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LEAF-TARGETED FERREDOXIN-NADP$^+$ OXIDOREDUCTASE (FNR)

Electron Transfer Properties and Thylakoid Association in *Arabidopsis thaliana*

by

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LIST OF ORIGINAL PUBLICATIONS

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II Lintala M, Allahverdiyeva Y, Kangasjärvi S, Lehtimäki N, Keränen M, Rintamäki E, Aro EM, Mulo P. (2009) Comparative analysis of leaf-type ferredoxin-$NADP^+$ oxidoreductase isoforms in *Arabidopsis thaliana*. The Plant Journal 57:1103-1115.

III Benz JP, Stengel A, Lintala M, Lee YH, Weber A, Philippar K, Gügel IL, Kaieda S, Ikegami T, Mulo P, Soll J, Bölter B. (2009) *Arabidopsis* Tic62 and ferredoxin-NADP(H) oxidoreductase form light-regulated complexes that are integrated into the chloroplast redox poise. The Plant Cell 21:3965-3983.

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ABBREVIATIONS

APX	ascorbate peroxidase
ATP	adenosine triphosphate
AUC	analytical ultra centrifugation
BN	blue native
CET	cyclic electron transfer around PSI
Chl	chlorophyll
Cyt	cytochrome
F1, F2	first and second generation of crossing, respectively
FAD	flavin adenine dinucleotide
Fd	ferredoxin
FNR	ferredoxin-$NADP^+$ oxidoreductase
FQR	ferredoxin-plastoquinone reductase
GR	glutathione reductase
His	histidine
HMW	high molecular weight
IEF	isoelectric focusing
LHC	light harvesting complex
MDH	malate dehydrogenase
MV	methyl viologen
NADPH	nicotinamide adenine dinucleotide phosphate
NDH	NAD(P)H dehydrogenase
NMR	nuclear magnetic resonance
NPQ	non-photochemical quenching of Chl fluorescence
NR	nitrate reductase
OEC	oxygen evolving complex
P680, P700	reaction center Chl of PSII and PSI, respectively
PAGE	polyacrylamide gel electrophoresis
PC	plastocyanin
pI	isoelectric point
PQ	plastoquinone
PS	photosystem
PTOX	plastid terminal oxidase
q-RT-PCR	quantitative real time polymerase chain reaction
R1	repetative sequence at Tic62 C-terminus
RNAi	RNA interference
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
T-DNA	transfer DNA
Tic	translocon of the inner envelope membrane of chloroplast
Toc	translocon of the outer envelope membrane of chloroplast
Trx	thioredoxin
VDE	violaxanthin de-epoxidase

ABSTRACT

Photosynthetic reactions are divided in two parts: light-driven electron transfer reactions and carbon fixation reactions. Electron transfer reactions capture solar energy and split water molecules to form reducing energy (NADPH) and energy-carrying molecules (ATP). These end-products are used for fixation of inorganic carbon dioxide into organic sugar molecules. Ferredoxin-$NADP^+$ oxidoreductase (FNR) is an enzyme that acts at the branch point between the electron transfer reactions and reductive metabolism by catalyzing reduction of $NADP^+$ at the last step of the electron transfer chain.

In this thesis, two isoforms of FNR from *Arabidopsis thaliana*, FNR1 and FNR2, were characterized using the reverse genetics approach. The *fnr1* and *fnr2* mutant plants resembled each other in many respects. Downregulation of photosynthesis protected the single *fnr* mutant plants from excess formation of reactive oxygen species (ROS), even without significant upregulation of antioxidative mechanisms. Adverse growth conditions, however, resulted in phenotypic differences between *fnr1* and *fnr2*. While *fnr2* plants showed downregulation of photosynthetic complexes and upregulation of antioxidative mechanisms under low-temperature growth conditions, *fnr1* plants had the wild-type phenotype, indicating that FNR2 may have a specific role in redistribution of electrons under unfavorable conditions. The heterozygotic double mutant (*fnr1xfnr2*) was severely devoid of chloroplastic FNR, which clearly restricted photosynthesis. The *fnr1xfnr2* plants used several photoprotective mechanisms to avoid oxidative stress.

In wild-type chloroplasts, both FNR isoforms were found from the stroma, the thylakoid membrane, and the inner envelope membrane. In the absence of the FNR1 isoform, FNR2 was found only in the stroma, suggesting that FNR1 and FNR2 form a dimer, by which FNR1 anchors FNR2 to the thylakoid membrane. Structural modeling predicted formation of an FNR dimer in complex with ferredoxin. In this thesis work, Tic62 was found to be the main protein that binds FNR to the thylakoid membrane, where Tic62 and FNR formed high molecular weight complexes. The formation of such complexes was shown to be regulated by the redox state of the chloroplast. The accumulation of Tic62-FNR complexes in darkness and dissociation of complexes from the membranes in light provide evidence that the complexes may have roles unrelated to photosynthesis. This and the high viability of *fnr1* mutant plants lacking thylakoid-bound FNR indicate that the stromal pool of FNR is photosynthetically active.

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1. INTRODUCTION

1.1. Description of chloroplasts

Plants and other photosynthetic organisms are able to convert light to chemical energy in photosynthetic electron transfer reactions. In these reactions, light energy and electrons originating from water molecules are used for reduction of $NADP^+$. Concomitantly, electron transfer reactions create a proton gradient across the thylakoid membrane, which is used to run the production of ATP. The energy stored in these molecules is used by all living organisms to perform their physiological functions.

Chloroplasts are characteristic organelles of photosynthetic eukaryotic organisms. Apart from photosynthesis, many other important biosynthetic reactions occur (at least partly) in chloroplasts. According to endosymbiotic theory, about 1.5 billion years ago a eukaryotic host cell engulfed a free-living photosynthetic prokaryote, an ancestor of present-day cyanobacteria, which during evolution became fully integrated into the metabolism of photosynthetic eukaryotes (Hedges et al., 2004; Yoon et al., 2004). Chloroplasts are surrounded by a double lipid bilayer – an envelope composed of the outer and the inner membrane – and they contain a specialized internal membrane system, the thylakoid membrane, where the photosynthetic electron transfer reactions occur (Figure 1). Thylakoid membranes are highly organized into appressed membranes, called grana stacks, and non-appressed membranes called stroma thylakoids, which connect the grana stacks. The soluble compartment inside the sack-like thylakoid membranes is called the lumen (Figure 1). Most of the metabolic reactions – e.g. photosynthetic carbon fixation reactions – take place in the stroma, which is a soluble compartment of the chloroplasts that surrounds thylakoid membranes (Figure 1).

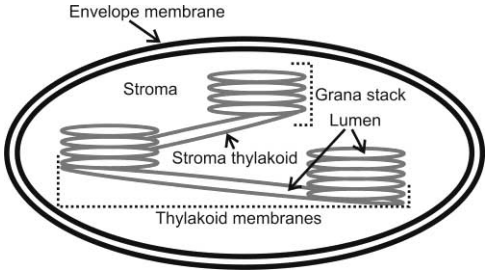

Figure 1. Schematic representation of a chloroplast. Chloroplasts are surrounded by a double membrane, the outer and inner envelope membranes. Thylakoid membranes are arranged in piled grana stacks and connecting stroma thylakoids. The soluble compartment around the thylakoid network is called the stroma and the soluble compartment enclosed by thylakoid membranes is called the lumen.

During the evolution of chloroplasts, the majority of genes originating from the bacterial ancestor of the chloroplasts were lost or transferred to the nucleus of the host cell (Martin and Herrmann, 1998; Kleine et al., 2009). Since the circular genome of chloroplasts, the plastome, contains less than 100 protein-coding genes and the chloroplast proteome is estimated to consist of 3,500–4,000 polypeptides (Leister 2003), several mechanisms for re-targeting and transport of nuclear-encoded, cytosol-translated, but chloroplast-located proteins had to be developed. Most of the nuclear-

encoded chloroplast proteins are transported to chloroplasts through the general import apparatus, which is composed of translocons of the outer and inner envelope membranes of chloroplasts (Toc and Tic, respectively; Vothknecht and Soll, 2005). Import-competent forms of the proteins, 'preproteins', contain an N-terminal transit peptide, which is needed for sorting and recognition and is cleaved off by the stromal processing peptidase after import (Bruce, 2001).

1.2. Protein import apparatus

1.2.1. The Toc complex

At the outer envelope membrane, preproteins are recognized by the Toc complex and import is initiated in a GTP/GDP-dependent manner (Agne and Kessler, 2009). The core of the Toc complex is composed of three proteins: Toc159, Toc75, and Toc34 (Schnell et al., 1997; Schleiff et al., 2003), which form a complex that is responsible for recognition of the preprotein and its translocation across the outer envelope membrane. In addition, two more proteins with less well known functions, Toc64 and Toc12, are associated with the Toc complex (Sohrt and Soll, 2000; Becker et al., 2004a; 2004b).

1.2.2. The Tic complex

Although seven proteins have been identified so far in the Tic complex, the exact composition of the complex has not yet been resolved. Tic110 is one of the most abundant proteins in the inner envelope membrane and it is able to form a cation active channel (Balsera et al., 2009). In addition to Tic110, Tic20 has also been predicted to form a protein-selective channel at the inner envelope membrane (Heins, et al 2002; Kessler and Blobel, 1996; Balsera et al., 2009; Ma et al., 1996; Kouranov and Schnell, 1997). Interaction of the preprotein with the Tic complex and translocation through the inner envelope membrane requires high concentrations of ATP (Grossman et al., 1980). The motor complex of Tic has been predicted to be composed of Tic40 and stromal chaperones with ATPase activity (Chou et al., 2003). Tic22 is a peripheral subunit located at the intermembrane face of the inner envelope membrane (Kouranov et al., 1998). It has been shown to interact with Toc64 and Toc12 (Becker et al., 2004b), indicating that it may be involved in promoting the contact site between the Toc and Tic complexes, or it may have a role as a preprotein receptor at the intermembrane space.

For efficient protein import, additional regulatory subunits have evolved. Based on their redox-active domains, Tic32, Tic55 and Tic62 have been predicted to form a redox regulon of the Tic complex, which can sense and quickly react to signals that give information about chloroplast status. Tic32 and Tic62 belong to an extended family of short-chain dehydrogenases containing NADP(H)-binding domains and showing enzymatic activity (Chigri et al., 2006; Stengel et al., 2008). The composition of the Tic complex changes according to the stromal $NADP^+$/NADPH ratio, as reduced conditions dissociate Tic32 and Tic62 from the Tic complex (Chigri et al., 2006; Stengel et al. 2008). Additionally, calcium signaling may regulate protein import since

Tic32 has been found to interact with calmodulin (Chigri et al., 2006). Tic55 is a member of a small family of non-heme oxygenases containing a Rieske-type iron-sulfur cluster (Caliebe et al., 1997), and recently it was found to be a target of thioredoxins (Bartsch et al., 2008), thus adding one more potential regulatory point to the import machinery.

1.2.2.1. Tic62

Tic62 has two structurally and functionally distinct domains (Küchler et al., 2002; Balsera et al., 2007; Stengel et al., 2008). The N-terminal part of the protein contains an NADPH binding site and belongs to a well-conserved group of dehydrogenases, whereas the C-terminus contains repetitive sequence modules consisting of 30 amino acids with a random coiled structure, including a highly conserved KPPSSP motif (Küchler et al., 2002; Balsera et al., 2007). With the yeast two-hybrid system, ferredoxin-$NADP^+$ oxidoreductase (FNR) was found to interact strongly with the repetitive module of the C-terminus of Tic62 (Küchler et al., 2002). Interaction of Tic62 with the membranes is dependent on the N-terminus, while the sequences between N- and C-terminal regions are needed for interaction of Tic62 with the Tic complex (Stengel et al., 2008).

