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PERIODICUM BIOLOGORUM
VOL. 114, No 1, 25–31, 2012

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Review

Balancing chloroplast redox status – regulation of FNR binding and release

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Key words: photosynthesis, linear electron flow, retrograde signalling, electron partitioning, stromal redox status

Received February 6, 2012.

Abstract

Working in synchrony, photosynthetic charge separation, electron transfer, and redox reactions generate proton motive force necessary for the synthesis of ATP and funneling of electrons toward stromal reducing equivalent NADPH. The last step of electron transfer from ferredoxin to $NADP^+$ is catalyzed by ferredoxin-$NADP^+$ oxidoreductase (FNR). Two proteins, TROL (thylakoid rhodanese-like) and Tic62 (62 kDa component of the translocon at the inner envelope of chloroplasts), have been characterized and shown to form dynamic complexes with FNR. Inactivation of TROL leads to changes in efficiency of electron transfer and induction of non-photochemical quenching. TROL-deficient plants have changed nuclear gene expression with up-regulation of NADPH-dependent malic enzyme, which can form NADPH in an alternative pathway. Thus, NADPH synthesis, mediated by FNR-TROL interaction, may be the source element in metabolic retrograde signal-transduction pathway linking light reactions with nuclear gene expression.

INTRODUCTION

Oxygenic photosynthesis encompasses light reactions taking place in the thylakoid membranes, and carbon fixation reactions in soluble stroma. The photosynthetic machinery must be structurally and functionally extremely dynamic to enable flawless primary production under a wide spectrum of environmental conditions. The molecular mechanisms of these dynamic changes are presently largely uncharacterised, in particular because various auxiliary proteins linking photosynthesis with physiological responses are still not characterized. Cooperation of two photosystems in the chloroplast thylakoid membranes produces a linear electron flow (LEF) from H_2O to $NADP^+$. Maintenance of the poised state of the photosynthetic system is acquired by the interplay between cyclic (CEF), linear, and pseudocyclic electron transport pathways *(1)*. All three pathways involve activity of PSI that transfers electrons from the plastocyanin located in the thylakoidal lumen to the stromal ferredoxin (Fd), mediated by three subunits, C, D and E, of the so-called stromal ridge of PSI *(2)*. In the reduced state Fd provides electrons for the ferredoxin:NADP+ oxidoreductase (FNR), which produces NADPH in a linear pathway *(3)*, for the ferredoxin-thioredoxin reductase (FTR), which catalyses the reduction of chloroplast thioredoxins *(4),* for feeding of the CEF *(1)* or, alternatively, electrons can be transferred to superoxide, the terminal acceptor in pseudocyclic pathway *(1)*. The generation of NAD(P)H

and ATP, which are cells' energetic equivalents, is crucial for all biosynthetic processes within chloroplasts. These two types of molecules are the principal energetic links between membrane-associated redox reactions and metabolism in the cell soluble compartments. They are generated simultaneously in the chloroplast during light-dependent electron transport and photophosphorylation and utilized in the reductive assimilation of inorganic elements into organic compounds, from which ATP and reductant can be regenerated by oxidative phosphorylation in the mitochondria, which enables the reducing power of NAD(P)H to be converted into ATP. LEF produces a fixed ATP/NADPH ratio, and each metabolic pathway directly powered by photosynthesis consumes different fixed ATP/NADPH ratios. Chloroplasts have very limited pools of ATP and NADPH and since mismatches in ATP/NADPH ratios rapidly (within seconds) inhibit photosynthesis *(5)*, chloroplasts must balance the production and consumption of both ATP and NADPH by augmenting production of the limiting intermediate (e.g. by CEF) or dissipating the intermediate in excess.

FERREDOXIN:NADP$^+$ OXIDOREDUCTASE

FNR is a key enzyme of photosynthetic electron transport required for generation of reduction equivalents. It transfers electrons from ferredoxin to NADP$^+$ and reducing power derived this way may be further used for carbon assimilation (Calvin-Benson cycle), amino acid, lipid and chlorophyll biosynthesis or reduction of stromal redox-active components. FNR is a ubiquitous flavin adenine dinucleotide (FAD)-binding enzyme that has been identified in various organisms including heterotrophic and phototrophic bacteria, in mitochondria and plastids of higher plants and algae, as well as apicoplasts of some intracellular parasites *(6)*. FNR harbors one molecule of noncovalently bound FAD as a prosthetic group *(3)* and it catalyzes reversible electron transfer between reduced Fd to NADP$^+$ for production of NADPH according to the reaction $2Fd_{red} + NADP^+ + H^+ \leftrightarrow 2Fd_{ox} + NADPH$.

