Turun yliopisto
University of Turku

CHARACTERIZATION OF LEAF-TYPE FERREDOXIN-NADP$^+$ OXIDOREDUCTASE (FNR) ISOFORMS IN *ARABIDOPSIS THALIANA*

Nina Lehtimäki

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. AI osa - tom. 490 | Astronomica - Chemica - Physica - Mathematica | Turku 2014

University of Turku

Faculty of Mathematics and Natural Sciences
Department of Biochemistry
Laboratory of Molecular Plant Biology

Supervised by

Dr. Paula Mulo
Laboratory of Molecular Plant Biology
Department of Biochemistry
University of Turku
FI-20014 Turku, Finland

Reviewed by

Professor Paula Elomaa
Department of Agricultural Sciences
University of Helsinki
FI-00014 Helsinki, Finland

Professor Poul Erik Jensen
Department of Plant and Environmental Sciences
University of Copenhagen
DK-1871 Frederiksberg C, Denmark

Opponent

Dr. Caroline Bowsher
Faculty of Life Sciences
University of Manchester
M13 9PT, United Kingdom

Cover picture by Anna Lepistö

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-5748-4 (PRINT)
ISBN 978-951-29-5749-1 (PDF)
ISSN 0082-7002
Painosalama Oy - Turku, Finland 2014

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred in the text by their Roman numerals.

I Lehtimäki N, Lintala M, Allahverdiyeva Y, Aro EM, Mulo P. (2010) Drought stress -induced upregulation of components involved in ferredoxin-dependent cyclic electron transfer. Journal of Plant Physiology, 167:1018-22.

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 Lintala M*, Lehtimäki N*, Benz JP, Jungfer A, Soll J, Aro EM, Bölter B, Mulo P. (2012) Depletion of leaf-type ferredoxin-$NADP^+$ oxidoreductase results in permanent induction of photoprotective mechanisms in Arabidopsis chloroplasts, The Plant Journal, 70:809-817.

IV Lehtimäki N*, Koskela MM*, Dahlström KM, Pakula E, Lintala M, Hanke G, Rokka A, Battchikova N, Salminen TA, Mulo P (2014) Post-translational modifications of leaf-type ferredoxin-$NADP^+$ oxidoreductase in Arabidopsis chloroplast. Manuscript.

*Equal contributions

Publication I has been reprinted by kind permission of Elsevier.

Publications II and III have been reprinted by kind permission of Blackwell publishing.

ABBREVIATIONS

2D	two-dimensional
ATP	adenosine triphosphate
BN	blue native
CET	cyclic electron transfer
Chl	chlorophyll
Cyt	cytochrome
ETC	electron transfer chain
FAD	flavin adenine dinucleotide
FD	ferredoxin
FNR	ferredoxin-$NADP^+$ oxidoreductase
FQR	ferredoxin-plastoquinone reductase
HPLC	high-performance liquid chromatography
IEF	isoelectric focusing
RNAi	RNA interference
LET	linear electron transfer
L	leaf-type
LHC	light harvesting complex
MS	mass spectrometry
MV	methyl viologen
NADPH	nicotinamide adenine dinucleotide phosphate
NDH	NAD(P)H dehydrogenase
NPQ	non-photochemical quenching of Chl fluorescence
P_{700}	reaction center Chl of PSI
PAGE	polyacrylamide gel electrophoresis
PC	plastocyanin
PGR5	proton gradient regulation 5
PGRL1	proton gradient regulation like 1
pI	isoelectric point
PQ	plastoquinone
PS	photosystem
PTM	post-translational modification
R	root-type
RT-qPCR	real time quantitative polymerase chain reaction
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
T-DNA	transfer DNA
Tic	translocon of the inner envelope membrane of chloroplast
Toc	translocon of the outer envelope membrane of chloroplast
TROL	thylakoid rhodanese-like
WT	wild-type

ABSTRACT

Life on earth is based on sunlight, which is captured in chemical form by photosynthetic reactions. In the chloroplasts of plants, light reactions of photosynthesis take place at thylakoid membranes, whereas carbon assimilation reactions occur in the soluble stroma. The products of linear electron transfer (LET), highly-energetic ATP molecules, and reducing power in the form of NADPH molecules, are further used in the fixation of inorganic CO_2 molecules into organic sugars. Ferredoxin-$NADP^+$ oxidoreductase (FNR) catalyzes the last of the light reactions by transferring electrons from ferredoxin (FD) to $NADP^+$. In addition to LET, FNR has been suggested to play a role in cyclic electron transfer (CET), which produces ATP without the accumulation of reducing equivalents. CET is proposed to occur via two putative routes, the PGR5-route and the NDH-route.

In this thesis, the leaf-type FNR (LFNR) isoforms LFNR1 and LFNR2 of a model organism, *Arabidopsis thaliana*, were characterized. The physiological roles of LFNRs were investigated using single and double mutant plants. The viability of the single mutants indicates functionality of both isoforms, with neither appearing to play a specific role in CET. The more severe phenotype of low-temperature adapted *fnr2* plants compared to both wild-type (WT) and *fnr1* plants suggests a specific role for LFNR2 under unfavorable growth conditions. The more severe phenotype of the *fnr1 x fnr2* (F1 generation) plants compared to single mutants reflects down-regulated photosynthetic capacity, whereas slightly higher excitation pressure indicates mild over-excitation of electron transfer chain (ETC). However, induction of CET and various photoprotective mechanisms enable adaptation of *fnr1 x fnr2* plants to scarcity of LFNR. The *fnr1 fnr2* plants (F2 generation), without detectable levels of LFNR, were viable only under heterotrophic conditions. Moreover, drought stress induced acceleration of the rate of P_{700}^+ re-reduction in darkness was accompanied by a concomitant up-regulation of the PGR5-route specific components, PGR5 and PGRL1, demonstrating the induction of CET via the PGR5-route. The up-regulation of relative transcriptional expression of the *FD1* gene indicates that the FD1 isoform may have a specific function in CET, while no such role could be defined for either of the LFNR isoforms.

Both the membrane-bound and soluble LFNR1 and LFNR2 each appear as two distinct spots after 2D-PAGE with different isoelectric points (pIs), indicating the existence of post-translational modifications (PTMs) which do not determine the membrane attachment of LFNR. The possibility of phosphorylation and glycosylation PTMs were excluded, but all four LFNR forms were shown to contain acetylated lysine residues as well as alternative N-termini. N-terminal acetylation was shown to shift the pI of both LFNRs to be more acidic. In addition, all four LFNR forms were demonstrated to interact both with FD1 and FD2 *in vitro*.

TIIVISTELMÄ

Elämä maapallolla perustuu auringonvaloon, jota kasvien viherhiukkasissa tapahtuvat yhteyttämisreaktiot sitovat kemialliseen muotoon. Yhteyttämisen valoreaktiot tapahtuvat tylakoidikalvoilla ja hiilensidontareaktiot liukoisessa stroomassa. Lineaarisen elektroninsiirron tuotteet, korkeaenergiset ATP-molekyylit sekä pelkistävä voima NADPH-molekyylien muodossa, käytetään epäorgaanisten CO_2-molekyylien sitomiseen orgaanisiksi sokereiksi. Ferredoksiini-$NADP^+$-oksidoreduktaasi (FNR) katalysoi reaktiota, jossa ferredoksiini (FD) pelkistää $NADP^+$:n NADPH:ksi. FNR:n on ehdotettu toimivan myös syklisessä elektroninsiirrossa, joka tuottaa ATP:ta ilman NADPH-molekyylien muodostumista. Syklisen elektroninsiirron on ehdotettu tapahtuvan kahden mahdollisen reitin (PGR5- ja NDH-reitin) kautta.

Tässä väitöskirjassa tutkittiin lituruohon (*Arabidopsis thaliana*) lehtityypin FNR:n (LFNR) isoentsyymien (LFNR1 ja LFNR2) fysiologisia tehtäviä käyttäen mutanttikasveja, joista isoentsyymejä koodaavista geeneistä toinen (*fnr1*- ja *fnr2*-kasvit) tai molemmat on inaktivoitu. *fnr1*- ja *fnr2*-kasvien elinkyky viittaa molempien isoentsyymien toiminnallisuuteen. Lisäksi molemmat isoentsyymit osallistuvat samankaltaisesti sykliseen elektroninsiirtoon. Alhaiseen lämpötilaan sopeutuneiden *fnr2*-kasvien pieni koko ja alhainen klorofyllipitoisuus verrattuna villityyppiin ja *fnr1*-kasveihin viittaa LFNR2-isoentsyymin erityiseen fysiologiseen rooliin epäsuotuisissa kasvuolosuhteissa. Hidaskasvuisten ja kloroottisten *fnr1 x fnr2* (F1-sukupolvi) – kasvien fotosynteettisen koneiston alassäätelystä huolimatta elektroninsiirtoketju on lievästi ylivirittynyt. Kuitenkin syklisen elektroninsiirron sekä useiden valolta suojaavien mekanismien aktivoituminen mahdollistaa niiden sopeutumisen LFNR:n vähäiseen määrään. F2-sukupolvi (*fnr1 fnr2*), joilta LFNR puuttuu kokonaan, ovat elinkykyisiä vain heterotrofisissa kasvuolosuhteissa. Kuivastressissä $P700^+$:n uudelleenpelkistymisen nopeuden kiihtyminen pimeässä sekä PGR5-reitille spesifisiä proteiineja koodaavien *PGR5*- ja *PGRL1*-geenien ilmenemisen lisääntyminen osoittaa syklisen elektroninsiirron kiihtyneen PGR5-reitin kautta. *FD1*-geenin ilmenemisen lisääntyminen transkriptitasolla suhteessa *FD2*-geeniin viittaa FD1-isoproteiinin mahdolliseen spesifiseen rooliin syklisessä elektroninsiirrossa, kun taas kummallekaan LFNR isoentsyymille ei sellaista voitu määrittää.