1.3. Photosynthesis

Photosynthesis is separated spatially and functionally in two parts. In thylakoid membranes, light-driven electron transfer reactions use electrons from water molecules to create reducing energy and ATP. Concomitantly, molecular oxygen is released to the atmosphere as a by-product of photosynthesis. In the Calvin-Benson cycle, NADPH and ATP are used in a series of assimilatory reactions to fix inorganic carbon dioxide (CO_2) to organic carbohydrates in the stroma. Although light is indispensable for photosynthesis, it also has a high potential to damage the photosynthetic machinery as it creates exceptionally oxidizing and reducing components. To avoid light-induced damage, plants have evolved various photoprotective mechanisms, which help in maintaining the photosynthetic activity (Niyogi, 1999).

1.3.1. The photosynthetic machinery and linear electron transfer reactions

The thylakoid-embedded pigment-protein complexes, photosystems (PS) I and II, together with their light harvesting antennae, and cytochrome b_6f complex (Cyt b_6f) are responsible for gathering light energy, converting it to chemical energy, and transferring electrons to acceptors in the stroma (Figure 2A; for a review see Merchant and Sawaya, 2005). PSII is a multi-subunit pigment-protein complex that catalyzes electron transfer from water to plastoquinones (PQ). Chlorophyll molecules in PSII and in the light harvesting complex of PSII (LHCII) absorb light energy, leading to excitation of the reaction center chlorophyll of PSII (P680). P680* (* denotes the excited molecule) donates electrons onward to the electron acceptors of PSII, finally reducing the PQ pool in the thylakoid membrane. Highly oxidizing $P680^+$ drives the complex reactions of water splitting in the oxygen evolving complex (OEC), which is

located on the lumenal side of PSII (Renger and Renger, 2008). Oxidation of water leads to a release of molecular oxygen and protons into the lumen. Electrons released from water are used to fill the electron hole of $P680^+$. From PSII, electrons are transferred further via the PQ pool to the Cyt b_6f complex. In the Cyt b_6f complex, one of the two electrons from fully reduced and protonated PQH_2 is transferred to the small lumenal electron carrier protein plastocyanin (PC), and the other back to an oxidized plastoquinone in the so-called Q-cycle. PC mediates electrons to PSI where the oxidized reaction center chlorophyll of PSI ($P700^+$) is the electron acceptor. Electrons in PSI are transferred from excited P700* via various electron carriers to the stromal side of the thylakoid membrane, and are used for reduction of the small soluble protein ferredoxin (Fd). In the last step of linear photosynthetic electron transfer reactions, the flavoenzyme ferredoxin-$NADP^+$ oxidoreductase (FNR) reduces $NADP^+$ to NADPH. Water splitting and the action of the electron transfer reactions lead to the formation of a proton gradient (ΔpH) across the thylakoid membrane, which is used to drive ATP synthase and production of ATP (Dekker and Boekema, 2005). Photosynthetic protein complexes are composed of nuclear- and plastome-encoded proteins, and therefore the mechanisms for coordination of gene expression in the plastid and the nucleus are required to maintain organelle function.

The photosynthetic protein complexes are not uniformly arranged in the thylakoid membrane. PSII and LHCII are mainly located in grana stacks whereas PSI, LHCI, and ATP synthase are located in stroma lamellae (Andersson and Anderson, 1980; Danielsson et al., 2004). The Cyt b_6f complex, however, is distributed more evenly along the thylakoid membrane (Albertsson et al., 1991; Vallon et al., 1991).

1.3.2. Alternative electron transfer routes

In addition to the linear photosynthetic electron transfer reactions, cyclic and pseudo-cyclic electron transfer reactions – coupled to ATP synthesis without net accumulation of reducing energy – also take place in chloroplasts. The relevance and molecular composition of these reactions is not yet clear, but the reactions could balance the NADPH/ATP ratio to meet the metabolic demands of chloroplasts (Shikanai, 2007).

1.3.2.1. Cyclic electron transfer around PSI

Cyclic electron transfer reactions (CET) share many components with linear electron flow, such as Cyt b_6f, PC, PSI, and Fd (Figure 2B). Although mutational analysis has previously provided evidence for the importance of CET in terrestrial C3 plants (Munekage et al., 2004), the ultimate physiological significance and routes of cyclic electron transfer are still enigmatic. The NDH (NAD(P)H dehydrogenase) complex mediates electron transfer from NADPH to the PQ pool and via the Cyt b_6f complex back to PSI (Rumeau et al., 2007). In addition, the NDH complex plays a role in chlororespiratory reactions, in which PQ-mediated reduction of molecular oxygen to water via the plastid terminal oxidase (PTOX) takes place in darkness (Peltier and Cournac, 2002; Rumeau et al., 2007). Another Fd-dependent CET route has been postulated to contain a still uncharacterized ferredoxin-plastoquinone reductase (FQR, Bendall and Manasse, 1995), which mediates electrons directly (or via the Cyt b_6f

complex) from reduced Fd to the PQ pool (Shikanai, 2007). The Fd-dependent route is not yet well characterized, since only the PGR5 (Munekage et al., 2002) and PGRL1 (DalCorso et al., 2008) proteins – which are known to have rather regulative roles than catalytic roles – have been identified in this pathway.

1.3.2.2. Electron donation to molecular oxygen and antioxidative systems in the chloroplast

Besides cyclic electron transfer, molecular oxygen is thought to be one of the major alternative acceptors of photosynthetic electrons (Asada, 1999; Ort and Baker, 2002). Oxygen can be reduced by PTOX, which uses electrons from the PQ pool, but electrons can also be directed to molecular oxygen from PSI in the Mehler reaction (Figure 2C; Mehler, 1951). In this reaction the reduction of oxygen is only partial, leading to production of reactive oxygen species (ROS). Even low levels of ROS can inhibit photosynthesis by oxidizing the SH residues of thiol-modulated enzymes, e.g. various Calvin-Benson cycle enzymes (see below), so immediate detoxification of ROS is indispensable to maintain photosynthetic activity. In addition, ROS have the capacity to irreversibly damage the lipids, proteins, pigments, and nucleic acids. Plant chloroplasts comprise enzymatic and non-enzymatic antioxidative mechanisms, which help plants cope with oxidative stress (Niyogi, 1999). In the so-called water–water cycle, enzymatic and non-enzymatic antioxidative components reduce oxygen step-by-step to water in a safe way to avoid accumulation of ROS (Figure 2C). The superoxide radical (O_2^-) is the reactive product of one-electron reduction of oxygen, and it is dismutated spontaneously or via catalysis by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2; Asada, 2000). Fast scavenging of H_2O_2 prevents reaction of H_2O_2 with transition metals (Fe, Cu) or with O_2^-, which would lead to the formation of highly reactive hydroxyl radicals (HO·). Ascorbate preoxidase (APX) enzymes catalyze the reduction of H_2O_2 to water using ascorbate as an electron donor. In addition to APX and ascorbate, other molecules – such as peroxiredoxins and glutathione – also scavenge H_2O_2. Reduction of H_2O_2 by APX leads to the formation of an oxidized form of ascorbate, monodehydroascorbate (MDA), which is reduced back to ascorbate in the ascorbate-glutathion cycle (Figure 2C). An enzyme called monodehydroascorbate reductase (MDAR) catalyzes the reduction of MDA to ascorbate. Alternatively, two MDA molecules can be disprotonated to ascorbate and dehydroascorbate (DHA), which is then reduced to ascorbate by glutathione. The energy for MDAR and reduction of glutathione comes from NADPH (Figure 2C).

ROS are also produced in PSII. Light absorption in PSII and LHCII causes excitation of Chl molecules to the singlet excitation state (^{1}Chl). ^{1}Chl can convert to triplet Chl (^{3}Chl) through intersystem crossing. ^{3}Chl can then interact with O_2 to produce reactive singlet oxygen ($^1O_2$) (Krieger-Liszkay, 2004). $^1O_2$ can cause oxidative damage to the photosynthetic machinery and membrane lipids. Dangerous triplet chlorophyll can be quenched by carotenoids that are bound to the antenna system. In addition, carotenoids together with α-tocopherol located in the thylakoid membrane can scavenge singlet oxygen (Kruk et al., 2005).

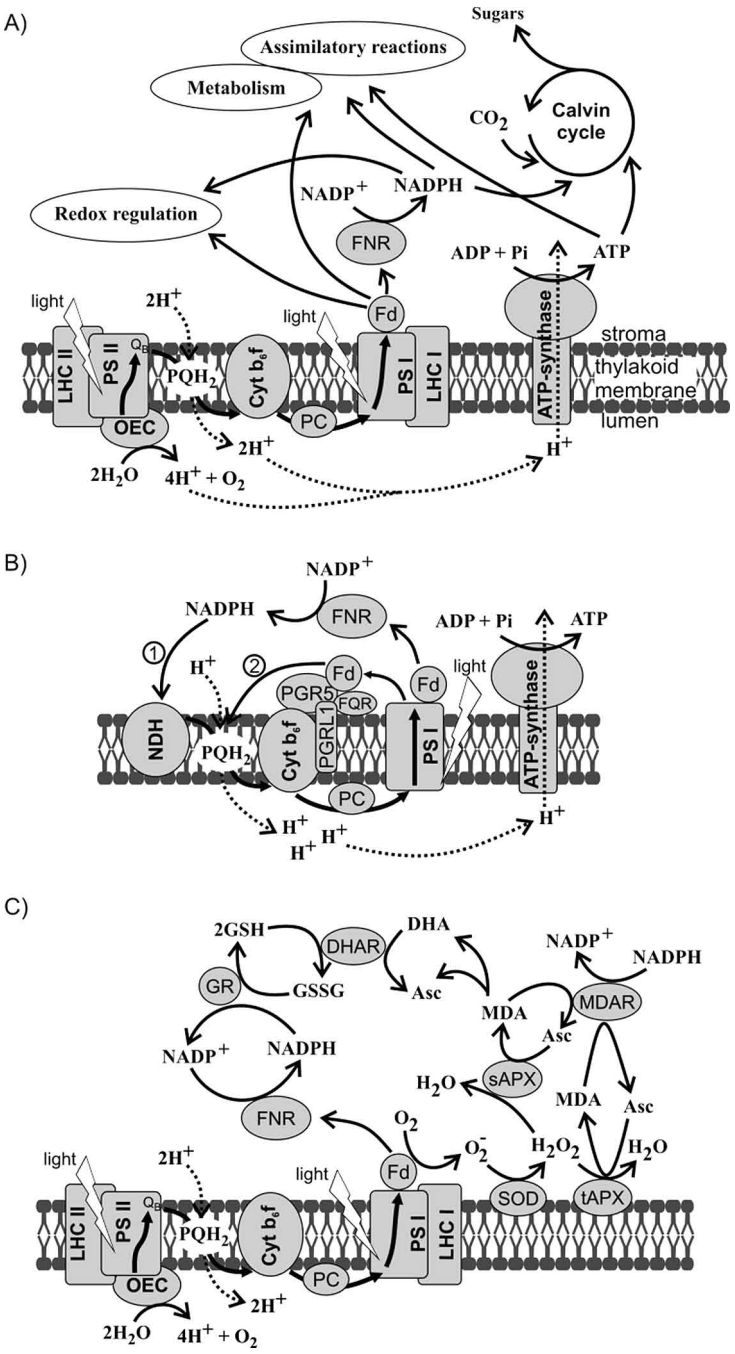

Figure 2. Simplified schematic representation of electron transfer and ROS quenching reactions in chloroplasts. A. Linear photosynthetic electron transfer reactions and stromal reactions. B. Putative cyclic electron transfer reactions around PSI: (1) NDH-dependent pathway and (2) Fd-dependent pathway. C. Pseudo-cyclic electron transfer to molecular oxygen and ROS quenching mechanisms. See text for details.