Plant-type FNRs are hydrophilic proteins with a molecular weight of approximately 35 kDa. Studies have shown that FNR consists of two structural domains connected by a loop *(7)* – a topology that is highly conserved in all chloroplast FNRs. The amino-terminal domain of FNR comprizes a β-barrel, consisting of six antiparallel β-strands, a loop and an α-helix, and contains the binding domain for the FAD cofactor. Binding of FAD is a prerequisite for membrane binding of FNR *(8)*. The domain at the carboxyl-terminus contains a five-stranded parallel β-sheet surrounded by six α-helices, and is involved in NADP$^+$ binding *(9)*. The large shallow cleft between the two domains accommodates the Fd *(10)*. Also involved in the interaction with Fd to form the Fd-FNR complex, is the flexible amino-terminus of FNR, which has been revealed by nuclear magnetic resonance and mutagenesis studies *(11)*. FNR in higher plants is encoded by a small nuclear gene family, consisting of one to three *FNR* genes with up to 80% homology, and is synthesized in the cytosol. Its precursor contains an amino-terminal transit peptide, targeting the protein to the chloroplasts *(12)*. FNR has been found in the soluble stroma, as well as at the thylakoid membranes and at the chloroplast inner membrane. Both the membrane-bound and the soluble pool of FNR are photosynthetically active *(13)*. Also, both pools form Fd complexes of the same stability, though *in vitro* experiments showed that the rate constant of NADP$^+$ photoreduction is much higher in the membrane-bound than in the soluble complexes *(14)*. Furthermore, *fnr* knock-out mutants, which do not contain membrane-bound FNR, exhibit normal photosynthetic activity, which indicates that the soluble FNR *in planta* really is photosynthetically competent *(15)* and that the solubility of FNR is not the decisive determinant of enzymatic activity.

CEF supplies the ATP needed for driving the CO_2 concentrating mechanism in the C_4 plants. In C_3 plants, under normal physiological conditions, CEF might have a role in adjusting the stoichiometry of ATP/NADPH generated by photosynthesis *(16)*. In CEF, electrons are transferred from PSI to Cyt b_6f complex via Fd, with associated formation of proton gradient. PQ is reduced by Fd or NADPH via one or more enzymes collectively called PQ reductase, rather than by PSII, as in LEF. From hidroplastoquinone (PQH_2), electrons return to PSI via the Cyt b_6f complex. Thus, CEF around PSI produces ATP without accumulation of NADPH. FNR has been identified as a component of the Cyt b_6f complex *(17)* and therefore its role in CEF has been assumed, but not yet defined.

Reactive oxygen species (ROS) are constant by-products of PSII and PSI activity in chloroplasts of higher plants. They serve as signalling components, but are harmful when produced in excess. In order to investigate the role of FNR in oxidative stress responses in higher plants, research has been done on wheat. Results show that even though the content of FNR mRNA and protein decreased in response to oxidative stress induction *(18)*, the production of ROS caused a significant release of FNR from thylakoid membranes. As a result, NADP$^+$ photoreduction capacity was reduced, possibly in order to maintain the NADPH/NADP$^+$ homeostasis in affected plants *(18)*. In addition, it has been shown that FNR dissociates from thylakoides during drought stress *(19)*, and that thylakoid protein complexes containing FNR dissasemble after high light exposure *(20)*.

Since it has been proposed that binding of FNR to the thylakoid membrane regulates the enzyme activity *(21)*, possible binding partners for FNR have been suggested and investigated, and even direct membrane attachment was speculated. Although interactions between several photosynthetic protein complexes and FNR, such as Cyt b_6f *(17, 22)*, PSI *(23)* or NDH complex *(24)* have been shown, up until recently no clear biochemical evidence of direct interaction has been provided for any of them. It was demonstrated recently that FNR interacts specifically with two chloroplast proteins, Tic62 and TROL, via

a conserved Ser/Pro-rich motif. Both Tic62 and TROL seem to act as molecular anchors for FNR, because they form high molecular weight complexes with FNR at the thylakoid membrane.

