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Energy Conductance from Thylakoid Complexes to Stromal Reducing Equivalents

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1. Introduction

Oxygenic photosynthesis is the basis of heterotrophic life on Earth. It produces carbohydrates and oxygen and may be dived into two sets of reactions: light reactions taking place in the thylakoid membranes, and carbon fixation reactions in soluble stroma. The light reactions involve highly reactive species, and if not controlled properly, they can produce deleterious reactive oxygen species. The structure and function of photosynthetic machinery must be extremely dynamic to enable flawless primary production under a wide spectrum of environmental conditions. The molecular mechanisms behind these dynamic changes remain largely uncharacterized, in particular because various auxiliary proteins linking photosynthesis with physiological responses are still missing.

Cooperation of two photosystems in the chloroplast thylakoid membranes produces a linear electron flow (LEF) from H_2O to $NADP^+$. Efficient photosynthetic energy conversion requires a high degree of integration and regulation of various redox reactions in order to maximize the use of available light and to minimize damaging effects of excess light (Allen, 2002). The interplay between cyclic (CEF), linear, and pseudocyclic electron transport pathways is required for maintaining the poised state of the photosynthetic system (Allen, 2003). In the over-oxidized state there is no electron flow while in the over-reduced state photooxidation can cause damage to photosystems and eventually death. Common to all three pathways is the activity of PSI that transfers electrons from the plastocyanin located in the thylakoidal lumen to the stromal ferredoxin (Fd). This transfer is mediated by three subunits, C, D and E, of the so-called stromal ridge of PSI (Nelson and Yocum, 2006). In the reduced state Fd provides electrons for the ferredoxin:NADP+ oxidoreductase (FNR), which produces NADPH in a linear pathway (Carrillo & Ceccarelli, 2003), for the ferredoxin–thioredoxin reductase (FTR), which catalyses the reduction of chloroplast thioredoxins (Shaodong et al., 2007), for feeding of the CEF (Allen, 2003) or, alternatively, electrons can be transferred to superoxide, the terminal acceptor in pseudocyclic pathway (Allen, 2003). The generation of reducing power is crucial for all biosynthetic processes within chloroplasts. NAD(P)H and ATP may be considered cell's energetic equivalents and are the principal energetic links between membrane-associated redox reactions and metabolism in the cell soluble compartments. These two types of molecules are generated simultaneously in the chloroplast during light-dependent electron transport and photophosphorylation. They are utilized in the reductive assimilation of inorganic elements (carbon, nitrogen, sulphur) into cellular matter, 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. The synthesis of ATP and NADPH in linear electron flow is tightly coupled and if the substrates for the ATP synthase (ADP, inorganic phosphate) become limiting, then the proton motive force builds up, inhibiting electron transfer to $NADP^+$. Likewise, if $NADP^+$ is limiting, photosynthetic electron carriers become reduced, slowing electron transfer and associated proton translocation, thus limiting ATP synthesis (Kramer & Evans, 2006). Linear electron flow 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 (Kramer & Evans, 2006), 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.

2. Ferredoxin: $NADP^+$ oxidoreductase

Final electron transfer from ferredoxin to $NADP^+$ is accomplished by the ferredoxin:$NADP^+$ oxidoreductase (FNR), a key enzyme of photosynthetic electron transport required for generation of reduction equivalents. 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 (Ceccarelli et al., 2004). In higher plants FNR is encoded by a small nuclear gene family and has been found in various chloroplast compartments: at the thylakoid membrane, in the soluble stroma, and at the chloroplast inner envelope. Both the membrane-bound and the soluble FNR pools are photosynthetically active.

2.1 Structure and localization of chloroplast FNRs

FNR harbors one molecule of noncovalently bound FAD as a prosthetic group (Arakaki et al., 1997; Carillo & Ceccarelli, 2003) and it catalyzes reversible electron transfer between reduced Fd to $NADP^+$ for production of NADPH according to the reaction $2Fd_{red} + NADP^+ + H^+ \leftrightarrow 2Fd_{ox} + NADPH$. The FAD cofactor of FNR functions as an one-to-two electron switch by reduction of FAD to a semiquinone form $FADH^{\cdot}$, followed by another round of reduction to $FADH^-$, and hydride transfer from $FADH^-$ to $NADP^+$.