The non-photochemical quenching of chlorophyll fluorescence (NPQ) dissipates excess excitation energy as heat in LHCII, and it is considered to be an important photoprotective mechanism preventing the formation of singlet oxygen and overreduction of intersystem electron carriers. Acidification of the thylakoid lumen, due to a high proton gradient across the thylakoid membrane, promotes NPQ by activating violaxanthin de-epoxidase enzyme (VDE) and by protonating the PsbS protein. VDE catalyzes the conversion of violazanthin to antheraxanthin and zeaxanthin (Niyogi et al., 1998; Li et al., 2000; 2002). In turn, protonation of PsbS induces structural changes in the LHCII (Li et al., 2000; Kiss et al., 2008), which accompanies quenching with the help of zeaxanthin.

1.3.3. Reductive metabolism in the stroma

The end products of the electron transfer reactions are mostly used in the Calvin-Benson cycle. This is composed of 11 stromal enzymes that fix atmospheric carbon dioxide (CO_2) into carbon skeletons that are used directly for starch and sucrose biosynthesis (Raines, 2003). Additionally, the reducing power from ferredoxin and NADPH together with ATP are used in various other biosynthetic, assimilatory, and redox reactions including fatty acid biosynthesis, nitrite and sulfate reduction, and amino acid biosynthesis (Figure 2A).

The activity of several enzymes is regulated via redox exchange of thiol groups (S-S ↔ 2SH) according to the reducing environment. Thioredoxins (Trx) are small universal proteins containing active thiol/disulfide exchange motif that can reduce thiol groups in enzymes. In chloroplasts, the thioredoxin-dependent regulation is closely connected to photosynthesis, as the enzyme that reduces thioredoxins, the ferredoxin:thioredoxin reductase (FTR; Buchanan and Balmer, 2005), accepts electrons from reduced ferredoxin. Four enzymes of the Calvin-Benson cycle are activated through light-dependent reduction by Trx, making Trx regulation indispensable for efficient photosynthesis. In addition, the chloroplasts enzyme NADPH-dependent malate dehydrogenase (MDH) is activated by Trx (Scheibe, 2004). NADP-MDH shuttles NADPH from chloroplasts to the cytosol. The enzyme is activated in light by Trx and inhibited by $NADP^+$, thus switching off its own activity when all the NADPH is used for assimilatory processes.

1.3.4. Ferredoxin

Ferredoxins (Fd) are small iron-sulfur [2Fe-2S] cluster-containing electron carrier proteins that are important electron distributors in photosynthetic and non-photosynthetic plastids. In photosynthetic tissue, Fd is reduced by PSI and, in addition to $NADP^+$ reduction via FNR, electrons from Fd can be directed to various metabolic and signalling processes. Apart from its function in linear electron transfer reactions, Fd has been predicted to have a role in cyclic electron transfer reactions around PSI, and it is an important component of redox regulation via the Trx system in chloroplasts. In addition FNR (NADPH) is capable of reducing Fd, which then donates electrons to the reductive metabolism including nitrogen assimilation (nitrite

reductase), sulphur assimilation (sulfite reductase), amino acid synthesis (Gln-oxoglutarate amino transferase), and fatty acid synthesis (fatty acid desaturase). In higher plants, a nuclear gene family encodes distinct leaf-type and root-type ferredoxin isoforms, which have different redox potentials – being specific either for FNR or for other enzymes (Bertini et al., 2002; Onda et al., 2000; Hanke et al., 2004).

1.3.5. Ferredoxin-NADP$^+$ oxidoreductase

The enzyme ferredoxin-NADP$^+$ oxidoreductase (FNR) harbors non-covalently bound flavin adenine dinucleotide (FAD) as a redox center, which functions as a general electron splitter, transferring electrons between obligatory one- and two-electron carriers (Arakaki et al., 1997; Pschorn et al., 1988). FNR catalyzes the reversible electron transfer reaction between two independent Fd molecules and a single molecule of NADP(H). In plant plastids, FNR can be assigned to leaf-type (or photosynthetic; LFNR) and root-type (or non-photosynthetic; RFNR) isoforms. LFNR catalyzes the final step of photosynthetic electron transfer reactions reducing NADP$^+$ to NADPH (Shin and Arnon, 1965; Arakaki et al., 1997), whereas RFNR catalyzes the reverse reaction, oxidizing NADPH to provide reduced Fd for various enzymes. The molecular size of FNR is around 33 kDa and it has two structural domains (Pschorn et al., 1988). The redox-active FAD is tightly associated with the N-terminal part of the protein while the NADP$^+$-binding domain is in the C-terminal part. However, based on the crystal structure (Bruns and Karplus, 1995; Deng et al., 1999; Kurisu et al., 2001; Dorowski et al., 2001), the tertiary structure of FNR is folded so that the FAD and NADP$^+$ binding sites are in close proximity. To ensure efficient electron transfer between the substrates, Fd is also bound in close proximity to FAD and NADP$^+$ (Martínez-Júlvez et al., 1999; Kurisu et al., 2001).

In *Arabidopsis thaliana*, FNR proteins are encoded by a small family of nuclear genes. Two genes, At5g66190 and At1g20020, code for the leaf-targeted forms of FNR (LFNR1 and LFNR2, respectively) and two genes, At4g05390 and At1g30510, code for root plastid-targeted forms of FNR (RFNR1 and RFNR2, respectively) (Hanke et al., 2005). In my thesis, I studied the LFNRs in *Arabidopsis thaliana* and the thesis will only deal with the leaf-type FNRs. Several LFNR isoforms have been isolated and characterized from maize, Arabidopsis and wheat. In maize leaves, three isoforms of FNR vary in location; ZmFNR1 is restricted to the thylakoid membranes, ZmFNR3 is a soluble enzyme in the stroma, and ZmFNR2 is present in both fractions (Okutani et al., 2005). Hanke and co-workers (2005) identified the two leaf-targeted FNR isoforms in green tissue of Arabidopsis plants, both of which have more than 80% identity to maize leaf FNR. In Arabidopsis, the two leaf-targeted FNR isoforms (FNR1 and FNR2) are present in chloroplasts in three different compartments: the thylakoid membrane, the stroma, and the inner envelope membrane (Hanke et al., 2005; Küchler et al., 2002). The membrane-bound pool can be divided further into tightly bound and loosely bound pools (Matthijs et al., 1986). The loosely bound pool of FNR can be washed out from thylakoids with salt, whereas the tightly bound pool needs detergent treatment to dissociate from the membrane (Matthijs et al., 1986). The physiological role of the different FNR pools is not clear, but it has been postulated that the

thylakoid-bound pool of FNR is photosynthetically the most active one (Forti et al., 1983; 1984). In wheat (*Triticum aestivum*) leaves, the acidic and more basic isoforms of leaf-type FNRs are present. Post-translational modifications of the FNR isoforms result in the formation of eight distinct spots after 2-dimensional (2-D) SDS-PAGE (Grzyb et al., 2008a; Gummodova et al., 2007; Moolna and Bowsher, 2010). These different wheat FNR isoforms differ in their response to physiological parameters of chloroplast maturity, nitrogen regime, and oxidative stress (Moolna and Bowsher, 2010).

FNR is a soluble protein and it has been predicted to associate with the thylakoid membrane by way of various protein complexes or binding proteins. Early studies proposed the presence of a 17.5-kDa binding protein (Carrillo and Vallejos, 1982; Vallejos et al., 1984), which forms a trimeric complex by binding two FNR subunits at the thylakoid membrane (Ceccarelli et al., 1985). Another small 10-kDa protein, called connectein, was found to be involved in thylakoid binding of FNR in maize (Shin et al., 1985), but the detailed identity of these proteins has not been resolved. Furthermore, FNR has been postulated to be connected to the thylakoid membrane via the PsaE subunit of PSI (Andersen et al., 1992). This interaction was believed to bring FNR in close proximity to ferredoxin, which is docked to PSI via PsaD, a subunit located close to PsaE on the stromal side of PSI. The interaction between FNR and the NDH complex has been suggested to allow the NDH complex to use NADPH in chloroplasts for cyclic electron transfer (Quiles and Cuello, 1998; Guedeney et al., 1996). Also, co-purification of FNR with the Cyt b_6f complex (Zhang et al., 2001) and association with the PGRL1 protein (DalCorso et al., 2008) are presumed to affiliate FNR with cyclic electron transfer reactions. In addition to the proposed binding proteins, direct association of FNR with membranes has also been suggested (Grzyb et al., 2007b).

2. AIMS OF THE STUDY

Genome sequencing of *Arabidopsis thaliana* has revealed that several chloroplast proteins, including FNR, are encoded by a small family of nuclear genes. The FNR gene family encodes two highly homologous leaf-targeted FNR isoforms. In chloroplasts, FNR is located in different pools but the functional differences between the soluble stromal pool and the membrane-bound pool are unclear. The attachment site of FNR at thylakoid membranes has been under intense study for decades. Despite the fact that there have been several putative interaction candidates, the mode of interaction has remained elusive. In my thesis work, I aimed (i) to determine whether the two leaf-targeted FNR isoforms in *Arabidopsis thaliana* have specific functions in the chloroplasts – one, for example, being involved in linear electron transfer reactions and the other in cyclic electron transfer reactions; (ii) to clarify the functional differences between soluble and membrane-bound pools of FNR; and (iii) to study in more detail how the binding of FNR to the thylakoid membrane is mediated.

3. METHODOLOGICAL ASPECTS

3.1. Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia was used in most of the experiments. Additionally, pea (*Pisum sativum*) was used for biochemical experiments in Paper III. Transfer DNA (T-DNA) insertion mutant plants *fnr1* and *tic62* were purchased from the SALK and GABI-Kat collections (Alonso et al., 2003; Rosso et al., 2003) and RNAi silencing line *fnr2* was purchased from AGRIKOLA (Hilson et al., 2004). In the T-DNA insertion lines (*fnr1a, fnr1b, tic62-1,* and *tic62-2*), expression of the respective gene is prevented by insertion of a foreign DNA sequence into the coding region of the gene. In RNAi line *fnr2*, expression of the gene is silenced by a gene-specific RNAi construct inserted randomly in the genome. *fnr1/FNR1 fnr2(RNAi)*, hereafter referred as *fnr1xfnr2*, was produced by crossing *fnr1* and *fnr2* plants and represents the F1 generation. *fnr1 fnr2* double-mutant plants were screened from the segregating F2 generation. Photoautotrophic and heterotrophic growth conditions and the light treatments of the plants are described in the respective papers.