Tic62

During its import into chloroplasts from the site of synthesis on cytosolic rybosomes, FNR interacts with Tic62 protein, a 62 kDa component of the »Translocon at the inner envelope of chloroplast« *(25, 26, 27, 20)*. Proteomics studies have identified Tic62 in the chloroplast envelope, stroma and thylakoid fraction *(20)*. Furthermore, Tic62 at the thylakoid membrane was found in several high molecular mass protein complexes (250–500 kDa), and it was shown to be tightly associated with both chloroplast FNR isoforms *(20)*. Topology studies on Tic62 revealed that its N-terminus binds pyridine nucleotides, while the stroma exposed C-terminus contains repetitive, highly conserved FNR-binding domains *(25)*. The presence of the FNR-binding domains of the Tic62 protein is limited to vascular plants *(26)* and it occurs in different numbers dependent on the plants species. Tic62 from *Pisum sativum* (PsTic62) contains three Pro/Ser-rich repetitive motives at the C-terminus, S-P-Y-x(2)-Y-x-D/E-L-K-P(2)-S/T/A-S/T-P-S/T-P, involved in the binding of FNR *(25)*. PsTic62 homolog in *A. thaliana*, encoded by a single-copy gene (*At3g18890*), shows approximately 60% identity for the deduced mature sequence and has a calculated molecular weight of 62.1 kDa *(25)*. AtTic62 contains four repetitive motives at the C-terminus, but it has been shown previously that only one repeat is sufficient for the binding of FNR *(25, 26)*.

The function of FNR in the Tic complex has been suggested to link redox regulation to chloroplast protein import *(25)*. Also, diurnal changes in the chloroplast redox poise may control import characteristics of the organelle *(28)*. It was shown that Tic62 changes its localisation depending on the oxidation of the stroma, i.e. the $NADPH/NADP^+$ ratio. In conditions of stromal oxidation, i.e. low $NADPH/NADP^+$ ratio, Tic62 shuttles to the chloroplast inner membrane and strongly assotiates to the Tic complex *(27)*. FNR shows similar shuttling behaviour, so the Tic62-FNR interaction depends on the chloroplast redox state *(27)*. It was also shown that lack of Tic62 and the subsequent lack of Tic62-FNR complexes does not affect plant phenotype or any of the photosynthetic properties *(20)*, which suggests that these complexes probably participate in processes other than photosynthesis. Membrane-bound Tic62-FNR complexes were shown to be most abundant in the dark, while exposed to light, these complexes disassembled. Also, *in vitro* alkalization of isolated thylakoids resulted in dissociation of FNR and Tic62 *(20)*. It should be noted that the pH of chloroplast stroma varies from being slightly acidic in complete darkness to more alkaline at saturating light *(29)*, and that FNR activity is lower in acidic than alkaline environment *(30)*. These results imply that Tic62 acts as a chaperone for FNR, while recruitment and binding of FNR to the thylakoid membrane may be a method of storing and protecting the flavoenzyme from degradation in the dark, when photosynthesis does not occur *(20)*.

TROL

Jurić et al. *(31)* identified TROL (thylakoid rhodanese-like protein), a novel component of non-appressed thylakoid membranes which is responsible for anchoring of FNR. It is a 66 kDa nuclear encoded component of thylakoid membranes, containing two transmembrane helices and a centrally positioned (inactive) rhodanese domain. TROL is an integral membrane protein, firmly attached to the thylakoid membrane and cannot be extracted from the membrane by high salt, urea or high pH treatments *(31)*. TROL possesses a unique fusion of two distinct modules: a centrally positioned rhodanese-like domain, RHO, which is found in all life forms, and a C-terminal single hydrophobic FNR-binding region, ITEP, which is ascribed to vascular plants *(26)*. It is hypothesized that both N- and C-terminal parts of TROL face the stroma, while RHO faces the thylakoid lumen *(31)*. A closer investigation of the TROL protein sequence revealed an interesting region upstream of the ITEP domain. The Pro-Val-Pro repeat-rich region was designated PEPE and is presumed to be exposed into the stroma. It consists of two identical repeats, followed by a possible PVP hinge and is proposed to introduce flexibility in the helix that may result in kink and swivel motions of FNR-binding region.