Chloroplast FNR proteins are hydrophilic proteins with a molecular weight of approximately 35 kDa. Sequence similarity of FNRs from various plant species varies from 40% to 97% (Arakaki et al., 1997), and especially regions involved in FAD and $NADP^+$ binding share high degree of identity. The topology of all chloroplast FNRs is highly conserved, consisting of two distinct domains connected by a loop (Dorowski et al., 2001), which shows the biggest variance between the species. The N-terminal domain (ca. 150 amino acids) is made up of a β-barrel structure built of six antiparallel β-strands and capped by an α-helix and a long loop and is involved in FAD binding, while the C-terminal domain (ca. 150 amino acids) consists of a central five-stranded parallel β-sheet surrounded by six α-helices and is mainly responsible for binding of $NADP^+$ (Karplus et al., 1991). Fd is bound to

the large, shallow cleft between the two domains (Pascalis et al., 1993). The amino acids essential for the formation and activity of the Fd-FNR complex have been identified in detail and nuclear magnetic resonance and mutagenesis studies have further revealed that the flexible N-terminus of FNR is also involved in the interaction with Fd (Maeda et al., 2005).
FNR is synthesized on cytosolic rybosomes as a precursor containing an amino-terminal transit peptide, which is responsible for targeting the protein to the chloroplasts (Newman & Gray, 1988). Upon import of FNR into chloroplasts cleavage of the transit peptide by a stromal processing peptidase occurs, followed by the interaction with stromal chaperones Hsp70 (heat shock protein of 70 kDa) and Cpn60 (chaperonin of 60 kDa), which assist in the proper folding of FNR (Tsugeki & Nishimura, 1993), and FAD incorporation, which is required for maintenance of the native structure. Binding of FAD is also a prerequisite for membrane binding of FNR (Onda & Hase, 2004). Regulation of the enzyme activity has been proposed to occur by binding of FNR to the thylakoid membrane (Nakatani & Shin, 1991). Although soluble and membrane-bound FNR form a complex with Fd with the same dissociation constant, the rate constant of $NADP^+$ photoreduction has been shown to be much higher in the membrane bound than in the soluble complex *in vitro* (Forti & Bracale, 1984). But, since the *Arabidopsis fnr1* knock out mutant does not contain any membrane-bound FNR and still possesses normal photosynthetic performance, it may be concluded that *in planta* the soluble FNR is photosynthetically competent (Lintala et al., 2007), and thus the solubility of FNR itself is not a crucial determinant of enzyme activity.

2.2 FNR gene families

In higher plants, chloroplast-targeted FNR is encoded by a small nuclear gene family with one to three *FNR* genes sharing approximately 80% homology with each other. The chloroplast FNR proteins seem to be at least partly redundant, but they also possess unique properties, which are probably required for adjustment of chloroplast metabolism according to changes in the ambient environment (Mulo, 2011). FNR gene families have been well studied in the dicot C_3 plant *Arabidopsis thaliana* (thale cress, Hanke et al., 2005), monocot C_3 plant *Triticum aestivum* (wheat, Gummadova et al., 2007; Grzyb et al., 2008), and monocot C_4 plant *Zea mays* (maize, Okutani et al., 2005).
In *Arabidopsis* two genes, *At5g66190* and *At1g20020*, encode the two distinct ~ 32 kDa leaf isoforms FNR1 and FNR2 (Hanke et al., 2005; Lintala et al., 2007). Both genes are predominantly expressed in the rosette leaves, whereas only minor amount of mRNA could be detected in the stems, flowers and siliques, and no FNR proteins could be detected in the root tissue (Hanke et al., 2005). Chloroplast FNR1 has been shown to be more abundant in the membrane fraction (Hanke et al., 2005), especially at the stroma thylakoids (Benz et al., 2009), whereas FNR2 accumulates in higher amounts in the soluble stroma (Hanke et al., 2005). Indeed, FNR1 serves as a membrane anchor to FNR2, since upon inactivation of *FNR1* all the chloroplast FNR (FNR2) exists as a soluble protein (Lintala et al., 2007). It is very likely that FNR *in vivo* exists as a (hetero)dimer (Hanke et al., 2005). Recently, also formation of large (~ 330 kDa) FNR oligomers, devoid of other proteins, has been documented (Grzyb et al., 2008). Inactivation of one chloroplast *FNR* isoform did not result in upregulation of the expression of the other, neither at the level of transcription nor translation (Lintala et al., 2007; Lintala et al., 2009). Inactivation of either *FNR* gene resulted in general down-regulation of the photosynthetic machinery, but neither of the isoforms showed any specific function in LEF or CEF of photosynthesis, or other alternative electron transfer

reactions (Lintala et al., 2007; Lintala et al., 2009). Growth of the *fnr* knock-out plants under unfavorable conditions revealed a unique role for FNR2 in redistribution of electrons to various redox reactions (Lintala et al., 2009). Beside two leaf-type FNR, two root-type FNR isoenzymes, encoded by genes *At1g30510* and *At4g05390* are present in *Arabidopsis* as well (Hanke et al., 2005). The growth of *Arabidopsis* on different nitrogen regimes induced differential expression of the two chloroplast *FNR* genes showing that multiple FNR isoenzymes have variable metabolic roles and differentially contribute to nitrogen assimilation (Hanke et al., 2005). Studies *in vivo* have revealed that suppression of FNR expression leads to increased susceptibility to photo-oxidative damage, impaired plant growth and lowered photosynthetic activity of transgenic plants (Hajirezaei et al., 2002; Palatnik et al., 2003). On the other hand, overexpression of FNR results in slightly increased rates of electron transport from water to NADP+ and increased tolerance to oxidative stress (Rodriguez et al., 2007).

