitats (Waycott *et al.*, 2002; Chambers *et al.*, 2008). Monophyly of Embryophyta is supported by both morphological and molecular comparisons (Kenrick and Crane, 1997). The two most complex groups of the Charophyta, the coleochaetales and the charales (stoneworts), both of which are characterized by true multicellular organization and oogamous sexual reproduction, have been variously invoked as sisters to land plants (Bowman, 2013), as well as the structurally simpler Zygnematophyceae (Ruhfel *et al.*, 2014). Within the embryophytes, liverworts are the most basal group, followed by mosses, and then hornworts, and vascular plants sharing a sister-group relationship (Wellman, 2014). Vascular plants appeared ~410 Mya and then diverged into several lineages, only two of which survive: the euphyllophytes (ferns and seed plants) and the lycophytes (Kenrick and Crane, 1997).

Prevalence of the *fld* gene in each taxon of the photosynthetic eukaryotes is indicated in Fig. 3 by size and colour codes. The gene is found in all major algal taxa including green and red algae, as well as those groups derived from them through secondary and tertiary endosymbiosis. Notorious exceptions are glaucophytes and euglenids, although only one sequenced genome is available from the former group (*Cyanophora paradoxa*) and none from the latter. On the other hand, analysis of genome databases confirmed the absence of this flavoprotein in algal species from most taxa. Within *Viridiplantae*, all the organisms in which the presence of Fld was confirmed belong to the chlorophytes. No charophyte genome is yet available, and no Fld sequence could be retrieved from the expressed sequence tag collections of this group of algae (Timme *et al.*, 2012). The absence of the *fld* gene in the genomes of land plants is confirmed in the more than 30 fully sequenced genomes (www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html). They include the lycophyte *Selaginella moellendorffii*, a "primitive" vascular plant, as well as the moss *Physcomitrella patens*, a bryophyte, phylogenetically closer to the first green algal lineage that successfully colonized terrestrial habitats.

Although the "Middle Age" of plant evolution (from charales and choleochaetales to gymnosperms) is largely empty of hard data, the absence of Fld sequences in that vast range of species suggest that the gene was lost earlier, most likely when streptophytes split from the chlorophytes (Fig. 3). More extensive genetic and genomic analyses of the "missing links" (charophytes, liverworts, hornworts, ferns) will be needed to elucidate this question.

Geographical distribution: environmental constraints

Although each environment has complex chemistry and physics, it is accepted that iron levels tend to be high in freshwater and coastal habitats, owing to suspended sediments and aerial inputs from land (Jickells *et al.*, 2005). In contrast, iron deficiency is predicted in as much as 40% of the open ocean, notably in the Southern Ocean as well as in the equatorial and north Pacific (Moore *et al.*, 2001). Iron utilization also posed a serious challenge to plants after land colonization, but of a different nature. Iron is the fifth most abundant element, 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 media (Guerinot and Yi, 1994; Kobayashi and Nishizawa, 2012). It is worth noting that alkaline calcareous soils represent about one-third of the planet's cultivable land (Guerinot and Yi, 1994).

The phylogenetic distribution described in the previous section indicates that retention of the *fld* gene along the

path of evolution has been disparate, even among species of the same genera, such as in the chlorophytes *Bathycoccus* (Monier *et al.*, 2012; Moreau *et al.*, 2012; Vaulot *et al.*, 2012) and *Ostreococcus* (Derelle *et al.*, 2006; Palenik *et al.*, 2007; http://genome.jgi-psf.org/OstRCC809_2).

As already indicated, Fld presence shows a clear bias towards iron-deficient habitats (Erdner *et al.*, 1999). Conversely, Fld absence is a common feature in species isolated from iron-rich environments. In fact, all freshwater algal species with completely sequenced genomes lack the gene (Merchant *et al.*, 2007; Blanc *et al.*, 2010; Prochnik *et al.*, 2010; Curtis *et al.*, 2012; Price *et al.*, 2012; Bogen *et al.*, 2013). The same pattern holds true for cyanobacteria (Toulza *et al.*, 2012).

The enigma of flavodoxin disappearance from the plant genome: a hypothesis

The preceding discussion suggests that the major adaptive advantage of Fld in marine habitats seems to be increased tolerance to iron starvation, a frequent stress in open oceans. Indeed, iron deficiency is regarded as a main selective pressure which determined the fate of many genes in phototrophs (Rocap *et al.*, 2003; Palenik *et al.*, 2006). It is therefore surprising that Fd was retained in all photosynthetic eukaryotes in spite of its lability to iron starvation and oxidants, whereas Fld disappeared from the plant genome. The causes underlying this outcome are unclear, and the life history of other Fe-containing proteins (and their replacements) has been entirely different. For instance, cytochrome c_6 was in the end displaced by plastocyanin (De la Rosa *et al.*, 2006), and Cu/Zn-containing superoxide dismutases eventually became the dominant isoforms of this type of enzymes in eukaryotes (Miller, 2012).