Sekä kalvoon sitoutunut että liukoinen LFNR1 ja LFNR2 esiintyvät kaksisuuntaisen geelielektroforeesin jälkeen kahtena erillisenä muotona, joilla on erilaiset isoelektriset pisteet (pI). Tämä viittaa molempien isoentsyymien translaation jälkeiseen modifioimiseen, joka ei näytä määrittävän kalvoon sitoutumista. Kaikki neljä LFNR-muotoa sisältävät asetyloituneita lysiinejä sekä erilaisia proteiinin aminopäitä, mutta eivät fosforylaatiota tai glykosylaatiota. N-terminaalisen asetylaation osoitettiin siirtävän molempien LFNR-isoentsyymien isoelektristä pistettä happamammaksi. Lisäksi kaikki neljä LFNR-muotoa kykenevät vuorovaikutukseen sekä FD1:n että FD2:n kanssa *in vitro*.

TABLE OF CONTENTS

1 INTRODUCTION

1.1 Photosynthesis

Photosynthesis is the basis of all life on the earth: the light reactions of photosynthesis capture energy from sunlight and transfer it into a chemical form. The carbon assimilation reactions of photosynthesis further use this energy to produce carbohydrates from CO_2. Via photosynthesis, plants, cyanobacteria and other oxygenic photoautotrophs offer molecular oxygen and organic carbon sources to heterotrophs. Photosynthesis is an essential research subject – when the whole reaction chain is properly understood, this knowledge provides possibilities for both improved food and clean energy production.

1.1.1 Photosynthetic machinery and linear electron transfer

In higher plants photosynthesis takes place in specific organelles known as chloroplasts. Light reactions occur at the thylakoid membranes and carbon assimilation reactions occur in the soluble part of the chloroplast, the stroma (Fig. 1). In the light reactions of photosynthesis, energy from sunlight is captured via two photosystems, photosystem II (PSII) and photosystem I (PSI). Electrons originally held in water molecules are transferred via the large membrane-embedded pigment-protein complexes, PSII, cytochrome b_6f (Cyt b_6f), and PSI, to ferredoxin (FD) and further, for the reduction of $NADP^+$ to NADPH via the ferredoxin-$NADP^+$ oxidoreductase (FNR) enzyme (Fig. 2).

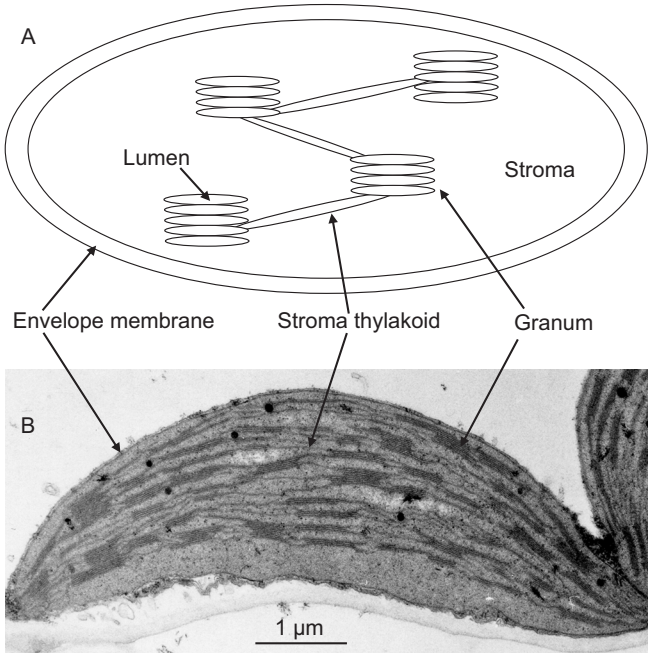

Figure 1. Outline of the chloroplast ultrastucture. Schematic illustration (A) and an electron micrograph of an ultrathin section (B) of a chloroplast. The outer membrane, intermembrane space and the inner membrane comprise the envelope that surrounds the soluble compartment, the stroma. Thylakoid membranes exist as stroma thylakoids and as stacks of thylakoids, called grana (*singular* granum). The soluble part inside the thylakoids is called the lumen.

During linear electron transfer (LET) protons are transferred to the lumen (Fig. 2). This results in a proton gradient, which is discharged via ATP synthase to produce highly-energetic ATP molecules. Products of light reactions, ATP and NADPH, are utilized mainly in carbon assimilation reactions, but also in other biosynthetic and regulatory reactions in chloroplasts. Here, the metabolism downstream of PSI is described in detail, since the FNR enzyme is the subject of this work.

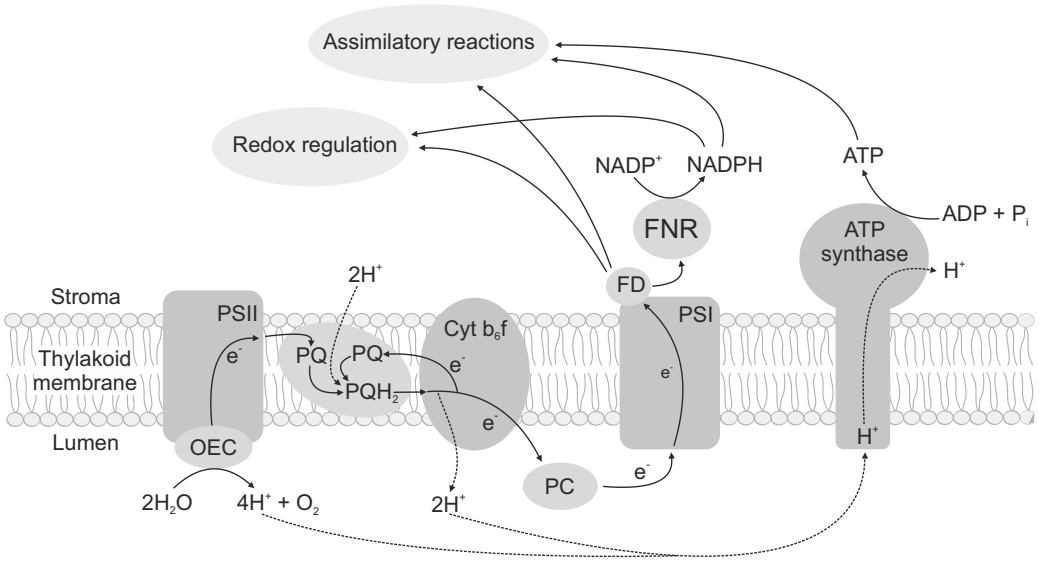

Figure 2. A schematic illustration of the linear electron transfer in oxygenic photosynthesis. Linear electron transfer reactions and stromal reactions are shown in outline. The oxygen evolving complex (OEC) catalyses reactions, which split water to oxygen and protons. Electrons are transferred via the pigment-protein complexes (PSII, Cyt b_6f and PSI), plastoquinone (PQ) pool and soluble plastocyanin (PC) to FD and further to number of stromal reactions. See text for details.

1.1.1.1 Ferredoxin

In organisms performing oxygenic photosynthesis, electrons are channelled from PSI to the stromal FD molecules. These FD molecules are small soluble proteins (plant-type ~ 10 kDa), which each contain a [2Fe-2S] cluster and have a negative redox potential of -300 − -460 mV (Williams-Smith and Cammack, 1977). Subunits of the PSI complex that interact with FD have been identified. It has been proposed that PSAC participates in electron transfer, whereas PSAD assists FD into its binding site and PSAE stabilizes the complex (Fischer, et al., 1998; Setif, et al., 2002). The FD molecule is guided to the catalytic site of a target enzyme via ionic interactions between negatively charged amino acid residues of FD and positively charged amino acid residues of the target enzyme. This interaction is further strengthened by the

removal of water molecules between FD and the interacting enzyme (Hase, et al., 2006).

In higher plants there are several genes encoding FD proteins. In *Arabidopsis thaliana* (Arabidopsis) six different FD encoding genes have been identified. Two of these encode photosynthetic proteins, FD1 (*At1g10960*) and FD2 (*At1g60950*), two encode root-type FDs, and the remaining two are FD-like proteins, FDC1 and FDC2, which have longer C-termini as compared to FD1 and FD2 (Voss, et al., 2011). Whilst the main photosynthetic isoform, FD2, constitutes as much as 90-99% of the whole FD pool in leaves (Hanke, et al., 2004; Voss, et al., 2008), the minor, FD1, isoform could be essential in CET (Hanke and Hase, 2008). The distinct Arabidopsis FD isoforms could also have specific roles in other metabolic reactions, such as electron distribution to assimilation of nitrate and sulphate as well as biosynthesis of chlorophyll (Chl) (Hanke and Mulo, 2013).