Based on isoelectric focusing and SDS-PAGE the proteome of wheat has revealed four distinct leaf FNR isoforms that can be divided into two groups, FNRI and FNRII. Both groups contain two proteins, which differ from each other by truncation of the N-terminus (Gummadova et al., 2007; Grzyb et al., 2008). It has been demonstrated that the presence of mature wheat FNR proteins with alternative N-terminal start points, differing by a three amino acid truncation in pFNRI and a two amino acid truncation in pFNRII, have statistically significant differences in response to the physiological parameters of chloroplast maturity, nitrogen regime, and oxidative stress (Moolna et al., 2009). It has been suggested that these isoforms may be crucial to the regulation of reductant partition between carbon fixation and other metabolic pathways. Also, differences in the N-terminus of the wheat FNR isoforms seem to result in changes of FNR activity, subchloroplast localization as well as affinity of FNR to different Fd isoforms (Moolna et al., 2009). Results of Moolna et al. (2009) also suggest that four pFNR protein isoforms are each present in the chloroplast in phosphorylated and nonphosphorylated states, probably as a response to physiological parameters.

The genome of maize codes for three distinct leaf FNR genes that share 83–92% homology with each other, and are present in the leaves at approximately equivalent concentration. FNR1 is found at the thylakoid membrane, FNR3 is an exclusively soluble stromal protein, while FNR2 has a dual location (Okutani et al., 2005). The activity of FNR2 is similar to FNR3, and higher than that of the FNR1, and the mode of interaction between Fd(s) and the FNR isoforms is dependent on both pH and redox status of the chloroplast (Okutani et al., 2005). It has also been proposed that leaf FNR isoenzymes 1 and 2 are relatively more abundant under conditions of high demand for NADPH (Okutani et al., 2005).

2.3 FNR and the cyclic electron flow

FNR functions in the crossing of various electron transfer pathways. Besides its confirmed involvment in the last step of the LEF from water via PSII, plastoquinone (PQ) pool, Cyt b_6f complex, PSI, Fd and FNR to $NADP^+$, the role of FNR in cyclic electron transfer has not yet been defined. 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. It is generally accepted that

CEF supplies the ATP needed for driving the CO_2 concentrating mechanism in the C_4 plants. Recently, the significance of CEF has been shown in C_3 plants as well, where under normal physiological conditions CEF might have a role in adjusting the stoichiometry of ATP/NADPH generated by photosynthesis (Munekage et al., 2004). FNR has been identified as a component of the Cyt b_6f complex (Clark et al., 1984).

Four possible routes of cyclic electron transfer have been proposed so far and may operate in parallel. **NAD(P)H dehydrogenase (NDH)-dependent route**, in which electrons are first transferred from NADPH to NAD(P)H dehydrogenase-1 complex, and secondly to the PQ pool (Kramer & Evans, 2006; Mulo, 2011). The redox reactions are coupled to proton translocation in two ways. First, protons are taken up on the negatively charged side of the membrane during quinone reduction and released on the positive side of the membrane during quinol oxidation. Four protons should be translocated for each electron transferred through the cycle, two via the reduction and oxidation of PQ and the Q cycle and two more via the NDH proton pump (Kramer & Evans, 2006). **Fd-dependent route**, in which electrons are funneled from Fd to Cyt b_6f complex via a partly uncharacterized route involving PGR5 and PGRL1 proteins, which work together to catalyze cyclic electron flow, and possibly FNR and hypothetical FQR (ferredoxin-plastoquinone oxidoreductase) (Munekage et al., 2002; Kramer & Evans, 2006). **Nda2, a type 2 NAD(P)H:PQ oxidoreductase route** is active in some green algae and conifers that lack the chloroplast NDH complex. In *Chlamydomonas*, PQ reduction in CEF has been proposed to occur via a Nda2 (Desplats et al., 2009). It is related to those found in bacteria and mitochondria and does not pump protons. Since type 2 complexes are structurally much simpler than complex I (one subunit with a single flavin cofactor compared with at least 11 protein subunits, nine FeS clusters, and a flavin), Nda2 may be less efficient in energy balancing (Kramer & Evans, 2006). **The Cyt b_6f complex and FNR route** uses the PQ reductase site of the Cyt b_6f complex to reduce PQ (Zhang et al., 2004). Electron transfer to Q_i probably involves the newly discovered heme c_i, which allows electrons to flow from Fd or FNR to the bound PQ (Zhang et al., 2004). This pathway probably involves the formation of a special cyclic electron flow supercomplex (Iwai et al., 2010).