3.2. Analysis of chloroplast proteins

Total soluble proteins and thylakoid membrane proteins were isolated according to standard protocols described in the papers. Intact chloroplasts were isolated using Percoll step gradients, and chloroplasts and thylakoid membranes were subfractionated as in Paper III and references therein. Chlorophyll content of the isolated thylakoid membranes was measured as in Porra et al. (1989) and the protein content of soluble and membrane fractions was measured using the BioRad Protein Assay Kit. Distinct gel electrophoresis methods were used for protein separation. Denaturating SDS-PAGE was used to study the overall content of chloroplast proteins by staining gels with Coomassie blue, or detecting proteins by western blotting using protein-specific antibodies (see papers for details). For identification, proteins were separated by isoelectric focusing according to the isoelectric point of the proteins, followed by SDS-PAGE (Paper I). Thereafter, individual spots were identified by immunoblotting or by mass spectrometric analysis after silver staining of the gels (Paper I). Treatment of proteins with SDS denatures proteins and adds negative charge to unfolded polypeptides, which allows to separation of the proteins according to their molecular mass in SDS-PAGE. When proteins are separated in their native form, no ionic detergent (such as SDS) is applied to protein samples and they are therefore separated according to their native charge (isoelectric point). Native gel electrophoresis was used to separate the FNR isoforms from each other (Papers II, III, and IV). Blue native (BN) PAGE was used to separate protein complexes by mildly solubilizing the thylakoid membranes with non-ionic detergent prior to gel electrophoresis (Papers II, III, and IV). This allows to study the accumulation of thylakoid protein complexes in chloroplasts and analysis of complex formation of the proteins of interest when combined with immunodetection.

3.3. Measurements of photosynthetic parameters

PSII performance was measured as chlorophyll *a* fluorescence using PAM fluorometers (Walz) or with a Plant Efficiency Analyzer (PEA, Hansatech Instruments). The maximum quantum efficiency of PSII was monitored as F_V/F_M, excitation pressure of PSII (1-qP) as $1-[(F_M'-F_S)/(F_M'-F_0')]$, and quantum yield of PSII (Y(PSII)) as $(F_M'-F_S)/F_M'$. Non-photochemical quenching (NPQ) was determined as $[F_M-F_M']/F_M'$. Light-saturated rate of PSII oxygen evolution was measured by Hansatech oxygen electrode using DMBQ as an electron acceptor, while PSI electron transfer activity from $DCPIPH_2$ to methyl viologen (MV) was measured as oxygen consumption.

Cyclic electron transfer around PSI was estimated using three distinct methods. PAM-101/102/103 equipped with an ED-P700DW-E emitter-detector unit or JTS-10 (Biologic) was used to monitor the redox state of PSI reaction center chlorophyll P700 as changes in absorbance at 810 nm (820 nm with JTS-10), using 860 nm as reference wavelength (880 nm in the case of JTS-10) as reference. P700 was oxidized by 30-s FR light illumination of the dark-adapted leaves, and the subsequent re-reduction of $P700^+$ was monitored in darkness. The thermoluminescence AG signal was measured with the PSI Thermoluminescence System TL200/PMT (Photon Systems Instruments). Leaf discs were dark-adapted at +10°C for 30 s, illuminated with FR light for 60 s at the same temperature, and the thermoluminescence signal was recorded immediately after illumination. The transient post-illumination increase in chlorophyll fluorescence (F_0 rise) was measured after turning off the actinic light as described in Allahverdiyeva et al. (2005).

The CO_2 assimilation rate of the intact plants was determined with the Ciras-1 photosynthesis system (PP Systems) at atmospheric CO_2 concentration (360 ppm).

3.4. Enzyme activity measurements

FNR activity was measured as ferredoxin-dependent cytochrome C reduction assay in the presence of NADPH (Papers III and IV). In Paper I, the relative NADP-malate dehydrogenase (MDH) activity was measured by a spectrophotometric assay as previously described (Scheibe and Stitt, 1988). In Papers II and IV, the ascorbate peroxidase (APX) and glutathione reductase (GR) activities were measured as previously described (Foyer et al., 1989), with the modifications of Pätsikkä et al. (2002). In Papers I and II, nitrate reductase activity was measured as previously described (Marton et al., 1982).

3.5. Pigment analysis

Chlorophyll content per leaf area was measured as described in Inskeep and Bloom (1985). In Paper IV, chlorophylls, carotenoids, and α-tocopherol were extracted from

leaf discs using methanol and separated and analyzed according to Gilmore and Yamamoto (1991) using Agilent 1100 HPLC and Agilent ChemStation software.

3.6. Transcriptional analysis

In Paper I, the Arabidopsis cDNA microarray chips based on the GEM1 clone set from InCyte Genomics (http://www.incyte.com/) were used to study the gene expression profiles of the plants. RNA extraction, labeling, and hybridization were performed as in Piippo et al. (2006). For Paper II, Arabidopsis 24-K oligonucleotide arrays (MWG Biotech, http://www.mwg-biotech.com; ArrayExpress database accession number A-ATMX-2, http://www.ebi.ac.uk/arrayexpress) were used to study the gene expression profiles of the plants. cDNA synthesis, labeling, hybridization, and analysis were performed as in Kangasjärvi et al. (2008). For quantitative real-time PCR (q-RT-PCR) the cDNA synthesis was carried out with the iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories) and RT-PCR reactions were done using iQ SYBR Green Supermix (Bio-Rad Laboratories). Data analysis is described in Paper II.

3.7. Structural analysis of proteins

The structural model of FNR1 and FNR2 was constructed by comparing the amino acid sequences of Arabidopsis FNR1 and FNR2 to those of maize FNR1, FNR2, and FNR3, with the program MALIGN (Johnson and Overington, 1993) in the Bodil visualization and modeling package (Lehtonen et al., 2004) at the Department of Biochemistry and Pharmacy of Åbo Akademi University, Turku, Finland. Methods are described in more detail in Paper I. Chemical shift mapping by nuclear magnetic resonance (NMR) spectroscopy was used to study complex formation between FNR and the 30 amino acids repetitive C-terminal sequence of Tic62 at the Institute for Protein Research, Osaka University and CREST, Osaka, Japan. Analytical ultracentrifugation, atomic force microscopy, and electron microscopic images were used in the sedimentation equilibrium measurements for analytical ultracentrifugation (SE-AUC) to study the binding equilibrium of FNR and the C-terminus of Tic62 at the Institute for Protein Research, Osaka University and CREST, Osaka, Japan. Methods for NMR and AUC are described in more detail in Paper III.

3.8. Measurements of redox status of the plants

Relative ion leakage from detached rosettes was measured as described in Kangasjärvi et al. (2008) from plants with or without pretreatment with methyl viologen (MV). MV creates a high degree of oxidative stress by accepting electrons from PSI and donating them further to oxygen, thus resulting in formation of reactive oxygen species. High ion leakage from cells is a sign of oxidative stress-induced membrane damage and cell death. Ascorbate content of leaves was determined as in Foyer et al. (1983). Oxidative

modifications of thylakoid proteins were studied using the OxyBlot™ Protein Oxidation Detection Kit (Millipore).

3.9. Microscopic analysis of the leaf sections

Preparation and fixation of samples for light and electron microscopy were performed as in Pätsikkä et al. (2002)

3.10. *In vitro* transcription, translation, and preprotein import into chloroplasts

For *in vitro* transcription, the coding region of the genes (*FNR1*, *LHCB1.3*, and *GAP-B*) including transit peptide were cloned into the vector pSP65 (Promega) and transcription was carried as described in Firlej-Kwoka et al. (2008). Translation was performed using the Wheat Germ Extract Translation Kit (Promega) or the Flexi Rabbit Reticulocyte Lysate System (Promega) in the presence of [^{35}S]-methionine. Import into isolated chloroplasts was performed in the presence of ATP and [^{35}S]-labeled translation products at 25°C.

3.11. Protein interaction studies

DNA sequences corresponding to leaf-targeted FNR isoforms from Arabidopsis (Paper IV) and pea (Paper III), and to FBPase and C-terminus of AtTic62 from Arabidopsis, were cloned and used for transformation of (and expression in) *Escherichia coli*. The proteins were purified via their polyhistidine tags using Ni-NTA Sepharose (GE Healthcare) and eluted with imidazole. Proteins were then concentrated and the buffer was exchanged by dialysis prior to analysis.

Ectopically expressed and purified proteins were bound via their N- or C-terminal His-tag to Ni-NTA Sepharose blocked with egg albumin. [^{35}S]-Met labeled translation product of FNR (Paper IV) or concentrated stroma from *tic62* Arabidopsis plants (Paper III) were loaded onto columns. After incubation and subsequent washing, the proteins were eluted with imidazole (Paper IV). In Paper III, the proteins were eluted by sequential addition of salt, urea, and imidazole at increasing molarities. Eluted fractions were separated by SDS-PAGE and the proteins were detected either by autoradiography (Paper IV) or by immunoblotting (Paper III).

4. RESULTS

4.1. FNR isoforms in Arabidopsis chloroplasts

Two leaf-targeted FNR isoforms were detected from the thylakoid membrane of wild-type Arabidopsis as four protein spots after isoelectric focusing (IEF) followed by SDS-PAGE. Two spots with an isoelectric point (pI) of around 5 were identified as LFNR1, and two spots with a more basic pI were identified as LFNR2 by MALDI-TOF mass spectrometric analysis (Paper I). Apart from thylakoid membranes, the leaf-targeted FNR could also be found in the stroma (Paper I) and attached to the inner envelope membrane (Paper III). Leaf-targeted FNR isoforms have almost identical molecular mass, but the difference in pI allows separation of the FNR1 and FNR2 isoforms by native gel electrophoresis, and the presence of both isoforms in stroma and at thylakoid membranes could therefore be shown. The incomplete solubilization of the proteins did, however, prevent quantitative comparison of the isoforms (Paper II). The transcriptional analysis of *FNR* pool sizes from wild-type leaves by q-RT-PCR revealed that under standard growth conditions, the *FNR1* transcripts constitute 68% of the total *FNR* mRNA pool and *FNR2* transcripts constitute only 32% (Paper II). Separation of thylakoid protein complexes by blue native (BN) PAGE revealed that the thylakoid-bound FNR is present in several high molecular weight (HMW) complexes of around 200–500 kDa (Papers II and III).

4.2. Characterization of the mutant plants containing different amounts of FNR

In my thesis work, three Arabidopsis single-mutant plant lines were characterized. *fnr1* mutants are devoid of FNR1 protein, as the *FNR1* gene is interrupted by T-DNA insertion (Paper I). The expression of the other isoform, *FNR2*, was unchanged in the *fnr1* mutant plants, and consequently the total amount of FNR was clearly reduced when compared to wild-type plants (Papers I and II). In *fnr2* mutant plants, the total amount of FNR was also clearly smaller than in wild-type plants, and the RNAi construct of *FNR2* silenced specifically the expression of the *FNR2* gene, resulting in the loss of FNR2 protein with no marked downregulation (or upregulation) of the *FNR1* gene (Paper II). The third single-mutant plant, T-DNA insertion line *tic62*, lacking the Tic62 protein, was also characterized (Paper III). The amount of both leaf-targeted FNR isoforms was equally downregulated in the *tic62* mutant plants. In addition, double mutants from the crossing of *fnr1* and *fnr2* were characterized (Paper IV). The F1 generation, genotypically double heterozygous plants (hereafter referred as *fnr1xfnr2*) expressed only FNR1 protein, although in smaller quantity than the *fnr2* single mutant. These results indicate that one RNAi construct of *FNR2* is able to totally silence the expression of *FNR2* and that one functional gene of *FNR1* is not able to produce the protein at wild-type levels. Mutant plants completely devoid of leaf-type FNR (*fnr1 fnr2*), segregated from the F2 generation, could also be obtained (Paper IV).