1.1.1.2 Ferredoxin-$NADP^+$ oxidoreductase

The FNR protein (EC 1.18.1.2) is a soluble and ubiquitous flavin adenine dinucleotide (FAD) enzyme which catalyses electron transfer between two FD molecules and $NADP^+$/NADPH according to the formula: 2 FD_{red} + $NADP^+$ + H^+ ↔ 2 FD_{ox} + NADPH (Arakaki, et al., 1997). All plant-type FNRs have a similar structure that contains two domains of approximately 150 amino acids each (Fig. 3) (Karplus, et al., 1991). The site for FD interaction is between these two domains (De Pascalis, et al., 1993). The C-terminal domain binds $NADP^+$ and the N-terminal domain serves as a binding site for a non-covalently bound FAD (Karplus, et al., 1991), which is added to the FNR structure immediately following import into chloroplast. This addition of FAD has been shown to be essential for the membrane binding of FNR (Onda and Hase, 2004). FAD enables the transfer of electrons from two FD molecules to one $NADP^+$ molecule, first by reducing FAD to $FADH^-$, then further to $FADH^-$ and finally by transferring the hydride H^- to $NADP^+$ (Carrillo and Ceccarelli, 2003).

In higher plants, a small gene family encodes distinct FNR isoforms and at least two isoforms, acidic and basic, exist in chloroplasts (Gummadova, et al., 2007; Hanke, et al., 2005). In Arabidopsis, a small nuclear gene family encodes four FNR isoforms. The genes *At4g05390* and *At1g30510* encode the root-type FNR (RFNR) isoforms, RFNR1 and RFNR2, and *At5g66190* and *At1g20020* encode the leaf-type FNR (LFNR) isoforms, LFNR1 and LFNR2, respectively. RFNRs function in non-photosynthetic plastids transferring electrons from NADPH to FD, which further donates electrons to various bioassimilation and biosynthesis related routes such as nitrogen metabolism (Hanke, et al., 2005). LFNRs of ~ 32 kDa catalyse the last step in the photosynthetic electron transfer chain (ETC) by transferring electrons from reduced FD to $NADP^+$, thus producing NADPH molecules for stromal primary metabolism. The most essential of these metabolic pathways is that of carbon assimilation (Ceccarelli, et al., 2004). Both LFNR isoforms are expressed in the shoot tissue

whereas no expression has been detected in the roots (Hanke, et al., 2005). In Arabidopsis, LFNR1 is more acidic (pI 5.54) than LFNR2 (pI 6.19) (Hanke, et al., 2005). It has been shown that both LFNR isoforms occur as two forms with different isoelectric points (pI) at thylakoids (Lintala, et al., 2007), suggesting that both LFNRs are modified after translation. In addition to Arabidopsis, the FNR gene families have been described in maize (Okutani, et al., 2005; Twachtmann, et al., 2012), wheat (Gummadova, et al., 2007; Moolna and Bowsher, 2010; Bowsher, et al., 2011), and rice (Higuchi-Takeuchi, et al., 2011).

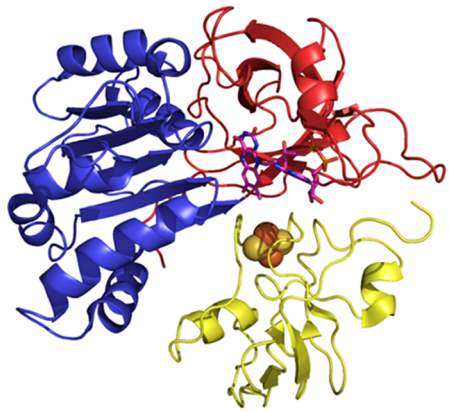

Figure 3. A structural model of LFNR in complex with FD. The N-terminal domain, which binds FAD, is shown in red. The C-terminal domain, which contains a binding site for $NADP^+$, is shown in blue. FD with a [2Fe-2S] cluster is shown in yellow. (From the courtesy of Dr. Tiina A. Salminen.)

LFNR isoforms are translated on cytosolic ribosomes and imported into the chloroplast. In chloroplasts, LFNR isoforms exist as a soluble pool in the stroma, but also as a membrane bound pool at thylakoids and at the inner envelope membrane of the chloroplast (Bohme, 1977; Fredricks and Gehl, 1982; Matthijs, et al., 1986; Lintala, et al., 2007; Kuchler, et al., 2002; Stengel, et al., 2008). The formation of a complex between the soluble or membrane-bound FNR and FD seems to occur with a similar dissociation constant, but regardless of this, *in vitro* experiments have shown that the membrane-bound form reduces $NADP^+$ molecules faster than soluble form (Forti and Bracale, 1984). Soluble LFNR is, however, shown to be photosynthetically active in Arabidopsis, as the mutant plants lacking thylakoid-bound LFNR are viable and are able to complete their life cycle successfully (Lintala, et al., 2007; Lintala, et al., 2014).

There is evidence that LFNR isoforms exist as a dimer (Kurisu, et al., 2001; Morales, et al., 2000; Zanetti and Arosio, 1980; Fredricks and Gehl, 1976) and it has been shown that in Arabidopsis LFNR1 is needed for membrane attachment of LFNR2 (Lintala, et al., 2007). Since FNR is not a transmembrane protein, there has been long-

term speculation surrounding its mechanism of membrane-binding, with the mode and location of binding potentially effecting both enzyme activity and targeted electron routes. According to previous suggestions, LFNR is able to interact with PSI via PSAE (Andersen, et al., 1992), Cyt b_6f (Clark, et al., 1984; Zhang, et al., 2001) and the NDH-complex (Guedeney, et al., 1996; Jose Quiles and Cuello, 1998). It has also been suggested that a base protein (Chan, et al., 1987; Vallejos, et al., 1984) or a small connectein protein (Shin, et al., 1985; Shin, et al., 1990) could mediate membrane binding. Moreover, LFNR may be co-located with glyceraldehyde-3-phosphate dehydrogenase, an enzyme functioning in the Calvin cycle, which suggests a high spatial concentration of NADPH molecules for carbon assimilation (Mulo, 2011).

Recently, however, it has been shown that both LFNR isoforms are able to form high molecular weight protein complexes with membrane-anchor proteins Tic62 (62 kDa subunit of the translocon of inner chloroplast membrane) (Kuchler, et al., 2002; Benz, et al., 2009) and TROL (thylakoid rhodanese-like protein) (Juric, et al., 2009). Both Tic62 and TROL proteins contain a specific FNR-binding domain(s) in their C-termini (Balsera, et al., 2007) and the binding is dependent on pH (Lintala, et al., 2014). Other potential LFNR-binding proteins lack this kind of motif. In addition, mutant plants lacking Tic62 or TROL are defective in binding of LFNR to membranes, whereas in *ndho* and *psae* mutant plants, which lack NDH-complex and functional PSI respectively, the LFNR-containing complexes at thylakoids accumulate normally (Lintala, et al., 2014). The *tic62 trol* double mutant plants completely lack membrane-bound LFNR, suggesting that LFNR binds tightly to thylakoids only via Tic62 and TROL (Lintala, et al., 2014). Accumulation of the Tic62-LFNR complexes is apparently not a direct prerequisite for photosynthesis, but Tic62 instead functions as a chaperone for LFNR, by protecting its stability and activity during photosynthetically inactive periods (Benz, et al., 2009). It may be instead, that the TROL-LFNR complexes have a connection to photosynthesis (Juric, et al., 2009). The *tic62 trol* double mutant plants possess no changes in the function of photosystems, but the $NADPH/NADP^+$ ratio of the mutant plants has decreased as compared to WT (wild-type) (Lintala, et al., 2014).

1.1.2 Alternative electron transfer routes

Electrons are channelled from the photosynthetic ETC to many essential metabolic routes. The main target of photosynthetically derived electrons is carbon assimilation. However, chloroplasts are also important sites for other metabolic reactions. FD-thioredoxin reductase mediated electrons affect the activation state of many enzymes (Schurmann and Buchanan, 2008), and assimilation of nitrogen and sulphur employs electrons from reduced FD (Hoff, et al., 1994; Hanke and Mulo, 2013). Moreover, catabolism and anabolism of Chl (Tanaka, et al., 1998; Reinbothe, et al., 2006), biosynthesis of phytochrome (Gohya, et al., 2006), as well as processing of fatty acids

(Wada, et al., 1993) require electrons from FD. In addition to these metabolic routes, a notable sink for photosynthetic electrons is molecular oxygen (Asada, 1999).

In addition to LET, cyclic electron transfer (CET) also takes place in the chloroplasts. In CET, electrons are channelled from PSI via FD back to the PQ-pool and Cyt b_6f complex. This creates a proton gradient and enables the production of ATP molecules without a concurrent accumulation of reducing equivalents (Arnon, et al., 1954; Joliot and Johnson, 2011). In cyanobacteria and unicellular algae, CET has an established role in metabolism (Mi, et al., 1995; Finazzi, et al., 2002) and in C_4 plants, CET creates additional ATP molecules in bundle sheath cells for the concentration of CO_2 (Rumeau, et al., 2007). Research on Arabidopsis mutants with impaired CET has demonstrated the significance of this route for normal growth and development (Munekage, et al., 2004), but still the comprehensive role and routes of CET in C_3 plants have remained controversial (Heber, 2002; Johnson, 2011; Joliot and Johnson, 2011; Allen, 2003). In higher plants, CET may be spatially separated from LET (Johnson, 2011). The process of LET is suggested to occur at the grana thylakoids, because the greatest fraction of PSII complexes exist in the grana stacks (Albertsson, 2001) and the diffusion of PQ is able to occur only in short distances (Kirchhoff, et al., 2000). Plastocyanin (PC) molecules could mediate the flow of electrons to PSI complexes located in grana margins and at stroma thylakoids (Joliot and Johnson, 2011). The CET process is instead proposed to take place in the stroma thylakoids (Joliot and Johnson, 2011).