2.4 FNR and oxidative stress tolerance

PSII and PSI in the chloroplasts of higher plants are potential sources of harmful reactive oxygen species (ROS). In *E. coli* FNR is involved in quenching of ROS (Krapp et al., 2002), and in methyl viologen resistant *Chlamydomonas reinhardtii* strains the steady state level of chloroplast FNR transcripts has been shown to be increased as compared to wild type (Kitiyama et al., 1995). Moreover, expression of plant FNR has been proven to restore the oxidative tolerance of a mutant *E. coli* (Krapp et al., 1997). The research on the participation of FNR in oxidative stress responses of higher plants has been performed on wheat and has shown that, in contrast to bacterial cells, the content of FNR mRNA as well as protein in higher plants rather decreases than increases in response to induction of oxidative stress (Palatnik et al., 1997). However, production of ROS results in marked release of FNR from the thylakoid membrane followed by reduction of $NADP^+$ photoreduction capacity, which might aim at maintaining the $NADP^+$/NADPH homeostasis of the stressed plants (Palatnik et al., 1997). Recently, it has been shown that FNR releases from the thylakoids in the plants suffering from drought stress (Lehtimaki et al., 2010), and the FNR containing thylakoid protein complexes disassemble upon high light illumination (Benz et al., 2009).

3. Supramolecular FNR complexes

It has been shown that FNR exists in soluble and membrane-bound forms (Palatnik et al., 1997; Lintala et al., 2007). Several potential FNR-binding partners have been discussed, which might be involved in membrane attachment of FNR. Various studies have shown interaction of FNR with the photosynthetic protein complexes Cyt b_6f (Clark et al., 1984; Zhang et al., 2001), PSI (Andersen et al., 1992) or NDH complex (Quiles & Cuello, 1998), but also interaction with glyceraldehyde-3-phosphate dehydrogenase, or direct membrane attachment have been suggested. However, until recently no exact protein partner responsible for FNR tethering has been identified. Two chloroplast proteins, Tic62 and TROL, were recently identified and shown to form high molecular weight protein complexes with FNR at the thylakoid membrane, and thus seem to act as molecular anchors of FNR to the thylakoid membrane. Tic62 and TROL have been shown to bind FNR by specific interaction via a conserved Ser/Pro-rich motif. In darkness, FNR forms large protein complexes at the thylakoids together with Tic62 and TROL. Similarly, Tic62 and presumably TROL bind FNR at the envelope. FNR is released from the membranes upon illumination.

3.1 Tic62

During its import into chloroplasts from the site of synthesis on cytosolic rybosomes, FNR has been found to interact with Tic62 protein, a 62 kDa component of the Translocon at the inner envelope of chloroplast (Küchler et al., 2002; Balsera et al., 2007; Stengel et al., 2008; Benz et al. 2009). Proteomics studies have identified Tic62 in the chloroplast envelope, stroma and thylakoid fraction (Benz et al., 2009). 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 (Benz et al., 2009). The N-terminus of Tic62 binds pyridine nucleotides, while the stroma exposed C-terminus contains repetitive, highly conserved FNR-binding domains (Küchler et al., 2002). Database searches have verified the presence of the FNR-binding domains only in the Tic62 protein of vascular plants (Balsera et al., 2007) and it occurs in different numbers dependent on the respective plants species.

The function of FNR in the Tic complex has been suggested to link redox regulation to chloroplast protein import. Indeed, *in vitro* experiments with compounds interfering either with NAD binding or NAD(P)/NAD(P)H ratio modulate the import characteristics of the leaf FNR isoforms: FNR1 is translocated preferentially at high NAD(P)/NAD(P)H ratio, while the translocation of FNR2 is not influenced by the redox status (Küchler et al., 2002). In maize, import of pre-FdI to chloroplast stroma is independent on illumination, while pre-FdIII and preFNR were efficiently targeted into stroma only in darkness (Hirohashi et al., 2001). These results imply that the diurnal changes in the chloroplast redox poise may control import characteristics of the organelle. It was recently shown that Tic62 shuttles between the soluble stroma and the chloroplast membranes, and that oxidation of stroma results in stronger association of Tic62 to the membrane fraction (Stengel et al., 2008). FNR shows similar shuttling behavior, and therefore the Tic62–FNR interaction is dependent on chloroplast redox state (Stengel et al., 2008). The lack of Tic62 and consequently the lack of Tic62–FNR complexes did not have any effects on the plant phenotype or photosynthetic properties, neither on LEF nor CEF (Benz et al., 2009), implying that the Tic62–FNR complexes serve for some other purpose(s) than photosynthesis. It has been observed that