Both LFNR isoforms represent photosynthetically functional FNRs, since both single *fnr* mutant plants were highly viable despite their reduced biomass accumulation (Figure 3; Papers I and II). In most cases, the *fnr1* and *fnr2* mutant plants resembled each other but frequently the *fnr2* mutants tended to express a slightly stronger phenotype than *fnr1*. *tic62* mutant plants, with somewhat downregulated amounts of both FNR isoforms, had wild-type appearance under all conditions tested (Paper III). The *fnr1xfnr2* plants (F1 generation), which had a significant deficiency in total FNR content, were viable under photoautotrophic conditions, although with a severely affected phenotype (Figure 3; Paper IV). The growth of *fnr1xfnr2* mutants was greatly retarded and chlorotic areas developed at leaf tips, resulting in death of the oldest leaves at a very early stage of plant development. Light micrographs from cross sections of wild-type and *fnr1xfnr2* leaves revealed that limited numbers of small chloroplasts were arranged towards anticlinal cell walls of the *fnr1xfnr2* leaves. However, the formation of granal stacks of thylakoid membranes in the *fnr1xfnr2* mutant was not affected. The *fnr1 fnr2* double-mutant plants, which were totally devoid of leaf-targeted FNR, were able to grow only heterotrophically (Paper IV). Despite having an external carbon source, these plants were extremely chlorotic and stunted, and the cellular structure of the leaves was greatly malformed, with disorganized mesophyll tissue and irregular cell morphology. Few thylakoid membrane-containing chloroplasts were observed in cells of *fnr1 fnr2* double-mutant plants, but the chloroplast structure also differed clearly from wild-type, as the mutant chloroplasts were swollen, were small in size, and contained abnormally loose thylakoid membranes.

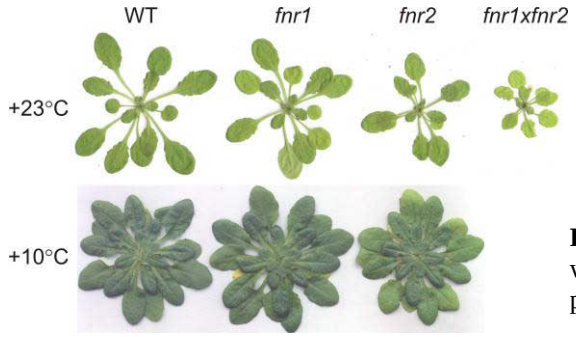

Figure 3. The phenotype of Arabidopsis wild-type (WT) plants and *fnr* mutant plants grown at +23°C and +10°C.

4.2.1. The photosynthetic properties of the mutant plants

Apart from small rosette size, all *fnr* mutant plants (but not the *tic62* mutant) accumulated less chlorophyll (Chl) than wild-type, resulting in a light green leaf color. *fnr2* mutant plants had slightly (but not significantly) smaller Chl content than *fnr1* mutants, whereas the Chl content of *fnr1xfnr2* mutant plants was clearly lower than that of the wild-type plants or either of the *fnr* single mutants (Table 1). Furthermore, the fresh weight and rosette size of the *fnr1xfnr2* mutants was clearly smaller than in the wild-type or the single *fnr* mutants (Figure 3; Table 1). Surprisingly, transcriptional

analysis revealed upregulation of photosynthetic genes in the single *fnr* mutant plants, such as those encoding the light harvesting antenna proteins of PSI and PSII. Genes encoding the small subunit of Rubisco, and a gene encoding plastocyanin protein were also upregulated in the *fnr1* and *fnr2* mutants relative to wild-type plants (Papers I and II). However, in line with the low chlorophyll content, photosynthetic pigment-protein complexes of all *fnr* mutant plants were downregulated, and less D1, PsaD, Lhcb1, and Lhcb2 accumulated in thylakoid membranes of the mutant plants than in wild-type plants (Table 1). Again, the accumulation of thylakoid proteins was most affected in the *fnr1xfnr2* mutant, although no differences in accumulation of ATP synthase subunits (CF1 or ATP-β) were detected between the wild-type and mutant plants (Papers I, II, and IV). Moreover, even though the amount of photosynthetic proteins was extremely low in *fnr1 fnr2* double-mutant plants, no such reduction in ATP synthase was observed (Paper IV). In line with the low accumulation of protein subunits of photosynthetic pigment-protein complexes, also small changes in the composition of entire, functional protein complexes were observed, as PSII and LHCII were found to assemble in smaller complexes in *fnr1* and *fnr2* mutant plants than in wild-type plants (Paper II).

The capacity of PSII in the mutant plants (*fnr* and *tic62*) was not different from that of the wild-type plants, although the PSII quantum yield (Y(PSII)) was slightly lower in the *fnr1xfnr2* mutant plants than in wild-type plants. However, when the susceptibility of PSII to high light irradiance was studied, clearly faster photoinhibition of PSII was detected in the *fnr1xfnr2* mutants, both in the presence and absence of lincomycin, which is a specific inhibitor of chloroplast protein translation (Paper IV). During high light illumination, the D1 protein in thylakoid membranes was also quickly degraded. Intriguingly, recovery from photoinhibition was equally fast in wild-type plants and *fnr1xfnr2* mutant plants. Although the size of the PQ pool and the excitation pressure of PSII (1-qP), which can be used to estimate the redox-state of the PQ pool, were smaller in the *fnr1* single mutant than in wild-type plants (Paper I), the PQ pool of the *fnr1xfnr2* mutant plants was more reduced than in wild-type plants (Paper IV). In addition, *fnr1xfnr2* mutants had a higher capacity for non-photochemical quenching (NPQ) under high actinic light illumination compared to wild-type plants (Paper IV). In line with increased NPQ, the de-epoxidation state of xanthophyl cycle pigments in *fnr1xfnr2* mutant plants was high, as the mutant plants accumulated high content of zeaxanthin even under standard growth conditions (Paper IV). Although the *tic62* mutants did not show any difference in Y(PSII), 1-qP, NPQ, or in accumulation of photosynthetic proteins when compared to wild-type plants, *TIC62* did show high co-expression with a number of photosynthetic genes (Paper III).

4.2.2. Alternative electron transfer reactions in the fnr mutant plants

No generally applicable methods to measure cyclic electron transfer pathways around PSI have been presented so far. In this thesis, three different methods were used to estimate the cyclic electron transfer capacity of the plants. First, the rate of cyclic electron flow was determined from the kinetics of absorbance changes at 810 nm resulting from $P700^+$ re-reduction in darkness after far red illumination. Both *fnr* single

mutants showed slower decay of $P700^+$ than the wild-type plants when grown under standard growth conditions (Paper I, data not shown for *fnr2*). Two other methods applied were the transient post-illumination increase in chlorophyll *a* fluorescence and the far-red light-induced thermoluminescence afterglow band, both of which have been suggested to measure mostly the NDH-dependent cyclic electron flow (Shikanai et al., 1998; Havaux et al., 2005a). Both latter methods gave results similar to that of $P700^+$ re-reduction, revealing reduced amplitudes of the representative curves (Paper II). These results showed that the single *fnr* mutant plants had a reduced ability to perform cyclic electron transfer. However, when the *fnr1* and *fnr2* single-mutant plants were grown under low-temperature conditions (+10°C), a faster decay in $P700^+$ re-reduction was observed compared to wild-type plants (Paper II). Similarly, in *fnr1xfnr2* mutant plants, the cyclic electron transfer – measured as $P700^+$ re-reduction – was found to be faster than in wild-type plants even under standard growth conditions (Paper IV). Moreover, the *fnr1xfnr2* mutant plants accumulated high levels of PGRL1 protein, which has been shown to have a role in Fd-dependent cyclic electron transfer reactions (DalCarso et al., 2008). No difference in $P700^+$ re-reduction or transient post-illumination increase in chlorophyll fluorescence was observed between *tic62* mutant and wild-type plants (Paper III).

Besides upregulated cyclic electron transfer reactions, other alternative electron acceptors were also upregulated in *fnr* mutant plants. Transcript profiling of *fnr1* and *fnr2* plants revealed upregulation of genes encoding nitrate reductase (NR), and several nitrate transporters, and glutamine synthases (Papers I and II), which use high amounts of reducing energy. Transcriptional upregulation was reflected in later steps of the metabolism and more nitrite accumulated in *fnr1* and *fnr2* leaves than in wild-type leaves, indicating an increased amount or activity of NR (Papers I and II). Based on the chlorotic phenotype of the *fnr* mutant plants and upregulation of plastid terminal oxidase (PTOX) in *fnr1xfnr2* mutants (Paper IV), the direction of photosynthetic electrons to molecular oxygen via PSI or the PQ pool appeared to be increased in the mutant plants. In single-mutant *fnr* plants, the capacity to cope with sudden exposure to oxidative stress was lower and more ions leaked from intact leaves after methyl viologen- (MV-) induced oxidative stress than from wild-type leaves (Paper II). In *fnr1xfnr2* mutant plants, an extensive ion leakage from leaves was evident even under steady-state conditions without any additional induction of oxidative stress (Paper IV).

4.2.3. Oxidative stress and the antioxidative properties of the mutant plants

In paper II, the acclimation of single-mutant *fnr* plants to oxidative stress and low-temperature growth conditions was investigated. Under standard growth conditions, in addition to increased ion leakage, single *fnr* mutant plants had higher levels of oxidized thylakoid proteins than wild-type plants. Acclimation of wild-type and single *fnr* mutants to low growth temperature concomitantly led to an increased tolerance to oxidative stress. Under low temperature conditions, only *fnr2* plants had a visual phenotype, low Chl content, and low accumulation of photosynthetic proteins, while *fnr1* plants had no distinct phenotype. In addition, under low temperature *fnr2* plants had increased tolerance to oxidative stress, as demonstrated by lower ion leakage from

cells compared to wild-type and *fnr1* plants. Moreover, low-temperature grown *fnr2* mutant plants contained less oxidized thylakoid proteins and accumulated more antioxidative enzymes compared to wild-type plants and *fnr1* mutant plants. The enhanced tolerance of low-temperature grown *fnr2* mutants to oxidative stress was reflected in the stromal redox status, since in the mutant leaves the ascorbate pool was in a more reduced state than in wild-type leaves.

The *fnr1xfnr2* mutants differed from single *fnr* mutants in terms of protection against oxidative stress (Paper IV). Already, the visual phenotype of the *fnr1xfnr2* mutant plants – the pale green color, stunted growth, and death of the oldest leaves at a very early state – indicated problems in the ability to cope with increased accumulation of ROS. Not only the upregulation of alternative electron acceptors (e.g. CET and O_2) but also the induction of various antioxidative mechanisms in *fnr1xfnr2* plants was quite obvious. More APX enzymes accumulated in the *fnr1xfnr2* chloroplasts, although no differences in total activity of APX or GR were detected compared to wild-type chloroplasts. Moreover, *fnr1xfnr2* mutant plants accumulated high relative contents of α-tocopherol and xanthophyll cycle pigments displaying high de-epoxidation state. In *fnr1xfnr2* mutant plants, a high content of zeaxanthin was detected even under standard growth conditions; yet, the xanthophyll cycle responded normally to changes in light intensity.

Table 1. Summary of the phenotype of *fnr* mutant plants grown at +23°C and +10°C.