During CET, electrons are channelled from the PQ pool to re-reduce P_{700}^{+} via Cyt b_6f and PC in a similar manner to LET. Electron transfer from FD to PQ may occur via the antimycin A sensitive PGR5- (proton gradient regulation 5) route (Tagawa, et al., 1963; Munekage, et al., 2004) or the NDH-route (Fig. 4). The PGR5-route, previously known as an FD- or FQR- (FD-plastoquinone reductase) route (Hanke and Mulo, 2013), has been suggested to be the principal CET pathway in C_3-plants, at least under standard conditions (Fig. 4, route 1) (Shikanai, 2007). The small soluble protein, PGR5, is attached to the thylakoids (Munekage, et al., 2002) and has been shown to be specifically involved in this route (Shikanai, 2007). The PGR5 protein may also regulate electron transfer between LET and CET (Nandha, et al., 2007). In the PGR5-route, electrons are transferred from FD to the PQ pool via the Cyt b_6f complex, which contains a c-heme suggested to be involved in CET (Shikanai, 2007). Alternatively, as Cyt b_6f is not antimycin A -sensitive, electrons may be funnelled directly to the PQ pool (Bendall and Manasse, 1995), possibly via the putative FQR enzyme (Cleland and Bendall, 1992). Recently, it was proposed that a thylakoid transmembrane protein, PGRL1 (proton gradient regulation like 1), could be the long-sought after FQR (Hertle, et al., 2013). The PGRL1 protein has previously been shown to interact with PGR5 in a physical and functional way in Arabidopsis (DalCorso, et al., 2008). New evidence indicates that PGRL1 is able to both form homodimers and heterodimers with PGR5 and bind a Fe-containing cofactor via redox-active cysteine residues (Hertle, et al., 2013). It has also been shown that PGRL1 is sensitive to antimycin A and can function

as FQR *in vitro* (Hertle, et al., 2013). In line with previous findings showing that LFNR is purified in complex with Cyt b_6f (Clark, et al., 1984; Zhang, et al., 2001; Okutani, et al., 2005), Joliot & Johnson (Joliot and Johnson, 2011) have proposed that LFNR binds to Cyt b_6f, thus offering a binding site for FD and enabling CET. Indeed, *in vitro* interactions between Arabidopsis PGRL1 and PSI, FD, LFNR and Cyt b_6f have been reported (DalCorso, et al., 2008), and there is evidence of PSI-Cyt b_6f-FNR-PGRL1 supercomplex formation in *Chlamydomonas reinhardtii* (Iwai, et al., 2010). It has been speculated that these complexes may be transient, and function as a switch between CET and LET (Joliot and Johnson, 2011).

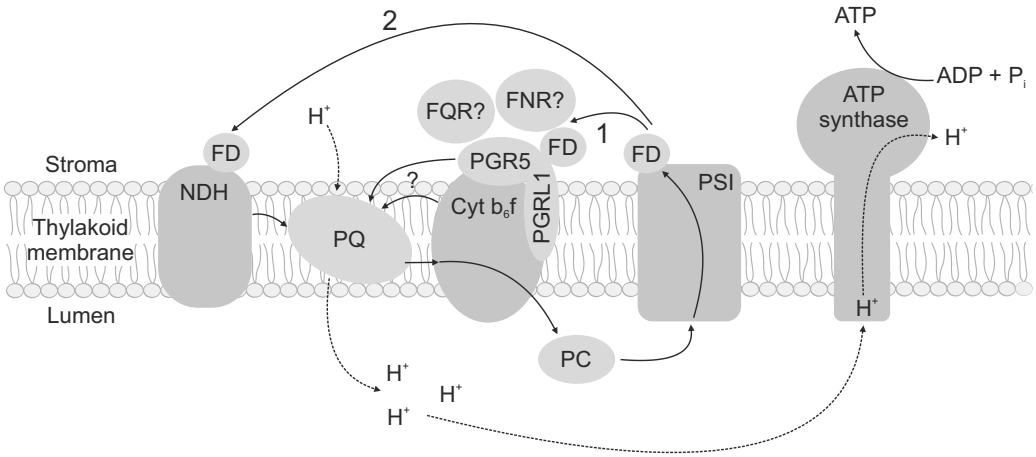

Figure 4. Putative cyclic electron transfer routes around photosystem I. Route 1 represents PGR5-route and route 2 NDH-route. See text for details.

The NDH-route transfers electrons via the NDH-complex, which is composed of several chloroplast and nucleus-encoded subunits (Fig. 4, route 2) (Suorsa, et al., 2009). The NDH-complex is involved in the electron transfer from the stromal side to the PQ pool (Shikanai, et al., 1998), and it may protect the photosynthetic machinery upon stress conditions (Rumeau, et al., 2007). In addition to CET, the NDH-complex is proposed to play a role in chlororespiration, where the PQ pool is protected from over-reduction by transferring electrons from PQ to molecular oxygen (Peltier and Cournac, 2002; Rumeau, et al., 2007). Previously, it was assumed that electrons are transferred in the NDH-route from NADPH molecules to the PQ pool via the NDH-complex, but recently a binding site for FD in NDH-complex, protein CRR31/NDHS, was identified (Yamamoto, et al., 2011). Specifically, the positive charge of arginine 193 in CRR31/NDHS is essential when FD is electrostatically interacting with the NDH-

complex (Yamamoto and Shikanai, 2013). There is also evidence for supercomplex formation between PSI and NDH-complex (Peng, et al., 2008). However, there is a contradiction between a low concentration of the NDH-complex (Sazanov, et al., 1996) and high rate of CET (Joliot and Joliot, 2002), implying that the role of this route is minor. Nevertheless, the NDH-route is proposed to be essential in C_4 photosynthesis and in cyanobacteria (Takabayashi, et al., 2005; Mi, et al., 1995).

Two main roles for CET have been suggested in C_3 plants. The first role validates the existence of CET under physiological conditions, whereby it could function to create the additional ATP molecules needed to increase the ATP/NADPH ratio (Allen, 2003; Joliot and Joliot, 2006). This is supported by the inability of LET to produce sufficient ATP molecules to create optimal ATP/NADPH ratio for carbon assimilation and by the fact that photorespiration increases the need for higher ATP concentration (Osmond, 1981). Indeed, controlling the switch between LET and CET might play a key role in adjusting the metabolism according to environmental cues (Niyogi, 1999; Kramer, et al., 2004), yet the exact mechanism of this control still remains unknown. The second suggested role for CET relates to adaptation in changing environmental conditions such as excess light, drought or low CO_2 (Clarke and Johnson, 2001; Golding and Johnson, 2003). Under high light conditions, induction of CET enhances the proton gradient, acidifying the lumen, which triggers the protection of both photosystems against photoinhibition (Joliot and Johnson, 2011). There are two proposed mechanisms for this protection, the first mechanism takes place at grana thylakoids via non-photochemical quenching (NPQ) (Heber and Walker, 1992; Joliot and Johnson, 2011), which results in dissipation of excess absorbed light energy as heat in a process including xanthophyll cycle (Shikanai, 2007) and PsbS (Li, et al., 2002). The second mechanism is linked to the acidification of the lumen, which induces oxidation of P_{700} reaction centres at stroma thylakoids and grana margins, thereby protecting PSI complexes against photoinhibition (Joliot and Johnson, 2011).

1.2 Post-translational modifications

Plants are sessile organisms which have to cope with fluctuating environmental conditions without changing their location. Post-translational modifications (PTMs) including proteolytic cleavage or addition of a modifying component to an amino acid residue allow a quick adjustment of metabolism. At present, more than 400 different modification types are identified (Khoury, et al., 2011). Among the most common modifications are phosphorylation, glycosylation, ubiquitination, methylation, and acetylation. PTMs affect proteins in various ways: activity of enzymes, subcellular localisation, stability and interaction with other molecules. It is shown that approximately 10% of the genes in Arabidopsis encode proteins related to phosphorylation and ubiquitination, and the plant protein kinase superfamily is larger when compared to other eukaryotes (Lehti-Shiu and Shiu, 2012). These findings imply that PTMs have a central role in plant metabolism.

In literature the term "PTM code" refers to the ensemble of all PTMs, including information on their quantity and sequence in certain metabolic process (Strahl and Allis, 2000; Cloutier and Coulombe, 2013). The most well-known example is the PTM code of histones, which includes combinatorial or sequential function of PTMs (Strahl and Allis, 2000). To date it is known that PTMs are located in the N- and C-termini of the four histones, changing charge (acetylation of lysine) or recruiting binding domains (acetylation, methylation and phosphorylation), thus affecting gene expression (Lothrop, et al., 2013). PTM codes for chloroplast proteins are yet to be deciphered.