the membrane-bound Tic62–FNR protein complexes were most abundant in the dark, while increase in light intensity resulted in the disassembly of the complex. Similarly, *in vitro* alkalization of isolated thylakoids dissociated FNR and Tic62 (Benz et al., 2009). It is important to stess that the interaction of Tic62 with FNR stabilizes the activity of the FNR protein (Benz et al., 2009) and that FNR activity is lower in acidic than basic environment (Lee et al., 2007). These results indicate that Tic62 acts as a chaperone for FNR, and protects the flavoenzyme from inactivation and degradation during the photosynthetically inactive periods, e.g. in darkness (Benz et al., 2009).

3.2 TROL

By using antisense and gene inactivation strategies Jurić et al. (2009) identified a novel component of non-appressed thylakoid membranes which is responsible for anchoring of FNR. TROL (thylakoid rhodanese-like protein) is a 66 kDa nuclear encoded component of thylakoid membranes required for tethering of FNR and sustaining efficient LEF in vascular plants. TROL contains two transmembrane helices and a centrally positioned (inactive) rhodanese domain (Jurić et al., 2009). As an integral membrane protein, TROL is firmly attached to the thylakoid membrane and cannot be extracted from the membrane by high salt, urea or high pH treatments (Jurić et al., 2009). 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 the vascular plants (Balsera et al., 2007). It is hypothesised that both N- and C-terminal parts of TROL face the stroma, while RHO faces the thylakoid lumen (Jurić et al., 2009). 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. It consists of two identical repeats, followed by a possible PVP hinge. In membrane proteins, prolines are known to have a structural role in transmembrane helices, where they distort the alpha-helix due to the loss of at least one stabilising backbone hydrogen bond. Thus, PEPE region, which is presumed to be exposed into the stroma, 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. (2002) that Tic62 interacts with FNR prompted the analysis of TROL protein sequence in search for the similar binding module. 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 (Küchler et al., 2002). 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 (Küchler et al., 2002). 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 (Küchler et al., 2002; Balsera et al., 2007). As TROL possesses almost identical domain to the Tic62 FNR-binding repeats, a modified yeast-two hybrid assay was used to confirm ITEP-FNR interaction (Jurić et al., 2009). Predictably, ITEP strongly binds to

the FNR protein, even eight times stronger than Tic62. Established high-affinity interaction with FNR, together with the reports on TROL abundance at the thylakoid membranes (Peltier et al., 2004), implies that we are probably not dealing with highly dynamical and fast-responding interaction, but with more quantitative-based and rather inert interaction. The size of the complex is, however, smaller (190 kDa) than the Tic62–FNR complexes (250-500 kDa), which is in line with only one FNR binding motif found in the amino acid sequence of *Arabidopsis* TROL protein, as compared to four of such motifs present in the sequence of *Arabidopsis* Tic62. Although *in vitro* experiments indicate that the interaction between TROL and FNR is stronger than the interaction between FNR and Tic62 (Jurić et al., 2009), the exposure of plants to high light intensity results in faster dissociation of FNR from the TROL–FNR complexes than from the Tic62–FNR complexes (Jurić et al., 2009).

Using a synthetic peptide, representing the conserved binding motif, called the FNR-membrane-recruiting-motif (FNR-MRM) found in Tic62 and TROL, Alte et al. (2010) determined the crystal structure of the FNR:peptide complex and concluded that the FNR-MRM induces self-assembly of two FNR molecules. Although FNR is commonly distributed among all three domains of life, FNR-MRM of both Tic62 and TROL exclusively exists in vascular plants, thus, membrane tethering of FNR by this motif seems to be a recent evolutionary invention (Alte et al., 2010). Whereas most TROL proteins comprise a single FNR-binding domain, its number varies to a higher extent in Tic62 proteins. However, the binding affinity to FNR did not change significantly when constructs comprising one or three FNR-interacting motifs were analyzed (Alte et al., 2010). This indicates that binding to each domain occurs independently of the other motifs and excludes cooperative binding effects.