	100 µmol photons $m^{-2}s^{-1}$, +23°C			100 µmol photons $m^{-2}s^{-1}$, +10°C	
	fnr1	*fnr2*	*fnr1xfnr2*	*fnr1*	*fnr2*
Chl	-	-	- -	+/-	-
Photosynthetic proteins	-	-	- -	+/-	-
Excitation pressure	-	n.a.	+	n.a.	n.a.
Cyclic electron transfer	-	-	+	+	+
Relative ion leakage	+	+	++	+/-	-

+ parameter is upregulated in the mutant plant indicated, as compared to wild type; - parameter is downregulated in the mutant plant relative to wild type; +/- no difference in parameter from wild type; a double symbol indicates up- or downregulation of the parameter in the mutant plant compared to wild-type and other *fnr* mutants; n.a. data not available.

4.3. FNR forms dimers and high molecular weight complexes at thylakoid membranes

As mentioned above, both FNR isoforms are found in the membrane and in soluble pool in wild-type chloroplasts. On the other hand, *fnr1* mutant plants have the FNR2 protein only in the soluble pool, while the FNR1 in *fnr2* and *fnr1xfnr2* mutant plants is distributed between both the thylakoid pool and the stromal pool of chloroplasts (Papers I, II, and IV). These results indicate that the FNR1 protein is required for thylakoid attachment of the FNR2 protein, most probably through (hetero)dimer formation. Crystal structures of *Anabaena* FNR and ZmLFNR1 from maize have been solved both in the "free state" and in complex with Fd (Kurisu et al., 2001; Morales et al., 2000; Serre et al., 1996). Both of these complexes contain two FNRs and one Fd.

We found that the other FNR, predicted to be in the "free state" in the maize structure, was able to associate with the FNR-Fd complex similarly to *Anabaena* FNR (Paper I; Kurisu et al., 2001). The ZmFNR dimer in complex with Fd was then used as a template when the structural model of the Arabidopsis FNR dimer was constructed. Computational analysis of the FNR proteins revealed that there were no structural hindrances that would prevent the formation of either homodimers or herterodimers of Arabidopsis FNR1 and FNR2 (Paper I). In paper IV, the dimer formation was tested by *in vitro* binding assay, in which either of the heterologously expressed and purified Arabidopsis FNR proteins was bound via its $(His)_6$-tag to nickel affinity matrix together with the *in vitro* translated [^{35}S]-labeled FNR isoform. The analysis of elution fractions revealed that, in line with the structural model, both homodimeric and heterodimeric associations in all combinations of FNR isoforms were equally possible, although very weak. The *in vitro* activities of FNR1 and FNR2, measured as Cyt C reduction assays, revealed that FNR1 was more active than FNR2 but there were no apparent difference in activity between monomeric/homodimeric and heterodimeric enzyme (Paper IV).

Not only FNR dimers but also larger protein complexes containing FNR were present in wild-type thylakoid membrane (Papers II, III, and IV). These complexes did not show co-migration with PSI, Cyt b_6f, or NDH complexes in BN-PAGE (Paper III), although in earlier studies these protein complexes were predicted to act as thylakoid platforms for FNR. In Paper III, Tic62 was shown to be present both at the inner envelope membrane and in the stroma, and additionally at the thylakoid membrane – and indeed, FNR was shown to have a highly similar migration pattern to that of Tic62 in BN-PAGE.

4.3.1. Interaction of Tic62 and FNR at the thylakoid membrane

When wild-type and *tic62* chloroplasts were fractionated into stroma and the envelope and thylakoid membranes, it was obvious that the amount of FNR was smaller in the membrane fractions of *tic62* than in those of the wild-type chloroplasts (Paper III). Further fractionation of thylakoid membranes by differential centrifugation to grana thylakoids, grana margins, and stroma lamellae revealed that in wild-type chloroplasts, both Tic62 and FNR are enriched in stroma lamellae although FNR in particular is distributed in other thylakoid fractions as well. In *tic62* chloroplasts, only small amount of FNR was present in the stroma lamellae fraction. The amount of FNR in *tic62* stroma was, however, at levels similar to those in wild-type stroma.

The *fnr* mutants were found to have a reciprocal molecular phenotype to that in *tic62* as in *fnr1* and *fnr2* mutants the total amount of Tic62 was reduced. Moreover, the formation of FNR and Tic62 high molecular weight (HMW) complexes at the thylakoid membrane was prevented in *fnr1*, *fnr2*, and *tic62* mutant plants, indicating that there is a very specific interaction between these proteins (Paper III). Freely running FNR was, however, detected in the lower part of BN gels in wild-type, *tic62*, and *fnr2* plants, indicating that FNR has the ability to bind to the thylakoid membrane also independently of Tic62. To test whether the mutant plants lacking one of the predicted FNR-interacting proteins would have differential accumulation of thylakoid-

bound FNR, the thylakoid proteins were applied to BN-PAGE. The mutant plants lacking PsaE, NdhO, or PGR5 protein did not show any changes in patterns of FNR in BN-PAGE compared to wild-type plants (Paper IV). However, the mutant plants lacking the newly discovered FNR binding protein TROL (Juric et al. 2009) lacked one of the smallest FNR complexes, which was different from FNR-Tic62 complexes (Paper IV).

4.3.2. Tic62 binds FNR in a high-salt insensitive manner at a site of FNR that is distinct from its catalytically important regions

The interaction between Tic62 and FNR was studied in Paper III. After high ionic strength washing of wild-type and *tic62* thylakoid membranes, it was obvious that FNR is bound more strongly to membranes in the presence of Tic62. Moreover, *in vitro* binding assays revealed that there was a strong interaction between Tic62 and FNR, since most of the FNR bound to His-tagged Tic62 could be eluted from Ni^{2+} matrix only by denaturation with urea.

Chemical shift mapping by nuclear magnetic resonance (NMR) spectroscopy together with analytical ultracentrifugation analysis at sedimentation equilibrium (AUC-SE) was used to investigate the interaction between FNR and Tic62. The C-terminal 30-amino acid repeat of Tic62 (R1 peptide) – which is known to be the specific FNR binding motif (Küchler et al. 2002) – instead of whole-length Tic62 was used in the studies because the high molecular mass of molecules often hampers the resolution and sensitivity of resonance. A number of spectra of uniformly labeled FNR changed significantly upon addition of R1 and strikingly many peaks of free FNR disappeared. At the same time, new peaks appeared at different resonance positions with strong binding mode. Chemical shift perturbations resulting from addition of R1 peptide were mapped onto the tertiary structure of FNR. The residues affected belonged to a large area on only one side of FNR, which was located on the side opposite to the Fd and $NADP^+$ binding areas. AUC-SE analysis revealed that FNR and R1 peptide form a complex with a stoichiometry of about 2:1. However, steric hindrances do not allow full saturation of FNR binding sites in the C-terminal part of Tic62, which contains several repeats depending on plant species (Balsera et al., 2007).

4.3.3. Regulation of Tic62-FNR complex formation

Regulation of formation of the Tic62-FNR complex at the thylakoid membrane was investigated in Paper III. It was found that the complex formation between FNR and Tic62 is regulated by light despite the total lack of any photosynthetic phenotype of *tic62* plants. At wild-type thylakoid membranes isolated from dark-adapted leaves, FNR was strongly present in HMW complexes together with Tic62. Light treatment of the leaves resulted in detachment of FNR from HMW complexes at the thylakoid membrane. Similarly, the amount of Tic62 also became reduced in HMW complexes but the effect was not as intense as for FNR. In order to mimic pH changes during the dark-to-light transition, the thylakoids were isolated from dark-adapted leaves and the pH was adjusted to different values. At pH 6, which mimics the stromal environment in

darkness, most of the Tic62 and FNR was present in the membrane fraction. Treatment of thylakoids with more alkaline pH released a proportion of Tic62 and FNR to the soluble fraction. Again, FNR was more easily dissolved from the thylakoid membrane than Tic62. Accordingly, the catalytic activity of heterologously expressed and purified FNR was measured *in vitro* in the presence and absence of varying amounts of recombinant Tic62 protein. The activity measured after overnight preincubation revealed a loss of most of the activity from samples containing only FNR, whereas the presence of full-length Tic62 or its C-terminus specifically retained most of the FNR activity. These results indicate that Tic62 is able to stabilize FNR.

5. DISCUSSION

5.1. Both FNR isoforms are functional, and partially redundant

The triple location of FNR in the stroma, at the thylakoid membranes, and at the inner envelope membrane was confirmed in this thesis work. As revealed in Paper I, both Arabidopsis isoforms at the thylakoid membrane are represented by two separate spots after 2-D gel electrophoresis. These protein spots corresponded to similar molecular masses, but there was a small shift in pI. This suggests that proteins undergo post-translational modifications; such changes are known to induce changes in the net charge of proteins. In wheat, the acidic and more basic isoforms of leaf-type FNRs are post-translationally modified by alternative N-terminal cleavage and/or by phosphorylation to form eight distinct isoforms in total (Gummadova et al., 2007; Moolna and Bowsher, 2010). Four putative phosphorylation sites on the surfaces of Arabidopsis FNR isoforms were predicted from structural modeling (Paper I). Moolna and Bowsher (2010) predicted slightly different phosphorylation pattern of Arabidopsis FNR based on the conservation of predicted phosphorylation sites between wheat and Arabidopsis FNRs. However, no evidence of *in vivo* phosphorylation of Arabidopsis or wheat FNR has been observed so far.

The high viability of both single *fnr* mutant plants indicates that both isoforms are functional and photosynthetically active. However, the phenotypic deficiencies of the single *fnr* mutant plants indicate that both isoforms are needed to guarantee efficient photosynthesis (Papers I and II). Mutant plants lacking the *FNR2* gene product and containing only a small quantity of FNR1 protein (*fnr1xfnr2*) are also viable under photoautotrophic growth conditions, whereas the mutant plants that are completely devoid of FNR are not able to grow autotrophically due to a serious lack of NADPH (Paper IV).

5.2. Lack of terminal photosynthetic electron acceptor leads to downregulation of the photosynthetic machinery

Downregulation of photosynthetic pigments and pigment-protein complexes in single and double *fnr* mutant plants indicates that depletion of FNR leads to downregulation of the entire photosynthetic machinery to avoid excess chlorophyll excitation, overreduction of the electron transfer chain, and production of ROS. The amount of FNR has been shown to be proportional to the degree of downregulation of photosynthetic protein complexes (Hajirezaei et al., 2002) and mutant plants that are totally devoid of FNR accumulate barely detectable amounts of photosynthetic thylakoid proteins (Paper IV).

Particularly in the early literature, it was emphasized that the thylakoid pool of FNR is the photosynthetically active one, whereas in the stroma the FNR pool would catalyze a reverse reaction towards reduction of ferredoxin (Forti et al., 1983; Forti and Bracale,

1984). Our results, however, clearly undercut these assumptions, as *fnr1* mutant plants – totally devoid of the thylakoid-bound pool of FNR – are highly viable (Papers I and II). Moreover, *tic62* mutant plants with a clear defect in the membrane-bound pool of FNR show no photosynthetic phenotype (Paper III). Accumulation of FNR at the thylakoid membrane in darkness and release of the protein to the stroma under illumination rather indicates that most of the thylakoid-bound pool of FNR is photosynthetically inactive (Paper III).