On the one side, the reasons for Fd conservation along the *Viridiplantae* lineage are probably related to its catalytic efficiency. Although Fld might be the preferred or specific electron donor in a few heterotrophic pathways (Freigang *et al.*, 2002; Puan *et al.*, 2005; Gutekunst *et al.*, 2014), Fd consistently displayed higher rates of electron transfer in photosynthesis-related reactions (Vigara *et al.*, 1998; Medina *et al.*, 1998; Meimberg and Mühlenhoff, 1999; Tognetti *et al.*, 2006). Possible reasons for this kinetic advantage have been discussed previously. Photosynthetic electron transport is usually one order of magnitude faster than most heterotrophic oxidoreductive processes, and increases in catalytic efficiency have also been observed for FNR after recruitment into the PETC (Carrillo and Ceccarelli, 2003). Then, if Fd did confer the highest rates of electron distribution to photosynthetic acceptors under iron-replete conditions, it could still hold enough selective value to warrant retention in the genomes of phototrophs, including those which spend most of their lifetime in iron-deficient habitats. Indeed, available evidence indicates that photosynthetic organisms become non viable below a certain threshold of Fd content (Mazouni *et al.*, 2003; Holtgrefe *et al.*, 2003), even in the presence of Fld (Poncelet *et al.*, 1998; Blanco *et al.*, 2011). The results suggest that Fld migh help to alleviate the symptoms of iron limitation and other environmental hardships, but that some residual Fd activity (or isoform) is required to ensure survival and reproduction. In other words, Fld does not provide full complementation of Fd in photosynthetic organisms. Although no definite proof for this contention is yet available, the evidence collected from mutant microorganisms and knocked-downed plants strongly support the essentiality of Fd for phototrophs.

The reasons behind Fld loss from the *Viridiplantae* are more obscure. Functional arguments cannot be invoked as the experimental evidence indicates that Fld expression in plants still confers selective advantages (Tognetti *et al.*, 2006). Disappearance of this valuable asset from the plant genome might be related to ecological adaptations to iron bioavailability and the successive stages of land colonization (Fig. 4). It is accepted that terrestrial plants evolved from coastal/freshwater macroalgae (charales, choleochaetales). We propose that

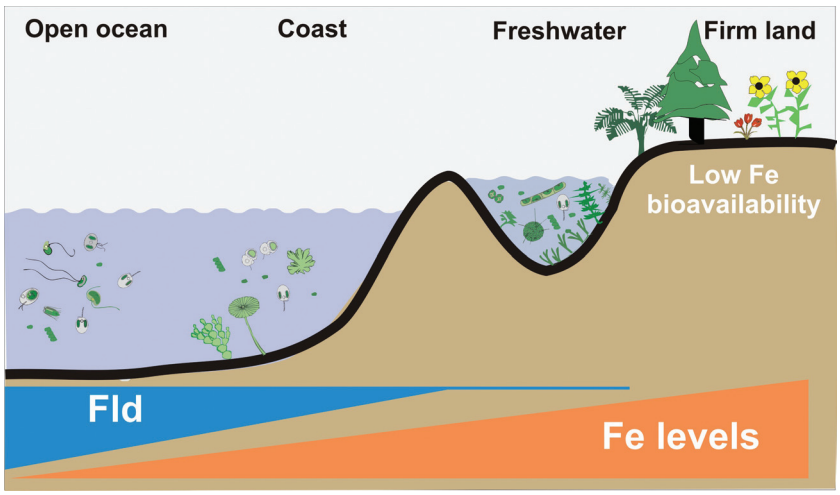

Fig. 4. The flavodoxin gene was lost along the evolution of *Viridiplantae* during the colonization of terrestrial habitats by streptophyte algae. The hypothesis assumes that plants originated from freshwater streptophytes which already lacked Fld. Cyanobacteria and algae thriving in iron-deficient oceanic environments usually contained the *fld* gene, whereas coastal and freshwater counterparts evolved in a habitat in which iron was plentiful and bioavailable, and the role of Fld as a backup of Fd was presumably not required. Typical cyanobacteria, algae, and plants are depicted schematically. The negative correlation between iron levels and Fld presence is shown in the bottom. They are intended to represent tendencies and do not depict actual iron/Fld contents.

these ancestors, thriving in an environment in which iron was both abundant and readily accessible, had no need to induce Fld expression to replace Fd (Fig. 4). Under such conditions, selection pressures for Fld retention as an adaptive trait might have been relaxed. Indeed, the geographical distribution of marine phototrophs shows that iron-rich coastal and freshwater regions are preferentially populated by Fld-lacking algae and cyanobacteria (Fig. 3), increasing the probability that plants evolved from algal precursors that had already lost the Fld gene. According to this hypothesis, the absence of Fld form the Embryophyta would be the result of a founder effect.

After colonization of the firm land, plants faced a novel type of challenge: how to get iron from an environment in which the metal was plentiful but not readily available. They adapted to the new situation by deploying a multigenic response in which many genes were recruited to operate at various levels, including rhizosphere acidification, reduction of Fe^{+3} to Fe^{+2}, metal chelation, and metal transport, all of them contributing to optimization of iron uptake (Kobayashi and Nishizawa, 2012). This strategy was successful enough to allow spreading of plant lineages throughout most of the planet. It is somehow surprising, however, that they were not able to recover the *fld* gene by, for instance, HGT from contemporary algae or bacteria. It should be borne in mind, however, that the evolution of land plants has been largely associated with expansion and diversification of existing gene families as the result of large-scale gene or whole-genome duplication events (Richardt *et al.*, 2007; Rensing *et al.*, 2008), rather than incorporation of novel genes and functions (Bock, 2010). In fact, examples of HGT to multicellular eukaryotes are rare (Rumpho *et al.*, 2008; Bock, 2010), and this limitation might have prevented recovery of an adaptive trait that provides ample stress tolerance when present in plants (Tognetti *et al.*, 2006, 2007). Although this conjecture is consistent with current observations, experimental validation is lacking and further research will be required to properly address this issue.

Note added in proof

In support of our hypothesis of an early disappearance of the flavodoxin gene in the Streptophyta, the first sequenced genome of a charophyte alga has been just published (Hori *et al.*, 2014), and it does not contain flavodoxin.

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