3.2.1 Redox regulation and tethering properties of TROL

Besides being involved in sulfur metabolism, rhodanese-like domains are implicated in redox regulation of various intracellular processes (Horowitz & Falksen, 1986; Horowitz et al., 1992; Nandi et al., 2000). It has been speculated that the rhodanese-like domain of TROL is involved in redox regulation of FNR binding and release (Jurić et al., 2009). It has been proposed that the redox regulation of FNR binding and release could be important for balancing the redox status of stroma with the membrane electron transfer chain. Such regulation could be important for prevention of over-reduction of any of these two compartments and maintenance of the redox poise (Jurić et al., 2009).

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 (70 kDa). Localization of the TROL precursor at the chloroplast envelope in its unprocessed form 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, similar to the shuttling of the Tic62 protein (Stengel et al., 2008).

FNR is supposed to be the key protein in transferring electrons to the final destination in LEF and *trol* plants indeed exhibit decreased LEF. On the basis of the investigation of membrane-bound pool of FNR and the 190 kDa-complex containing TROL and FNR in the wild-type plants grown under growth-light and high-light intensities (Jurić, 2010), a role for TROL as the FNR anchor could be proposed. TROL anchors and thus stabilises FNR during the night, possibly to prevent FNR from extracting electrons from NADPH molecules and compromising 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 (Benz et al., 2009). FNR could be gradually released by binding/releasing of certain signalling molecules to the luminal RHO domain, and electrons are transferred from ferredoxin to $NADP^+$ at normal rates (Figure 1). Under the high-light/excess-light intensities TROL discharges FNR, possibly because of binding/releasing of certain signalling molecules to the luminal RHO domain, and FNR now catalyses the reverse reaction of transferring electrons from excess NADPH to potential electron-acceptor molecules (Figure 1) (Jurić et al., 2010). The PEPE swivel that preceeds the ITEP domain could maneuver the bound FNR protein due to the proline-introduced flexibility. For instance, the free-moving PEPE swivel 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, if the binding of FNR to the thylakoids is a precondition for efficient LEF (Forti & Bracale, 1984), then the TROL-bound FNR molecules could be easily displaced from TROL to the already discovered FNR-binding membrane proteins or the unknown ones. This is in accordance with the clearly visible TROL-FNR complex under dark conditions and with its disappearance during light periods (Benz et al., 2009).

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 (Alte et al., 2010). The pH variations reflect differences of the chloroplast stroma between light and dark cycles (Alte et al., 2010): During light phases, when photosynthetic activity is high, protons are transported into the thylakoid lumen, leading to an alkaline 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 (Benz et al., 2009; Jurić et al., 2009). Light quantity can vary dramatically during the course of the day, 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 (Stengel et al., 2008). 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.

4. FNR, TROL and Tic62 *Arabidopsis* mutants

When the amount of FNR was artificially reduced by antisense or silencing techniques (Hajirezaei et al., 2002; Lintala et al., 2009), or by interruption of a *FNR* gene by T-DNA (Lintala et al., 2007; Hanke & Hase, 2008), the plants suffered from chlorosis and reduced photosynthetic activity, which finally resulted in reduced growth. Although the level of total NADP(H) was not affected in the mutants, the NADPH/$NADP^+$ ratio was strongly reduced (Hajirezaei et al., 2002; Hald et al., 2008). These mutants are prone to photo-oxidative damage, and suffer from oxidative stress (Palatnik et al., 2003; Lintala et al., 2009). The redox poise of the NADP(H) pool is also likely to regulate photosynthetic electron transfer activity in order to balance production and consumption of reducing equivalents, and thereby to limit production of ROS in the chloroplasts (Hald et al., 2008). Over-expression of FNR, however, did not markedly up-regulate the rate of $NADP^+$ photoreduction or CO_2 assimilation, but showed increased tolerance to photodamage (Rodriguez et al., 2007).