Methodology employed in PTM research has progressed in the latest decade, but most studies still concentrate on the identification of certain PTMs in certain proteins or protein populations. In order to solve the whole PTM code, it is important to quantitate PTMs and to take note of all different modifications in a certain context. The most commonly used method to characterize PTMs is mass spectrometry (MS), which provides molecular mass and fragmentation pattern for peptides of cleaved proteins, revealing PTMs that change the molecular weight of the peptides (Mann and Jensen, 2003). There are, however, several limitations when PTMs are studied via MS methodology. Only PTMs that are stable enough to remain with the peptide during fragmentation are able to be captured. Also, the completeness of the fragmentation pattern affects which modifications are revealed (Mann and Jensen, 2003). One key question is whether enough protein can be obtained for analysis, because a gene can result in numerous protein products as a result of alternative splicing and different modification status and, as a consequence, the amount of proteins in a certain modification state can be inadequate. The study of PTMs in a protein population can be completed using, for example, antibodies or metabolic dyes after first separating proteins on a gel (Mann and Jensen, 2003). It is important to note that neighboring PTMs may have an effect on the function of the antibody. It has been reported that methylation or acetylation of a nearby lysine residue prevents the recognition of phosphorylation (Lothrop, et al., 2013). To present, 2D gel electrophoresis is often used to separate proteins. Fairly good reproducibility and resolution is reached via performing the first dimensional separation using immobilized pH gradient (IPG) strips to separate proteins based on their charges (Gorg, 1993), followed by a second dimension separation on the basis of molecular weight.

1.2.1 Phosphorylation

Protein phosphorylation is one of the most well-known and common PTMs. Three amino acid residues – tyrosine (Tyr), serine (Ser) and threonine (Thr) – are typically known to be phosphorylated at their hydroxyl oxygen (Plaxton and McManus, 2008). From a methodological point of view, phosphorylated tyrosine is a stable modification whereas phosphorylated serine and threonine are moderately stable or labile (Mann and Jensen, 2003). One phosphorylated amino acid residue changes the molecular weight of the protein by 80 Da (Mann and Jensen, 2003). Phosphorylation is a reversible

PTM, the addition of a phosphate group is catalysed by protein kinases and the removal is catalysed by protein phosphatases. Environmental cues are able to be converted into internal signals in plant metabolism via the activation of specific kinases and phosphatases, and it is shown that phosphorylation takes place in every subcellular compartment in plant cells (Reiland, et al., 2009). As previously mentioned, a significant proportion of the genome of Arabidopsis is related to phosphorylation, and the large protein kinase superfamily underlines the importance of this PTM in regulation of plant proteins (Lehti-Shiu and Shiu, 2012). Phosphorylation and equally, dephosphorylation, often function as a switch by activating and inactivating enzymes. They play a central role in regulating molecular interactions, in signalling pathways, as well as affecting catalytic activity and subcellular localisation of proteins (Whitmarsh and Davis, 2000). It has also been shown that phosphorylation of the transit peptides of precursor proteins is essential for them to be imported efficiently into chloroplasts (Waegemann and Soll, 1996). Phosphorylation changes the charge of a protein, allowing the observation of the different phosphorylation states of a protein as a horizontal series of protein spots after isoelectric focusing (IEF) (Mann and Jensen, 2003). Different MS methods have been successfully employed to study the reversible phosphorylation of thylakoid proteins in chloroplasts (Fristedt and Vener, 2011).

1.2.2 N-glycosylation

Glycosylation denotes an enzymatic reaction where a carbohydrate group is added to a protein. This PTM is known to occur in all organisms (Spiro, 2002). One glycosylated protein may contain multiple carbohydrates in several different amino acid residues. N-linked glycosylation changes the molecular weight of the target protein by more than 800 Da, and it is methodologically labile or moderately stable by nature (Mann and Jensen, 2003). This type of glycosylation, where an oligosaccharide is added to an asparagine residue, is typical for excreted proteins (Mann and Jensen, 2003; Barrero-Gil and Salinas, 2013). The N-linked glycosylation process begins in the endoplasmic reticulum where a lipid linked glycan is transferred to an asparagine residue of a target protein and linked via an amide bond (Song, et al., 2013). There is a consensus site in amino acid sequence for N-glycosylation to occur: Asn-X-Ser/Thr (NxS/T), where X can be any amino acid with the exception of proline (Bause, 1983). It is estimated that glycosylation takes place on under 65% of the consensus sites of the secreted proteins, suggesting that other requirements for N-glycosylation also exist (Petrescu, et al., 2004). The N-glycan is modified further after it is linked to the protein by the co-operation of various enzymes, and modifications continue in the Golgi apparatus (Barrero-Gil and Salinas, 2013). Modification of an N-glycan to a more complex structure in the Golgi apparatus differs between plants and animals (Song, et al., 2013). It is known that certain N-glycans have a functional role in the endoplasmic reticulum, where the linked glycan functions as a sign of the quality concerning the protein folding state (Helenius and Aebi, 2004). In plants, N-glycosylation has been suggested

to affect subcellular localisation, secretion (Ceriotti, et al., 1998), folding, catalytic activity, thermostability (Lige, et al., 2001) and interactions with pathogens (Haweker, et al., 2010).

1.2.3 Acetylation and methylation

Acetylation is a common covalent post- or co-translational modification of eukaryotic proteins (Polevoda and Sherman, 2002), that is methodologically stable by its nature and changes the target protein molecular weight by 42 Da (Mann and Jensen, 2003). In protein acetylation, the acetyl group is transferred to the target protein from acetyl coenzyme-A (Cloutier and Coulombe, 2013). There are three types of protein acetylations that create the protein acetylome. In O-acetylation, the hydroxyl group of an internal serine or threonine residue is acetylated, and it is known that O-acetylation competes with phosphorylation at the same residues (Xing and Poirier, 2012). A second type of acetylation is N^{α}-acetylation where an acetyl group is linked to the α-amino group (N^{α}) of the N-terminal amino acid of a protein (Polevoda and Sherman, 2002). N^{α}-acetylation is a common irreversible co-translational protein modification in eukaryotes (Arnesen, et al., 2009). It is catalysed by N-terminal acetyltransferases that exist in all kingdoms (Polevoda and Sherman, 2002). It is unfeasible to predict potential N^{α}-acetylation sites because there is no simple consensus sequence (Pesaresi, et al., 2003). The biological significance of N^{α}-acetylation varies between target proteins (Pesaresi, et al., 2003). There is controversial data concerning the physiological roles of this PTM (Persson, et al., 1985) and only scarce information exists in relation to plants (Hoshiyasu, et al., 2013).

The third type of protein acetylation is N^{ε}-acetylation where an acetyl group is linked to the ε-amino group (N^{ε}) of an internal lysine residue (Singh, et al., 2010). This PTM affects the electrostatic status of the target protein by removing a positive charge (Glozak, et al., 2005). N^{ε}-acetylation is a reversible PTM, as lysine acetyl transferases catalyse the addition of acetyl group to lysine, whereas lysine deacetylases catalyse deacetylation (Xing and Poirier, 2012). There is evidence that in Arabidopsis N^{ε}-acetylation of histones play a central role in growth and development via the regulation of gene expression (Wu, et al., 2011) and in responses to environmental cues (Sheldon, et al., 2006). During the last decade N^{ε}-acetylation of non-histone proteins has been found from numerous proteins in mammalian cells and in bacteria, where it was found to affect enzyme activity and direction of carbon flux (Xing and Poirier, 2012). Two recent studies have revealed that N^{ε}-acetylation is also a widespread modification in Arabidopsis, and that proteins of various function and from different subcellular locations are N^{ε}-acetylated (Finkemeier, et al., 2011; Wu, et al., 2011).

Methylation is a stable modification which adds 14 Da to the molecular weight of the target amino acid (Mann and Jensen, 2003). Methyltransferase catalyses a transfer of a methyl group from a donor molecule, typically S-adenosylmethionine, to the target residue of a protein (Grillo and Colombatto, 2005). At present, several demethylase

enzymes have been shown to exist indicating that methylation is a reversible PTM (Mosammaparast and Shi, 2010). Lysine and arginine residues are common targets for methylation, but also the N-termini or C-termini of proteins, as well as the side chains of histidine, asparagine, glutamine, glutamic acid, aspartic acid and cysteine, may be targets (Cloutier and Coulombe, 2013). Lysine residues may be mono-, di- or trimethylated (Grillo and Colombatto, 2005). Identifying whether an amino acid residue contains acetylation or trimethylation of lysine is a good example of the challenges to studying PTMs: the difference in mass in MS methods is less than 0.04 Da. Methylation of arginine and lysine residues has been shown to affect the interactions, stability, localisation and enzyme activity of proteins (Ahmad and Cao, 2012; Paik, et al., 2007).

2 AIMS OF THE STUDY

In this thesis work, the target was to characterize the two isoforms of an essential enzyme, LFNR, functioning toward the end of the light reactions of photosynthesis. LFNR1 was characterized in earlier work (Lintala, et al., 2007), and this work was continued by using single mutant lines *fnr1* and *fnr2* (paper II), and also generations F1 (*fnr1 x fnr2*) and F2 (*fnr1 fnr2*) from their crossing (paper III). In addition, potential PTMs of LFNR1 and LFNR2 were a subject of analysis (paper IV). As LFNR is a suggested component of cyclic electron flow, my aim was to reveal whether the expression of *LFNR1* and *LFNR2* genes, as well as other putative components of different CET routes, was changed under drought stress when compared to standard growth conditions (paper I).

3 METHODOLOGICAL ASPECTS

3.1 Plant material, growth conditions and stress treatments

A. thaliana ecotype Columbia plants were used in all experiments. In papers II and III T-DNA insertion mutant line *fnr1* (SALK_085403) and RNAi mutant line *fnr2* (N204598 AGRIKOLA), and in paper III the F1-generation of their crossing (*fnr1 x fnr2*) and segregating F2-generation (*fnr1 fnr2*) were employed. Different *fnr* mutant lines were screened at DNA level by PCR analysis and at protein level by using native gel electrophoresis and a FNR specific antibody. Furthermore, in paper II, T-DNA insertion mutant lines *ndh-o* and *pgr5* were used. Generally plants were grown under 100 µmol photons $m^{-2}s^{-1}$ with 8-h light/16-h dark cycles at 23 °C. The *fnr1 fnr2* plants were grown heterotrophically as described in paper III. Specific growth conditions (in paper II low temperature), and treatments (in paper I drought stress, in paper III and IV high light stress) are described in paper in question.