Localization in non-appressed regions places TROL in the vicinity of the site of Fd reduction. TROL has been found in several complexes, indicating the presence of several TROL subpools in the thylakoid membrane. Only a 190 kDa complex appears to contain TROL in association with the FNR. Complexes at about 110 and 120 kDa indicate the existence of a small ligand which may be associated with other TROL domains, namely the large rhodanese-like domain which is predicted to be located in the thylakoid lumen. The findings of Küchler *et al. (25)* that Tic62 interacts with FNR prompted the analysis of TROL protein sequence in search for the similar binding module. Almost identical domain to the Tic62 FNR-binding repeats has been found in TROL. Modified yeast-two hybrid assay was used to confirm ITEP-FNR interaction *(31)*, and, as assumed, ITEP strongly binds to the FNR protein, even eight times stronger than Tic62. With its molecular weight of 190 kDa, the TROL-FNR complex is smaller than the Tic62-FNR complexes, whose molecular weight is 250-500 kDa, and that corresponds to the fact that there is only one FNR binding motif found in *Arabidopsis* TROL protein. Even though *in vitro* experiments indicate that the FNR binding site in TROL is a much stronger interaction site than the one in Tic62 *(31)*, in plants exposed to high light intensity FNR is discharged faster from the TROL-FNR than from the FNR-Tic62 complexes *(31)*.

A synthetic peptide, representing the conserved binding motif, called the FNR-membrane-recruiting-motif

(FNR-MRM) found in Tic62 and TROL, has been used for determination of the crystal structure of the FNR: peptide complex *(29)*. It has been shown that FNR--MRM induces self-assembly of two FNR molecules. The binding affinity to FNR did not change significantly when constructs comprising one or three FNR-interacting motifs were analyzed *(29)*, indicating that binding to each domain occurs independently of the other motifs and excludes cooperative binding effects.

Rhodanese-like domains are known to be involved in sulfur metabolism, and are also implicated in redox regulation of various intracellular processes *(32, 33)*. It has been proposed that the rhodanese-like domain of TROL is involved in redox regulation of FNR binding and release and could be important for balancing the redox status of stroma with the membrane electron transfer chain. Such regulation might be an important mechanism for prevention of over-reduction of any of these two compartments and maintenance of the redox poise *(31)*. TROL, as FNR, is mainly located at the stroma thylakoids, but it can be also found embedded in the chloroplast inner envelope membrane in the non-processed form