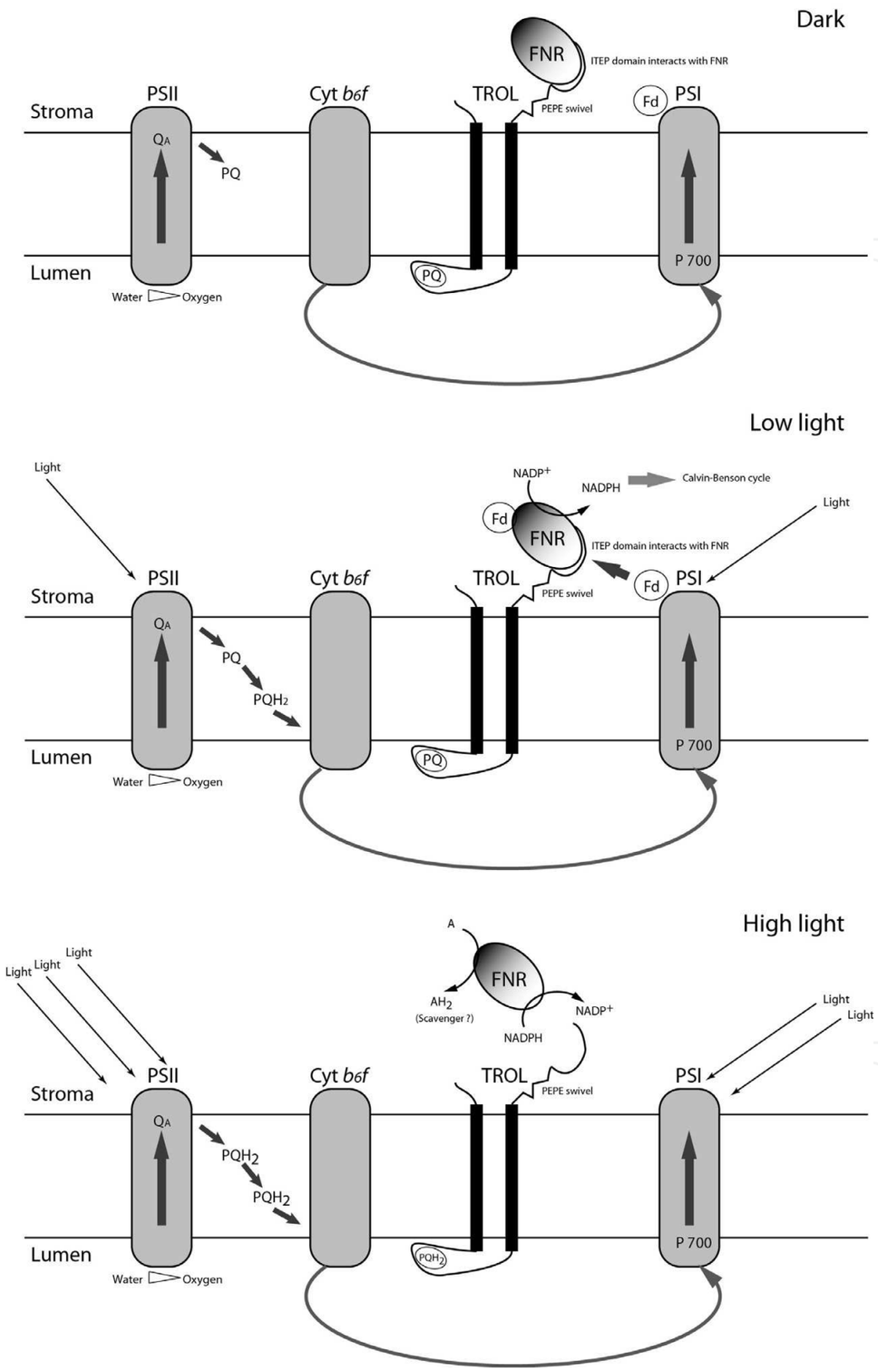

Fig. 1. The proposed mechanism of redox regulation of FNR binding and release. During the dark period, FNR is bound to the thylakoids *via* TROL. This stage could be sustained through the binding of small molecule, possibly oxidized PQ, to the RHO cavity. There is no

NADPH production. In conditions of growth-light, FNR is bound to the thylakoids *via* TROL and acts as an efficient NADPH producer. In conditions of saturating light, a molecule, possibly reduced PQ, competes for the RHO binding site and generates the signal for the FNR release, which, when soluble, acts as NADPH consumer. Protons are passed to an unknown scavenger A.

Overexpression of FNR in transgenic plants causes enhanced tolerance to photo-oxidative damage and herbicides that propagate reactive oxygen species (Rodriguez et al., 2007). On the other hand, antisense repression of FNR renders transgenic plants abnormally prone to photo-oxidative stress (Palatnik et al., 2003).

Analysis of *Arabidopsis* mutant lines indicates that, in the absence of TROL, relative electron transport rates at high-light intensities are severely lowered accompanied with significant increase in non-photochemical quenching (NPQ). If solubilization of FNR is necessary for the regulation of oxidative stress, then 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 (Hald et al., 2008). Thus, TROL might represent a missing 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 (Jurić et al., 2009).

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 (Jurić et al., 2009). 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 (Hald et al., 2008). Interestingly, NADP-malic enzyme 2 that catalyses the oxidative decarboxylation of malate, producing pyruvate, carbon dioxide and NAD(P)H in cytosol (Wheeler et al., 2005) was significantly up-regulated in TROL-deficient plants grown under growth-light conditions, thus providing the possible pathway of maintaining $NADP^+$/NADPH balance through the malate valve (Jurić et al., 2009). In this case, *trol* plants would act as efficient NADPH producers, and, in an effort not to hyperreduce the thylakoids, they would export the reducing energy in a form of malate to the cytosol.