5.3. Alternative electron transfer reactions are upregulated when the amount of FNR limits linear electron flow

Despite the fact that there has been intense research in the field, the role of FNR in cyclic electron transfer reactions has remained controversial. Discovery of the NDH complex from the thylakoid membrane of higher plant chloroplasts, and its co-migration with FNR in native gels indicated that FNR may function as an electron input unit for the NDH complex (Quiles and Cuello, 1998; Guedeney et al., 1996). However, the activity of NDH complex has been shown to be independent of FNR activity (Teicher and Scheller, 1998; Endo et al., 1997). Despite the discovery of multiple novel subunits in the NDH complex (see Suorsa et al., 2009 for a review), the electron input unit has not been identified. More recent publications have indicated the formation of cyclic electron transfer specific supercomplexes. NDH complex has been shown to form a supercomplex with PSI in plant chloroplasts (Peng et al., 2008; Sirpiö et al., 2009), and this was hypothesized to be the CET-acting form of NDH complex. However, the presence of FNR in this complex has not been proven and recently there have been speculations about electron donors other than NAD(P)H for NDH, such as Fd.

It has been suggested that FNR might function in cyclic electron transfer reactions, acting as a still uncharacterized ferredoxin-plastoquinone reductase (FQR). Firstly, FNR has been shown to have diaphorase activity (Bojko and Wieckowski, 1995; Melamed-Harel et al., 1984), and it has been shown to be able to donate electrons to the plastoquinone pool under *in vitro* conditions (Bojko et al., 2003). Secondly, FNR has been co-purified with the Cyt b_6f complex (Okutani et al., 2005; Zhang et al., 2001), suggesting the formation of a speculative supercomplex containing PSI, Cyt b_6f complex, and plastocyanin (Joliot and Joliot, 2002). This complex has, however, remained hypothetical due to the lack of biochemical identification until recently. Interaction of PGR5 with Fd, Cyt b_6, and PGRL1, and interaction of PGRL1 additionally with PsaD, FNR1, and FNR2 in the yeast 2-hybrid system and split-ubiquitine assays, caused authors to propose that a CET supercomplex containing PSI, Cyt b_6f, PGRL1, PGR5, Fd, and FNR (and predicted FQR) may indeed exist (DalCorso et al., 2008). However, such a complex has not been identified in plants. In addition, loss of PGR5 or PGRL1 did not affect the accumulation of PSI, Cyt b_6f, Fd ,or FNR; moreover, PGR5 and PGRL1 accumulated independently of each of the proteins and protein complexes previously mentioned (DalCorso et al., 2008). This means that Fd-dependent CET is able to operate without the formation of such a supercomplex, and/or

that the complex is only transient. Very recently, a CET-dependent supercomplex containing PSI-LHCI, LHCII, Cyt b_6f, FNR, and PGRL1 in near stoichiometric amounts was isolated from the unicellular green alga *Chlamydomonas reinhardtii* (Iwai et al., 2010). In Chlamydomonas, the induction of CET is closely connected to state transition (Rochaix, 2007), an event in which excitation energy is balanced by physical rearrangement of LHCII between PSII and PSI (Kargul and Barber, 2008). This isolated CET complex was shown to be able to mediate electron transfer between PSI and Cyt b_6f under state 2 conditions, indicating CET activity of the supercomplex.

When the amount of FNR is only partially limiting (as in the *fnr1*, *fnr2*, and *tic62* mutants), there is only minor downregulation or no change in cyclic electron transfer reactions (Papers I, II, and III). Altered CET capacity is most probably a secondary effect resulting from changes in the stromal redox state in *fnr* mutant plants. However, in the *fnr1xfnr2* mutant the content of the terminal acceptor clearly limits the linear electron transfer, and the reducing energy is directed to alternative electron transfer reactions, e.g. to Fd-dependent cyclic electron transfer and molecular oxygen via PTOX or via PSI in the Mehler reaction, to prevent major over-reduction of the inter-system electron carriers and production of ROS (Paper IV; see section 5.4). The same phenomenon can be seen when single *fnr* mutant plants grow under low-temperature conditions (Paper II). Most probably, the changes in the stromal redox state and an inability of ferredoxin to direct electrons to $NADP^+$ reduction constitute the driving force for the upregulation of cyclic electron transfer reactions in the *fnr* mutants.

LFNR isoforms can be divided into acidic and more basic forms, which show different responses to the nitrogen regime. Since FNR2 is expressed at high levels under high nitrate content in growth media, it is conceivable that FNR2 provides the additional reducing power required for assimilation of nitrate (Hanke et al., 2005). In Papers I and II, the upregulation of genes encoding enzymes involved in nitrogen metabolism was detected in both *fnr* mutant plants. Nitrate reductase activity is also increased in mutant plants relative to wild-type plants. In the cytosol, NR reduces nitrate to nitrite – which is a toxic compound, and is immediately imported to plastids where Fd-dependent nitrite reductase reduces it to ammonium. Ammonium fixation into amino acids is then performed by Fd-dependent glutamate synthase. Although nitrogen fixation in chloroplasts mostly uses Fd as a reducing power, assimilation of nitrate requires export of NADPH from chloroplasts to the cytosol via the malate shuttle. Low activity of NADPH-MDH in *fnr1* mutant plants (Paper I) is most probably due to an increased $NADP^+$/NADPH ratio, as it is known that a high content of $NADP^+$ reduces the activity of MDH by a feedback mechanism (Scheibe, 1991). Altogether, it is conceivable that the export of reductants to the cytosol is depressed due to deficiency in FNR which raises the question of how the energy needed to run NR in the cytosol is guaranteed in *fnr* mutant plants.

5.4. *fnr* mutant plants use various photoprotective mechanisms to prevent oxidative damage

Both photosystems produce reactive oxygen species under conditions where there is over-reduction of the PQ pool and loss of a final electron acceptor in stroma. Reduced levels of FNR are reflected in the redox status of the chloroplasts, and the *fnr* mutant plants show symptoms of oxidative stress. Plants have evolved various mechanisms to avoid excess light energy and light-induced damage to the photosynthetic apparatus. To avoid excessive absorption of light energy, chloroplasts are arranged towards anticlinal cell walls in *fnr1xfnr2* mutant plants. In all *fnr* mutant plants the chlorophyll excitation is additionally diminished by reducing the size of the light harvesting antennae and the chlorophyll content. The *fnr1xfnr2* mutant plants show increased NPQ under high actinic light intensities compared to wild-type. NPQ dissipates excess chlorophyll excitation as heat with the help of xanthophyll cycle pigments. In *fnr1xfnr2* the relative amount and the de-epoxidation state of xanthophyll cycle pigments are high – even under standard growth conditions – and the amount of zeaxanthin increases further with increasing light intensity (Paper IV). Apart from NPQ zeaxanthin has been shown to have other roles and in the *fnr1xfnr2* mutant plants high content of zeaxanthin can additonally be used, for example, to prevent lipid peroxidation (Havaux and Niyogi, 1999; Havaux et al., 2000; Baroli et al., 2003).

Accumulation of ROS is possible, despite the fact that plants have evolved various mechanisms to avoid and dissipate excitation energy and have the ability to direct excess electrons to alternative electron acceptors. ROS can, however, be scavenged by versatile antioxidative systems in chloroplasts. Under standard growth conditions, single *fnr* mutant plants can presumably adapt their photosynthesis and metabolism so that they can avoid excess production of ROS even without excessive upregulation of antioxidative systems. Adverse growth conditions, however, lead to enhanced protection against ROS in *fnr2* mutant plants. Similarly to low-temperature grown *fnr2* mutants, *fnr1xfnr2* mutants build up a defense system against ROS already when grown under standard growth conditions. In addition to upregulation of APX enzyme, which scavenges H_2O_2, a high relative content of α-tocopherol and xanthophylls and a high de-epoxidation state of xanthophyll pigments can help *fnr1xfnr2* plants cope with increased production of ROS. Xanthophylls and α-tocopherols can act as general photoprotective agents (Havaux et al., 2005b), as quenchers of singlet oxygen (Di Mascio et al., 1990) and as scavengers of fatty acid free radicals generated by lipid oxidation (Kamal-Eldin and Appelqvist, 1996). These protective mechanisms are, however, not enough to protect the *fnr1xfnr2* mutant plants completely from oxidative damage.

Efficient repair systems are necessary to maintain cellular functions, which make them part of the photoprotective system of plants. PSII reaction center protein D1 is prone to irreversible light-induced damage and must be replaced with a newly synthesized protein (Aro et al., 1993). The *fnr1xfnr2* plants are highly susceptible to high light-induced photoinhibition (Paper III, Figure 4). It can be concluded that the low chlorophyll content of *fnr1xfnr2* mutants is the main reason for fast photoinhibition, since more light can penetrate through leaves with scattered density of chlorophyll

(Pätsikkä et al., 2002). Despite fast photoinhibition, the recovery of PSII is highly efficient in *fnr1xfnr2* plants. High accumulation of α-tocopherol, as shown in Paper IV, may protect the chloroplast translation machinery responsible for the PSII repair cycle in *fnr1xfnr2*. In accordance with this result, Arabidopsis α-tocopherol deficient mutant plants have a slow PSII repair cycle (personal communication with E. Tyystjärvi).

Altogether, the loss of FNR results in changes in various photosynthetic and metabolic reactions, finally leading to defects in plant growth and development. Most of these changes are likely to be due to secondary effects, as a result of changes in electron transfer properties and stromal redox state. Figure 4 gathers together the results obtained using the *fnr* mutant plants and includes a proposal for the network that leads to the observed phenotype of the mutant plants.

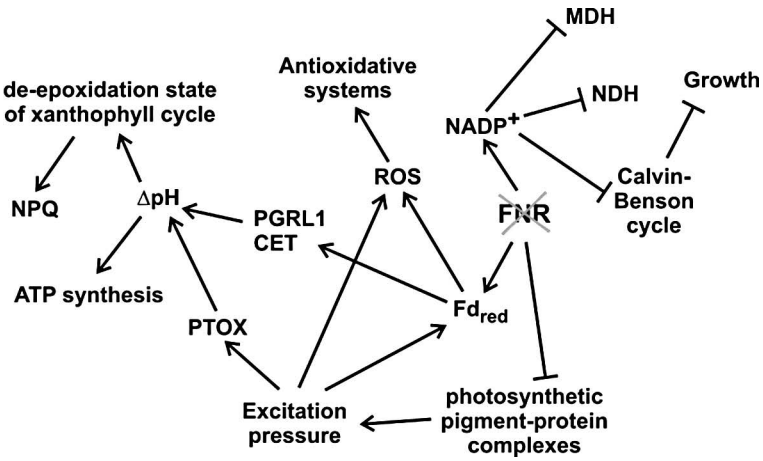

Figure 4. Schematic representation of the modulation of chloroplast functions by downregulation of FNR expression. Downregulation of FNR leads to induction (arrows) and suppression (lines with blunt ends) of various reactions in the chloroplast. Hindrance of $NADP^+$ photoreduction leads to downregulation of $NADP^+$-dependent enzymes (e.g. Calvin-Benson cycle enzymes). Defects in carbon fixation then lead to retardation of plant growth and to downregulation of linear electron transfer reactions as a feed-back mechanism. High excitation pressure leads to accumulation of reduced Fd (Fd_{red}) and upregulation of alternative electron transfer routes, such as cylic electron transfer (CET) and the Mehler reaction. Direction of electrons to oxygen in the Mehler reaction leads to enhanced production of reactive oxygen species (ROS). Alternative electron transfer reactions further promote proton gradient (ΔpH) formation across the thylakoid membrane. ΔpH allows sufficient accumulation of ATP synthase and keeps the de-epoxidation state of xanthophyll cycle pigments high, which then promotes non-photochemical quenching of chlorophyll fluorescence (NPQ).