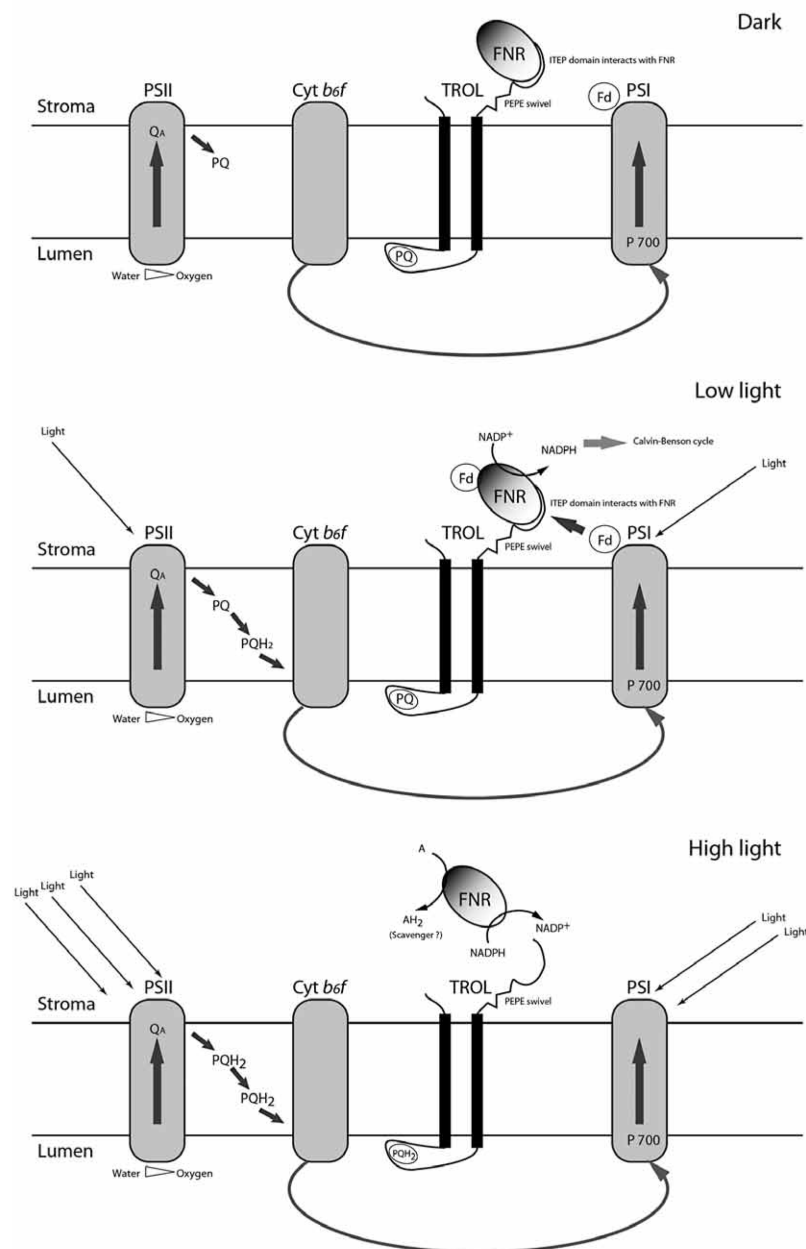

Figure 1. *The proposed mechanism of redox regulation of FNR binding and release. During the dark period TROL anchors FNR, possibly due to the binding of a s mall molecule, i.e. oxidized PQ, to the RHO cavity. There is no NADPH production. In conditions of growth-light, FNR is bound to the thylakoids via TROL and acts as an efficient NADPH producer. Under saturating light, a molecule, possibly reduced PQ, competes for the RHO binding site and generates the signal for the FNR release. Soluble FNR consumes NADPH and released protons are passed to an unknown scavenger A.*

(70 kDa), which indicates its possible role in electron transfer chain specific for this membrane. This dual localization might also be dependent on the $NADP^+$/NADPH ratio in the chloroplasts, as shown for the shuttling of the Tic62 protein *(27)*.

The investigation of the membrane-bound pool of FNR and the 190 kDa-complex containing TROL and FNR in wild-type plants grown under growth-light and high-light intensities *(34)* revealed a role for TROL as the FNR anchor. As an anchor, TROL stabilises FNR during the night, possibly to prevent extracting electrons from NADPH molecules which would compromise the downstream metabolic reactions. During the light period, under growth-light conditions, TROL anchors FNR and stabilises it, similar to association between Tic62 and FNR *(20)*. FNR could be gradually released by binding/releasing of certain signaling molecules to the luminal RHO domain, and electrons are transferred from ferredoxin to $NADP^+$ at normal rates (Figure 1). Under the influence of high-light/excess-light TROL releases FNR, possibly because of binding/releasing of certain signaling molecules to the luminal RHO domain and, as a consequence, FNR catalyzes the reverse reaction of transferring electrons from excess NADPH to potential electron-acceptor molecules (Figure 1) *(34)*. Flexible PEPE swivel that precedes the ITEP region could move FNR closer to the thylakoids to establish transient contacts with other transmembrane proteins, or it could move FNR away from the thylakoids. In addition, TROL-bound FNR molecules could be easily displaced from TROL to the already discovered FNR-binding membrane proteins or the unknown ones.

Perhaps the most interesting property of the FNR-Tic62/TROL interaction is the clear difference of the affinity at acidic (pH 6; KD ≈ 0.04 μM) compared to alkaline (pH 8; KD ≈ 3 μM) conditions *(29)*. The pH variations reflect differences of the chloroplast stroma between light and dark cycles *(29)*: during light phases, when photosynthetic activity is high, protons are transported into the thylakoid lumen, increasing stromal pH. By contrast, when photosynthesis ceases during dark phases the stromal pH decreases again. Under these conditions, Tic62 and TROL are predominantly associated with the thylakoid membrane where they recruit FNR into stable high-molecular-weight complexes *(20, 31)*. During the course of the day light quantity can vary dramatically, therefore requiring constant adjustment of the light harvesting processes and the enzymatic reactions. Changes in light quantities alter stromal pH as well as the amount of FNR bound to the thylakoid membranes. Also, the membrane attachment of FNR is influenced by the stromal redox state ($NADP^+$/NADPH ratio), which mimics variations in environmental conditions *(27)*. Therefore, reversible attachment of FNR to the thylakoid membrane via Tic62/TROL provides an elegant way to store redundant molecules, not required when photosynthesis is less active or dormant.