Also, in *trol* plants, genes encoding proteins involved in stress management are strongly up-regulated. As plant growth and development are driven by electron transfer reactions (Noctor, 2006), it is not surprising that leaf anatomy is altered in the knock-out. Furthermore, chloroplasts in the knock-out are small and have less developed thylakoids. 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 (Jurić et al., 2009).

When Tic62 was knocked out, formation of high molecular weight FNR protein complexes was hindered, while some free FNR still was detected at the thylakoids of *tic62* plants. The amount of FNR in the soluble pool, however, remained more or less constant. *Vice versa*, if either one of the chloroplast targeted FNR isoforms was missing the membrane binding of the Tic62 protein was prevented. Since no changes in the *FNR* gene expression or in the FNR

pre-protein import could be detected in the *tic62* plants, reduction of FNR level most probably resulted from differences in the turnover of FNR isoforms inside the chloroplasts (Benz et al., 2009).

In contrast to *tic62* plants, *trol* knock-out mutants have a clear photosynthetic phenotype (Jurić et al., 2009). The appearance of the *trol* plants is slightly smaller than the WT, but with no distinct differences in pigment composition. However, the mutant chloroplasts are small, irregular in morphology and show deteriorated thylakoid structure. The abnormalities in chloroplast structure are reflected on the function with marked differences in electron transfer rate under high light intensity (500 to 800 μmol photons m^{-2} s^{-1}). Since nonphotochemical quenching increases and variable chlorophyll fluorescence decreases in the *trol* leaves upon increasing illumination, it seems that the absence of TROL results in increased ability to dissipate excess absorbed energy.

The absence of TROL disables FNR from being tethered to the membrane, therefore a substantial amount of FNR remains soluble. Forti & Bracale (1984) demonstrated that the soluble form of FNR is very inefficient in $NADP^+$ photoreduction by isolated thylakoids. It has been shown in TROL-deficient plants that linear photosynthetic flow can be sustained until light intensity exceeds 250 μmol photons m^{-2} s^{-1}. As soluble FNR is no longer able to reduce $NADP^+$ at high rates, this could lead to over-reduction of the entire electron transport chain. In this case, NPQ modulation could be particularly important to prevent photo-damage caused by build-up of reduced electron carriers which block LEF before the lumen could be significantly acidified (Kanazawa & Kramer, 2002). In conclusion, TROL is necessary for maintenance of efficient LEF, induction of NPQ, as well as for redox-regulation of key thylakoid signal-transduction pathways. Furthermore, discovery of TROL provides new information for linking leaf and chloroplast morphogenesis with photosynthetic cues.

5. Conclusion

The discovery of TROL protein and its important role in tethering of the flavoenzyme FNR not only answers the old dilemma about the position at which this crucial photosynthetic enzyme is located, but opens new approaches for the investigations of oxidative stress management in chloroplasts. 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. Linking FNR with cellular energetics and possibly with retrograde signaling will also be investigated. Is TROL the source element in signal-transduction cascade linking photosynthesis with plant growth and cellular responses? TROL possesses several elaborate elements of signal transduction, namely rhodanese-like domain located in lumen, proline-rich swivel involved in signal attenuation, and FNR membrane recruitment motif. Functions of each of these domains will be investigated *in planta*, by using genetic transformation techniques. Global gene expression analysis revealed that genes depending on NADPH synthesis and availability are up-regulated in TROL deficient plants. Among them are NADP-malic enzyme and protochlorophyllide oxidoreductase B. How is TROL linked with malate shuttle enzymes? What is the role of TROL and Tic62 in the inner envelope membrane? Finally, other proteins following the same expression pattern in correlation analyses will be investigated for their ability to interact with TROL and Tic62. Supramolecular complexes of TROL and membrane yeast-two-hybrid screens will likely reveal so far overlooked elements of thylakoid signal transduction. An exciting quest for auxiliary proteins involved in fine-tuning of photosynthetic energy conversion lies ahead.

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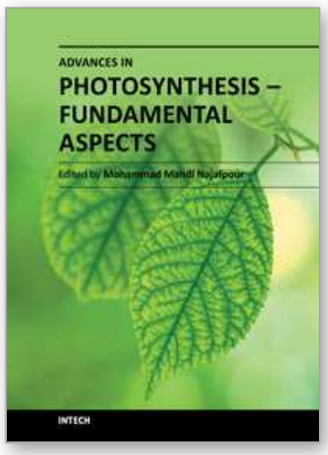

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