3.2 Measurement of photosynthetic properties

3.2.1 Analysis of cyclic electron transfer

The methodology for analysis of cyclic electron transfer is complicated, and there is no unambiguous and generally accepted way to measure it. As a cyclic process, there is no net flux of electrons and when measuring the flux through certain components of the ETC it should be noted that they also participate in LET (Johnson, 2011). In this work, CET around PSI was analysed via three distinct methods. In papers I, II and III the redox state of P_{700} from dark-adapted leaves was monitored by absorbance changes at 810 nm (820 nm with JTS-10) using 860 nm as reference (880 nm with JTS-10) employing a PAM-Fluorometer PAM-101/102/103 (Walz) with an ED- P_{700}DW-E emitter-detector or JTS-10 (Bio-Logic). In paper II, cyclic electron transfer was additionally studied via the transient post-illumination increase in Chl *a* fluorescence (F_0 'rise'), and by measuring the far-red light induced thermoluminescence afterglow (AG) signal using the PSI Thermoluminescence System TL200/PTM. The last two methods are proposed to illustrate the NDH-route of CET (Shikanai, et al., 1998; Havaux, et al., 2005).

3.2.2 Photosystem II

The electron transfer efficiency of PSII was studied using several methods in papers II and III. Oxygen evolution was monitored from extracted thylakoids under saturating white light using a Hansatech oxygen electrode to determine electron transfer activity from water to 2,6-dimethyl-p-benzoquinone (DMBQ). Chl *a* fluorescence was monitored using a Plant Efficiency Analyzer (Hansatech Instruments) or Dual-PAM

(Walz). The maximum quantum efficiency of PSII (F_V/F_M); excitation pressure of PSII, 1-qP (1-[($F_{M'}$-F_S)/($F_{M'}$-$F_{0'}$)]); quantum yield of PSII, Y(PSII) (($F_{M'}$-F_S)/($F_{M'}$)) and NPQ ((F_M-$F_{M'}$)/($F_{M'}$)) were computed using the determined fluorescence values. In paper III photoinhibition was measured as the F_V/F_M from leaves that were incubated either on water or on lincomycin, an inhibitor of chloroplast translation.

3.2.3 Photosystem I

The efficiency of PSI electron transfer was studied by monitoring oxygen consumption when electrons were funnelled from 2,6-dichloroindophenol ($DCPIPH_2$) to methyl viologen (MV) (paper II). Additionally, the quantum yield (Y(PSI)) and acceptor side limitation of PSI (Y(NA)) were analysed simultaneously by monitoring the redox state of P_{700} with Dual-PAM (Walz) (paper III).

3.2.4 CO_2 assimilation

CO_2 fixation in paper II was studied by measuring the CO_2 assimilation rate of whole plants under atmospheric CO_2 concentration and standard growth conditions using the Ciras-1 photosynthesis system (PP systems).

3.3 Transcriptional analysis

Gene expression analysis at transcript level was performed via quantitative real-time PCR (RT-qPCR) (iQ5, Bio-Rad Laboratories) as described in papers I, II and III, and via microarray analysis as described in paper II. Samples for both analyses were collected in the middle of the light period. The specificities of the primer pairs in RT-qPCR were tested prior to analysis using temperature gradient and melt curve analysis. In paper I PSBO1 (*At5g66570*), in paper II Actin 2 (*At3g18780*), and in paper III Sand family protein (*At2g28390*) and Tip41-like protein (*At4g32370*) were used as reference genes. Microarray analysis was conducted by using *Arabidopsis thaliana* 24-K oligonucleotide arrays (MWG Biotech) and the data was analysed by using the R software package LIMMA (http://www.r-project.org).

3.4 Analysis of proteins and pigments

Membrane-bound, soluble and total proteins were extracted as described in the papers in question. Purer extracts were obtained by chloroplast isolation using Percoll gradients, and further fractionation to samples containing membrane-bound or soluble proteins. Additionally, FD1 and FD2 bound protein samples were prepared in collaboration with Dr. Guy Hanke (University of Osnabrück) (paper IV). The FD1 or FD2 proteins were produced in *Escherichia coli*, purified and bound covalently to a sepharose resin. A crude extract of *A. thaliana* proteins interacted with the resin

following elution of FD1 or FD2 bound proteins. Samples were concentrated and desalinated using centrifugal filters (Amicon Ultra-2 10K, Millipore).

The protein contents of extracts were measured using BioRad Protein Assay Kit, and the Chl content of extracted thylakoid membranes as in Porra *et al*. (1989). The Chl content relative to leaf area was analysed via the method illustrated by Inskeep and Bloom (1985). In paper IV part of the protein extracts were treated with protease inhibitors (Complete, Roche Diagnostics) and/or phosphatase inhibitors (PhosSTOP, Roche Diagnostics) as described in the paper. Additionally in paper IV, certain protein extracts were treated using phosphatases (Lambda Protein Phosphatase and Alkaline Phosphatase CIP, New England BioLabs).

In paper III, pigment analysis including Chl *a* and *b*, neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, β-carotene and α-tocopherol, was performed using high-performance liquid chromatography (HPLC) after extraction under dim light and low temperature according to Lehtimäki *et al*. (2011).

Proteins or protein complexes were separated using several distinct gel electrophoretic methods. Non-denaturating PAGE (12% w/v acrylamide) was used in papers II and III to separate proteins in their native forms. Denaturating PAGE was performed using urea rich gels (15% w/v acrylamide, 6 M urea) or non-urea Next gels (Amresco Inc.). Thylakoid protein complexes were separated via Blue Native (BN) -PAGE as described by (Sirpiö, et al., 2007) and the BN gel strips were subjected to 2D-PAGE (15% acrylamide, 6 M urea) as described in paper II. In paper IV IEF was used to separate membrane-bound and soluble proteins according to their pIs employing Immobiline DryStrip (IPG) gels (GE Healthcare Life Sciences) with a non-linear pH gradient 3-11. After IEF, proteins were separated in second dimension using SDS-PAGE (14% w/v acrylamide) as described in paper IV.

Separated proteins, or in certain cases modifications of the proteins, were identified using various methods. Western blots and silver and Coomassie stainings were performed as illustrated in papers I-IV. Mass spectrometry analysis was performed for selected protein spots according to standard procedures. Glycosylated proteins were identified via staining with Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain kit (Molecular Probes) and visualizing under UV-light. Putative phosphorylation was analysed by using Pro-Q® Diamond Phosphoprotein Gel Stain kit (Molecular Probes) and Phos-tag™ Acrylamide (Wako Pure Chemical Industries, Ltd.). Oxidative modifications of membrane-bound proteins were identified by using OxyBlot™ Protein Oxidation Detection Kit (Millipore).

Structural modelling of the LFNR1 and LFNR2 proteins including their modifications in complex with a Tic62 peptide was completed in collaboration with Tiina A. Salminen (Åbo Akademi) using the crystal structure of *Pisum sativum* FNR as described in paper IV.

3.5 Microscopic analysis

In paper III, light and electron microscopy analyses from leaf tissue were performed according to Pätsikkä *et al*. (2002).

3.6 Analysis of oxidative stress

In papers II and III, ion leakage with and without MV, indicating cell death induced by oxidative stress, was studied as described by Kangasjärvi *et al*. (2008). Possible oxidative stress was further assayed via monitoring the activities of ascorbate peroxidase and glutathione reductase as illustrated in Foyer *et al*. (1983) with modifications of Pätsikkä *et al*. (2002). Additionally, the amount of ascorbate in leaf tissues was analysed based on the method by Foyer *et al*. (1983).

4 OVERVIEW OF THE RESULTS

4.1 LFNR isoforms in Arabidopsis chloroplasts

4.1.1 Location and amount of LFNR

In this thesis, the LFNR isoforms were characterized by using Arabidopsis WT and *fnr* mutant plants. Lintala *et al.* (2007) have characterized *fnr1* knock-out mutant plants and characterization was continued in this thesis by using *fnr2* knock-out plants (paper II) and F1 and F2 generations of the *fnr1* and *fnr2* crossing (paper III). The F1 generation (*fnr1/FNR1 fnr2(RNAi)*) is denoted as *fnr1 x fnr2*, and segregating F2 generation with no detectable amounts of LFNR as *fnr1 fnr2*. In WT plants, the proportion of *LFNR1* mRNA (68 %) was greater than the proportion of *LFNR2* mRNA (32%). In single *fnr* mutants the amount of total LFNR was decreased and transcriptional analysis indicated that neither isoform was up-regulated to compensate the lack of the other isoform. In *fnr1 x fnr2* plants there was only ca 50 % of LFNR when compared to WT plants. These mutant plants contained only LFNR1 isoform in stroma and at thylakoids, yet the amount of LFNR1 was less than that found in *fnr2* plants. Thus, a heterozygotic RNAi construct of *LFNR2* was sufficient to silence the expression of LFNR2 protein, whereas inactivation of one copy of the *FNR1* gene in mutants resulted in decreased accumulation of LFNR1 when compared to WT.