FNR, TROL AND TIC62 *ARABIDOPSIS* MUTANT PLANTS

In order to investigate FNR function in more detail, studies were done in which the *FNR* gene expression was interrupted by T-DNA *(15, 35)*, or affected by antisense and silencing techniques *(36, 37)*. Such mutant plants exhibited retarded growth due to chlorosis and decreased photosynthetic activity. Although total NADP(H) in transgenic plants did not change, the NADPH/$NADP^+$ ratio was strongly reduced *(36, 38)*. Also, antisense repression of FNR rendered mutant plants prone to photo-oxidative damage and injury due to oxidative stress *(39, 37)*.

In plants with knocked-out *tic62* gene, formation of high molecular weight FNR complexes was inhibited, though a low amount of free FNR could still be detected at the thylakoids. The soluble FNR pool remained more or less constant. Since in *tic62* plants no change in *FNR* gene expression or FNR import was observed, FNR levels were probably reduced because of the different turnover of FNR isoforms inside the chloroplast *(20)*.

Analysis of *Arabidopsis* mutant lines indicates that the absence of TROL severely lowers relative electron transport rates at high-light intensities which is accompanied with significant increase in non-photochemical quenching (NPQ). It was proposed that the soluble form of FNR participates in the regulation of oxidative stress. Thus, it is not surprising that, under high-light conditions, TROL-deficient plants exhibit increased rates of NPQ. This effect was also explained by recently proposed feedback redox regulation via the redox poise of the NADP(H) pool *(38)*. Therefore, TROL probably represents the thylakoid membrane docking site for a complex between FNR, ferredoxin and $NADP^+$. Such association might be necessary for maintaining photosynthetic redox poise and enhancement of the NPQ *(31)*. Inhibition of TROL accumulation by antisense expression results in quenching, which is higher than that of the wild-type plants, but lower than that of the TROL knock-out plants *(31)*. This demonstrates dosage effect of TROL and indicates that FNR binding to the thylakoid membranes is dependent on the availability of tethering sites and that the amount of soluble FNR directly influences NPQ.

It has been proposed that the balance between $NADP^+$ and NADPH regulates the photosynthetic electron transport at the level of cyt b_6/f complex in a feedback manner *(38)*. NADP-malic enzyme 2 that catalyzes the oxidative decarboxylation of malate, producing pyruvate, carbon dioxide and NAD(P)H in cytosol *(40)* was significantly up-regulated in TROL-deficient plants grown under growth-light conditions. This finding suggests the possible pathway of maintaining $NADP^+$/NADPH balance through the malate valve *(31)*. *Trol* plants could act as efficient NADPH producers, exporting the reducing energy in a form of malate to the cytosol to prevent the hyper reduction of the thylakoids. Also, in *trol* plants, genes encoding proteins involved in stress management are strongly up-regulated. As plant growth and develop-

ment are driven by electron transfer reactions *(41)*, it is not surprising that knock-out plants possess altered leaf anatomy and small chloroplasts with underdeveloped thylakoid system. These morphological changes reflect alterations in gene expression of a specific set of genes encoding chloroplast proteins. Many processes important for chloroplast morphogenesis could be influenced by NADPH production, or be dependent on metabolic retrograde signaling *(31)*.

The absence of TROL prevents tethering of FNR to the thylakoid membrane, leaving a substantial amount of FNR soluble. Soluble FNR is no longer able to reduce $NADP^+$ at high rates and, as the consequence, over-reduction of the entire electron transport chain might occur. Regulation of NPQ could be of great importance in prevention photo-damage caused by generation of reduced electron carriers which block LEF before the lumen could be significantly acidified *(42)*.

CONCLUSION

TROL protein is a bona fide thylakoid membrane docking site of crucial photosynthetic enzyme ferredoxin-NADP+ oxidoreductase. Apart from its role in photosynthetic energy conversion, FNR seems to play an important role in redox poising of both thylakoids and stroma. FNR in bacteria acts as an important scavenger of free radicals and it will be interesting to see if it possesses similar function in plant cells. Also, TROL could be the source element in signal-transduction cascade linking photosynthesis with plant growth and cellular responses. TROL possesses several elaborate elements of signal transduction; rhodanese-like domain located in lumen, proline-rich swivel involved in signal attenuation, and FNR membrane recruitment stretch. Supramolecular complexes of TROL and membrane yeast-two-hybrid screens may yet reveal so far overlooked elements of thylakoid signal transduction.

Acknowledgement: *The work of H.F., L.V. and L.H. has been funded by the Grant 098-0982913-2838 of the Ministry of Science, Education and Sports of the Republic of Croatia.*

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