5.5. Functional differences between FNR1 and FNR2

Although both Arabidopsis leaf-targeted FNR isoforms are functional, FNR1 and FNR2 are not totally redundant, which can be deduced from the phenotypic deficiencies of the mutant plants. Since the *fnr1* and *fnr2* mutants possess similar photosynthetic properties, it can be concluded that one isoform is not solely restricted

to linear electron flow and the other to cyclic electron flow. According to q-RT-PCR analysis of the FNR pool, FNR1 is more abundant than FNR2 (Paper II). Moreover, the activity of FNR1 is higher than that of FNR2 (Paper IV, Hanke et al., 2005). It is therefore surprising that the phenotype of the *fnr2* mutant is stronger than that of the *fnr1* mutant; thus the definition of specific roles of FNR1 and FNR2 still requires further study.

In an earlier study, FNR1 was found to be a high-salt eluting protein and FNR2 to be low-salt eluting protein, indicating different membrane binding abilities of the proteins (Hanke et al., 2005). Moreover, FNR1 was found to be more abundant in the membrane fraction than FNR2 (Hanke et al., 2005). These results were corroborated in my thesis work, and indeed I found that FNR1 is indispensable for thylakoid association of FNR in Arabidopsis. Attachment of FNR2 to the thylakoid membrane most probably occurs through heterodimer formation, FNR1 serving as a membrane anchor. In *Anabaena* (Morales et al., 2000) and in maize (Kurisu et al., 2001; Paper I), a possible formation of an FNR dimer – containing two FNR molecules binding one ferredoxin, forming a "front–front" dimer of FNR (see below) – was predicted based on crystallographic studies. According to structural modeling, the formation of FNR heterodimers and homodimers in Arabidopsis is equally probable (Paper I). The ability of the two FNR isoforms to form dimers was also studied *in vitro*. Formation of both heterodimers and homodimers was shown to be equally possible; nevertheless, the *in vitro* dimer formation was very weak (less than 5%). It would be interesting to investigate how addition of ferredoxin might affect FNR dimer formation *in vitro*. Moreover, it would be interesting to study whether the dimer formation would have any direct consequences, for example in changing the electron transfer properties of the enzyme.

5.6. Tic62 is the major thylakoid membrane anchor for FNR

Over the past three decades, various binding proteins and protein complexes have been proposed to tether FNR at the thylakoid membrane. However, identification of molecular interactions and the crystal structure of FNR and predicted binding proteins have constantly failed, keeping the question of how a soluble FNR is strongly bound to the thylakoid membrane still open.

In addition to dimers, high molecular weight complexes containing FNR were detected at the thylakoid membranes of wild-type plants (Paper II). Previously, FNR was thought to co-migrate with the PSI, the NDH-complex, and the Cyt b_6f complex in BN gels, but closer examination of the gels and immunoblots revealed that there was no clear co-migration of FNR with any of the above-mentioned complexes (Paper III). Moreover, the pattern of FNR complexes in BN gels in the plants lacking NdhO, PsaE, or PGR5 protein did not differ from that in wild-type plants (Paper IV). Interestingly, Tic62 showed a similar migration pattern to that of FNR in BN gels, and specific loss of FNR high molecular weight complexes from *tic62* thylakoids indicates that there is a strong interaction between FNR and Tic62 at the thylakoid membrane (Paper III).

FNR and Tic62 interact via a C-terminal repetitive sequence in Tic62 (the R1 peptide) and a novel binding site in FNR opposite to the $NADP^+$- and FAD-binding sites (Paper III). Crystallographic analysis of FNR has revealed a large hydrophobic cavity of unknown function far from the active sites (Bruns and Karplus, 1995; Serre et al., 1996). This region was predicted to be responsible for the membrane association of FNR, and may be the same as that shown to be essential for binding to the repetitive sequence of Tic62 (Paper III). The NMR and AUC-SE analyses revealed a binding stoichiometry of 2:1 for FNR and R1 peptide, respectively, possibly indicating binding of two FNR isoforms to one R1 peptide (Paper III). The strong binding stoichiometry between FNR and R1 peptide has now been shown by crystallographic studies also (unpublished results: personal communication with Prof. Soll). In that study, the FNR dimer without R1 peptide failed to form a crystal. This, and the very weak interaction between the two FNR isoforms detected by biochemical *in vitro* experiments (Paper IV), could be explained by the fact that the FNR front-front dimer requires the presence of an Fd molecule (unpublished results). The two different types of FNR dimers, one with Tic62 (via the back site of FNR) and the other with Fd (forming front-front dimer), would make possible the formation of large complexes containing only FNR, Tic62, and Fd as the C-terminus of Tic62 contains several repetitive sequences.

During the preparation of Paper III, an article describing the second FNR binding protein was published (Juric et al. 2009). TROL (thylakoid-rhodanese-like) protein is a novel nuclear-encoded integral thylakoid protein. It contains two modules: rhodanese-like domain and a C-terminal repetitive FNR binding sequence similar to that in Tic62. Unlike Tic62, which contains several repetitive sequences, TROL contains only one, which is, however, able to bind strongly FNR (Juric et al., 2009) and loss of TROL resulted in only a slight decrease in the content of FNR at the thylakoid membrane (Paper IV, Juric et al., 2009). In contrast to *tic62* mutant plants, T-DNA insertion mutants of TROL were found to have impaired capacity in linear electron transfer reactions, as deduced by relative ETR values and increased NPQ under high light conditions (Juric et al., 2009).

Using the FNR antibody and the *tic62* and *trol* mutant plants, we could identify bands consisting of FNR together with Tic62 and ones containing FNR together with TROL in BN gels (Paper IV). FNR and Tic62 form the four largest complexes, whereas FNR and TROL form only one smaller complex of molecular weight around 200 kDa. Most of the FNR is bound to thylakoid membranes via Tic62, and only a relatively small amount via TROL. A proportion of FNR is also present at thylakoid membranes as a monomer/dimer. Although Tic62 and TROL appear to be the only components that strongly bind to FNR at thylakoid membranes, more transient interactions of FNR with other proteins at the thylakoid membrane cannot be ruled out. Moreover, these interactions are most probably regulated differently to the interactions between FNR and Tic62 or TROL.

5.7. Interaction between FNR and Tic62 is regulated by the stromal redox state

FNR and Tic62 have been shown to shuttle between the stroma and the chloroplast membranes, depending on the stromal $NADP^+$/NADPH ratio under *in vitro* conditions; under oxidizing conditions, Tic62 and FNR are enriched in the membrane fraction whereas reduction of the NADP(H) pool leads to a release of Tic62 and FNR from the membrane pool to the stroma (Stengel et al., 2008). The interaction between Tic62 and FNR is also dependent on the stromal redox state, as they have been shown to interact more strongly under reducing conditions (Stengel et al., 2008). Interestingly, NADP(H)-dependent conformational changes of the N-terminal part of Tic62 have also been observed (Stengel et al., 2008), leading to speculation that the hydrophobicity of the protein may be altered by the NADP(H) co-factor, which might explain the attachment to and dissociation from the membrane. The stromal redox state also affected the localization of FNR and Tic62 *in vivo* (Paper III). In darkness the chloroplast stroma is slightly acidic, and illumination leads to acidification of the lumen and concomitant alkalization of the stroma. Dissociation of FNR from the high molecular weight complexes in light and rebinding in darkness suggests that there is light-dependent regulation of the FNR-Tic62 complexes. The FNR-TROL complex appears to be similarly regulated in light; the complex containing TROL and FNR is most abundant in darkness and disappears from light-treated thylakoids (Paper III).

5.8. Role of Tic62-FNR complexes at the thylakoid membrane and at the inner envelope membrane

The discovery of FNR at the inner envelope membrane – binding to Tic62, which is a part of the redox regulon of the Tic complex (Kuchler et al., 2002; Stengel et al., 2008) – led to the assumption that through FNR, the Tic complex could receive information about the redox status fo the stroma and regulate protein import accordingly. FNR could also conceivably act as an electron donor for a small, putative electron transfer chain in the Tic complex composed of Tic55, Tic32, and Tic62. The efficiency of import of several photosynthetic proteins, including FNR isoforms, was recently shown to increase *in vitro* at high $NADP^+$/NADPH ratio in the stroma (Stengel et al., 2009). However, the reduced amount of FNR in *tic62* mutant chloroplasts was not due to difficulties in import, since preFNR was imported into the chloroplasts of wild-type leaves as efficiently as into those of *tic62* mutant leaves (Paper III). On the other hand, in the single *fnr* mutant plants, genes encoding Lhcb and Lhca proteins are upregulated at the transcriptional level, whereas the content of corresponding proteins is downregulated in *fnr1* and *fnr2* mutant plants (Papers I and II). The downregulation of these proteins may be due to reduced import capacity – but also at other levels of regulation, such as translation, membrane integration, or the assembly of LHCII complex into the thylakoid membrane and the turnover rate of the protein in chloroplasts.

It can be concluded that the FNR-Tic62 complexes do not have any photosynthetic role, as the complexes were found to accumulate at the thylakoid membrane in darkness. Instead, Tic62 keeps FNR active during a long incubation time in darkness *in vitro*. The C-terminal part of Tic62 (containing the FNR-binding repetitive sequences) is relatively young from an evolutionary standpoint, being present only in flowering plants (Balsera et al., 2007). Since the plants completely lacking the thylakoid-bound FNR (Papers I and II) or the pool of FNR that associates with thylakoids via Tic62 (Paper III) were found to be healthy under the conditions used in the study, it is enigmatic that such a large amount of FNR is relocated to the thylakoid membrane in wild-type plants. There are a few possible reasons that could explain the interaction between FNR and Tic62 at the thylakoid membrane: (i) for storage and safe-keeping, (ii) for regulation of the amount of available enzyme in the stroma, or (iii) for some other as yet unknown function(s). It is obvious nowadays that the reducing energy in the form of reduced Fd and NADPH is used in several stromal metabolic, antioxidative, and signaling events in addition to the Calvin-Benson cycle. Thus, allocation of FNR for $NADP^+$ reduction must be quickly controlled under the ever-changing environment that plants experience in nature. The rebinding of FNR to the thylakoid membrane after plants are transferred from light to darkness occurs in a matter of minutes (unpublished results), indicating that quick removal of FNR from the stroma would allow other enzymes to use reducing power from Fd according to environmental cues. In addition, stabilization of FNR by Tic62 may indicate that Tic62 has a chaperone-related role in protecting the enzyme from adverse environmental conditions *in vivo*.

6. CONCLUDING REMARKS

In this thesis I have shown that both Arabidopsis leaf-targeted FNR isoforms are photosynthetically active, although loss of one isoform leads to downregulation of photosynthesis. The isoforms are highly redundant in terms of function but they cannot completely compensate for each other. FNR1 is indispensable for thylakoid association of FNR2 evidencing dimer formation between the isoforms. Tic62 was found to have a major role in tethering FNR to the thylakoid membrane. The thylakoidal pool of FNR was shown to have roles that are not necessarily involved in photosynthesis, and the soluble pool of FNR was shown to be photosynthetically active. The exact role of FNR and Tic62 (or TROL) complexes at the thylakoid membrane still requires investigation, but based on the results in this thesis, I can propose that Tic62 has a chaperone-like function – keeping FNR active at the thylakoid membrane during the photosynthetically inactive periods.

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