LFNR isoforms have nearly identical molecular mass but different pIs. Native-PAGE at pH 8.8 enabled separation of the LFNR isoforms, as LFNR1 with more negative charge (pI 5.54) migrates faster than LFNR2 (pI 6.19). In WT plants, LFNR1 and LFNR2 isoforms are both located in the soluble stroma and at thylakoid membranes, whereas in the *fnr1* plants LFNR2 isoform was located only in the stroma (Lintala, et al., 2007). In the *fnr2* plants, LFNR1 isoform was detected both in stroma and at thylakoids (paper II). By using BN-PAGE, LFNR of WT plants was localised in several large protein complexes at thylakoids. However, in *fnr2* plants the majority of thylakoid-bound LFNR was detected as free protein and only one protein complex contained LFNR (paper II).

4.1.2 Consequences of decreased LFNR expression on plant metabolism

4.1.2.1 Shortage of LFNR results in retarded growth of the plants

Lack of one, or a scarcity of both LFNR isoforms resulted in smaller rosette size, lower Chl content and higher Chl a/b ratio as compared to the WT plants (papers II and III). In single *fnr* mutants, these differences were smaller than in double mutant plants as compared to WT. However, *fnr2* mutants exhibited a somewhat more severe phenotype when compared to *fnr1* plants. For example, they contained chlorotic areas in leaves. Moreover, upon growth under low temperature (+10°C), *fnr1* plants were similar in

appearance to the thick and dark green –leaved WT plants, whereas *fnr2* plants differed from both, having a lower Chl content and higher Chl a/b ratio (paper II). Single *fnr* mutant plants were highly viable, whereas *fnr1 x fnr2* plants contained chlorotic areas in the tips of the leaves and the plants were pale green and showed retarded growth, suggesting disturbed autotrophic development. Indeed, the cells of *fnr1 x fnr2* plants contained fewer and smaller chloroplasts on anticlinal cell walls when compared to WT plants (paper III). Regardless of slower development and stress symptoms, *fnr1 x fnr2* plants completed their life cycles successfully. Plants with no detectable amounts of LFNR, *fnr1 fnr2*, were able to cope only on heterotrophic growth medium (2% w/v sucrose) (paper III). These plants were pale and yellowish with abnormal mesophyll, containing small cells and few small chloroplasts with loose thylakoid membrane structure.

4.1.2.2 Deficiency of LFNR results in down-regulation of photosynthetic machinery

The performance of PSII and PSI was analysed in *fnr* mutant plants (papers II and III). No significant differences in PSII or PSI activity of the single mutants were detected when compared to WT. However, the carbon assimilation was decreased especially in the *fnr2* plants, which was in line with the smaller size of rosette. In *fnr1 x fnr2* plants, the quantum yield of PSII electron transport, Y(PSII), was slightly lower and excitation pressure of PSII (1-qP) slightly higher than in WT under different actinic light intensities (paper III). In addition, NPQ was up-regulated under high actinic light intensities. Under standard conditions, the maximum quantum efficiency of PSII (F_V/F_M) was similar in *fnr1 x fnr2* and WT plants. However, PSII of *fnr1 x fnr2* plants was more vulnerable to photoinhibition under high light intensity, both with and without lincomycin, when compared to WT. Regardless of this severe photoinhibition, the recovery of PSII was as efficient in the *fnr1 x fnr2* plants as in WT plants. In the *fnr1 fnr2* plants, lower Y(PSI) and Y(PSII) indicated that PSs in *fnr1 fnr2* plants are substantially more susceptible to increased light intensity damage than those of WT plants.

The effects of a decreased amount of LFNR on the accumulation of other photosynthetic proteins were examined in all mutants. In both single *fnr* mutants, the expression of several light harvesting complex (*LHC*) encoding genes were up-regulated; although LHCB1 and LHCA1 proteins, representing light harvesting complexes, were less abundant than in WT plants (paper II). In single mutants, accumulation of proteins representing PSII (D1) and PSI (PSAD) was also impaired. Similarly to observed phenotypic differences, the low-temperature acclimated *fnr2* plants also differed from *fnr1* and WT plants at protein level, with amounts of D1, PSAD and LHCA1 proteins markedly decreased in *fnr2* plants. In *fnr1 x fnr2* plants, the amount of stromal proteins remained stable whereas the amounts of thylakoid proteins (LHCA1, LHCB1, D1 and PSAD) were clearly decreased. The exception to this down-regulation was ATP synthase (β-subunit), which showed no difference in

regulation between the double mutant and WT (paper III). According to BN-PAGE analysis, there were more PSII monomers and less PSII dimers and supercomplexes in the single mutants as compared to WT plants, which was consistent with flash fluorescence induction analysis (paper II). In addition, BN-PAGE analysis illustrated decreased amount of LHCII trimers and increased amounts of LHCII monomers.

4.1.2.3 Distribution of electrons in the *fnr* mutants

Targeting of electrons to alternative electron transfer routes was studied by focusing on the rate of re-reduction of P_{700}^+, which is considered as an indicator of CET (Bukhov, et al., 2004; Golding, et al., 2004). Under standard growth conditions, the single *fnr* mutant plants demonstrated both F_0 rise and thermoluminescence AG bands that indicated slower re-reduction of P_{700}^+ and suggested alterations in the CET route (paper II). In low-temperature grown single mutants, however, the re-reduction of P_{700}^+ was faster than in WT plants (paper II). High light treatment induced even faster re-reduction of P_{700}^+ both in WT and single mutant plants (paper II), but there were no significant differences between *fnr1* and *fnr2* mutant plants. In *fnr1 x fnr2* plants the re-reduction of P_{700}^+ was induced when compared to WT plants (paper III). The PGRL1 protein, representing the PGR5-route of CET, was substantially up-regulated (174 %) in *fnr1 x fnr2* plants in comparison to WT plants, indicating that CET may be induced in these plants via the PGR5-route. In transcriptional analysis performed to clarify whether the lack of either LFNR isoform affects other metabolic routes (paper II), genes encoding nitrate reductase were found to be up-regulated and leaves were found to contain more nitrite in *fnr2* plants in comparison to WT plants.

4.1.2.4 Scarcity of LFNR induces oxidative stress and photoprotective mechanisms

Chlorotic phenotype of mutant plants indicated oxidative stress, which was studied in *fnr1*, *fnr2* and *fnr1 x fnr2* plants by measuring relative ion leakage from leaves treated with MV (papers II and III). More ion leakage was detected from the cells of single mutant plants than from WT cells (paper II). Under low-temperature conditions, there was less ion leakage from single mutants, with *fnr2* plants being more resistant than WT and *fnr1* plants (paper II). In *fnr1 x fnr2* plants substantially higher ion leakage was observed even without MV-induction (paper III). Consistent with ion leakage evaluations, more oxidative modifications of thylakoid proteins were observed with OxyBlot™ Protein Oxidation Detection Kit in single mutants grown under standard conditions and less in low-temperature grown *fnr2* mutant plants as compared to WT plants (paper II). In single mutant plants, the photoprotective status was nearly unchanged under standard conditions. This was contrary to notable changes in *fnr1 x fnr2* mutant plants, in which several photoprotective mechanisms were induced. In *fnr1 x fnr2* plants, the amount of xanthophyll cycle pigments and β-carotene was increased in relation to total Chl amount (paper III). In addition, the de-epoxidation state of the xanthophyll cycle pigments and the relative amount of the lipid peroxidation quencher

α-tocopherol (Niyogi, 1999) were higher in *fnr1 x fnr2* plants when compared to WT plants. In line with signs of the lower oxidative stress in low-temperature adapted *fnr2* plants, as compared to *fnr1* and WT plants, induction of oxidative stress responses was evidenced by high accumulation of antioxidative ascorbate peroxidase and 2-cysteine peroxiredoxin enzymes, and by a more reduced state of ascorbate pool (paper II).

4.1.3 Post-translational modifications of LFNR isoforms

Both membrane-bound LFNR1 and LFNR2 form two distinct spots with different pIs, but nearly identical molecular weights, after 2D-IEF-PAGE, indicating the possibility of PTMs (Lintala, et al., 2007). Membrane-bound protein fractions gave additional, fainter spots in the proximity of the four dominant spots. These additional spots were confirmed to be either LFNR1 or LFNR2 isoform (paper IV, Fig. 2). In this thesis, the focus was on the identification of the PTMs of the main four spots (paper IV). It is important to note that in the soluble fraction all four LFNR forms were detected and thus it seems that PTMs do not determine membrane binding.

The possibility of phosphorylation of LFNR proteins was assayed, because it is the best characterized PTM in chloroplasts and the 2D-IEF-PAGE pattern of LFNRs is typical for phosphorylation (Mann and Jensen, 2003) (paper IV). In addition, both LFNR1 and LFNR2 contain potential phosphorylation sites on the surface of their structures (Lintala, et al., 2007), and phosphorylation of *P. sativum* FNR has been evidenced *in vitro* (Hodges, et al., 1990). Phospho-threonine, -serine and -tyrosine antibodies were used to detect potential phosphorylation of LFNR in Western blot analysis, but no signal was detected. Similarly, Pro-Q® Phosphoprotein Stain gave no signal from LFNR forms. Protein samples were treated with alkaline and Lambda protein phosphatases, which dephosphorylated photosynthetic proteins (paper IV, Fig. 3) to check whether some of the LFNR spots would disappear. However, phosphatase treatment or phosphatase inhibitor treatment had no effect on LFNR spot pattern. Additionally, the potential phosphorylation of LFNR was studied using Phos-tag™ acrylamide. Consistent with previous results, the electrophoretic mobility of LFNR remained the same after phosphatase treatment of the immunoprecipitated LFNR. According to these results, it seems that LFNR isoforms are not phosphorylated.

Recently, acetylation (Zybailov, et al., 2008; Wu, et al., 2011; Finkemeier, et al., 2011), methylation (Trievel, et al., 2003; Mininno, et al., 2012), and glycosylation (Faye, et al., 1993; Villarejo, et al., 2005) of chloroplast proteins have been reported and therefore these modifications were also subjects of this work (paper IV). There is a single conserved glycosylation site in all plant FNR proteins (Fig. 5) (paper IV). Prospective glycosylation was analysed using Pro-Q® Emerald Glycoprotein Stain and a fucose antibody, both of which gave a weak signal from LFNR forms from thylakoids (paper IV). However, these signals are likely to be nonspecific, as the same signals were detected from the membrane fraction of *cgl* mutant plants, which lack

complex N-linked glycans (von Schaewen, et al., 1993). In addition, no glycosylation was found from MS-data analysis (data not shown).

Acetylation and methylation status of membrane-bound LFNR forms was studied using MS-analysis (paper IV). Methylation could not be reliably detected, but all four LFNR forms possessed acetylated lysine (K) residues (N^{ε}-acetylation). The acidic LFNR1 spot contained acetylated K175 and K321 whereas in the basic LFNR1 only K321 was reliably observed as acetylated. Both acidic and basic LFNR2 spots were found to have acetylated K96 and K330. A structural model was used to illustrate the locations of acetylated lysine residues on the surface of LFNR molecules (Fig. 5). Intriguingly, although K321 and K330 are in the vicinity of NADP(H) binding site, based on structural modelling they are not likely to affect interaction with FD.

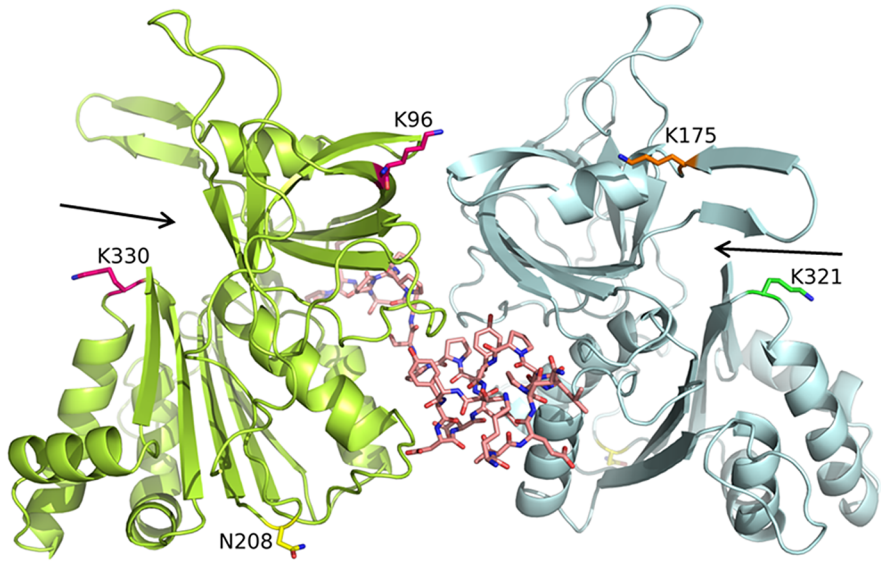

Figure 5. A structural model of LFNR1 and LFNR2 in complex with a peptide of their membrane anchor Tic62 pointing out locations of PTMs. LFNR1 is shown in turquoise, LFNR2 in green and a peptide of Tic62 peptide in pink. Acetylated lysine residues and conserved glycosylation site (N208) are shown. Interaction sites between LFNR and FD are pointed with arrows. (From the courtesy of Dr. Tiina A. Salminen)

In wheat, photosynthetic FNR isoforms undergo alternative N-terminal trimming, which affects their enzymatic activity (Gummadova, et al., 2007). The possibility of N-terminal trimming was studied using MS-analysis and N-terminal sequencing (paper IV). MS-analysis illustrated three possible starting sites for all forms, whereas N-

terminal sequencing enabled identification of one or two possible starting sites for the basic forms, whilst no data was gained for the acidic forms (table 1). The amount of peptides starting with alanine (A) was low based on MS-analysis, and this may explain why these peptides were not detected by N-terminal sequencing. In addition, according to MS-analysis, peptides starting with glutamine (Q) dominated relative to peptides starting with isoleucine (I) in the basic LFNR2 spot, which may explain the lack of the latter peptides in N-terminal sequencing. Moreover, MS-analysis revealed that the vast majority of N-terminally acetylated (N^{α}-acetylation) peptides were identified in the acidic spots, composed of peptides starting with glutamine (Q), valine (V) or isoleucine (I) residues (shown in bold in table 1).

Table 1. Alternative N-termini and detected N^{α}-acetylated residues of LFNR forms. All four forms contain three possible N-termini. The residues showing major N^{α}-acetylation are shown in bold. n/a denotes for data not available.

	LFNR1		**LFNR2**	
	acidic	*basic*	*acidic*	*basic*
MS	A/**Q/V**TTDTT	A/Q/VTTDTT	A/**Q/I**TTETD	A/Q/ITTETD
N-terminal seq.	n/a	Q/VTTDTT	n/a	QITTETD

Since the light-regulated redox state affects the accumulation of LFNR protein at thylakoids (Benz, et al., 2009), and because PTMs are known to be frequently affected by environmental cues, the pattern of LFNR spots was studied from plants treated under various light conditions (darkness; growth light, 100 µmol photons $m^{-2}s^{-1}$; and high light, 1000 µmol photons $m^{-2}s^{-1}$) (paper IV). Upon increasing light intensity, the intensities of both of the basic membrane-bound LFNR spots increased slightly, whereas the amounts of acidic membrane-bound spots decreased somewhat (paper IV, Fig. 5). Nevertheless, the most prominent changes were detected in the amounts of soluble LFNR spots, as the shift of the plants from darkness to light induced accumulation of both LFNR2 spots in the stroma at the expense of both LFNR1 spots. In addition to the characterization of potential PTMs, we analysed the binding capacity of different LFNR forms to two different FD isoforms, FD1 and FD2. However, all membrane-bound and soluble LFNR forms were able to bind both FD1 and FD2 proteins *in vitro* (paper IV, Fig. 6). Table 2 presents a summary of the studied PTMs in Arabidopsis LFNRs.

Table 2. Summary of studied PTMs and interaction with FD1 and FD2 in membrane-bound LFNR forms under standard growth conditions. - denotes absence of a PTM, + symbolises presence of a PTM or a detected interaction and n.d. stands for data not detected.

	LFNR1		**LFNR2**	
	acidic	*basic*	*acidic*	*basic*
Phosphorylation	-	-	-	-
N-glycosylation	- /n.d.	-/n.d.	- /n.d.	- /n.d.
Methylation	n.d.	n.d.	n.d.	n.d.
N^{ε}-acetylation	+	+	+	+
N^{α}-acetylation	+	-	+	-
Alternative N-termini	+	+	+	+
Binds FD1 *in vitro*	+	+	+	+
Binds FD2 *in vitro*	+	+	+	+

4.2 Cyclic electron transfer

4.2.1 Drought stress induces expression of components involved in the PGR5-route of CET

One aim of this work was to reveal whether the genes encoding potential CET-related components were differentially expressed after exposing Arabidopsis plants to drought, indicating their specific involvement in either the PGR5- or NDH-route of CET (paper I). Drought-stressed plants had a distinct phenotype of smaller size and accumulated anthocyanins compared to control plants. The faster re-reduction of P_{700}^{+} under drought conditions indicated induction of CET. The expression level of proteins representating the PGR5-route (PGRL1a, PGRL1b, PGR5) and NDH-route (NDHH), as well as putative CET-related proteins (LFNR1, LFNR2, FD1, FD2, PSAD1, PSAD2, PSAE1, PSAE2) from the stromal side of PSI, were studied both at transcriptional and translational levels using RT-qPCR and western blot analyses. Expression of *PGR5* and *PGRL1* was up-regulated both at transcript and protein level, whereas there was no change in the expression of *NDHH*. Both *LFNR1* and *LFNR2* were slightly up-regulated at transcript level, while the soluble LFNR protein level remained the same and the amount of membrane-bound LFNR was decreased in the drought-stressed plants as compared to the control. At transcript level, drought stress induced slight up-regulation of *FD1* and *PSAE2*, whereas both *PSAD* isoforms and *PSAE2* were slightly down-regulated. At protein level, PSAD was down-regulated and FD was up-regulated. The abundance of the whole *PGRL1* transcript pool increased under drought stress, and the amount of *PGRL1B* was increased relative to *PGRL1A*. Whilst the pool size of *LFNR* transcripts increased under drought stress, the ratios of isoforms remained the same. It is worth noting that the drought stress induced a reduction in all other studied transcript pools. Importantly, the relative amounts of *PSAE2* and *FD1* transcripts increased at the expense of their respective isoforms.

5 DISCUSSION

5.1 Do specific LFNR isoforms play distinct roles in CET?

Although suppression of CET upon chemical inhibition of LFNR has been reported decades ago (Shahak, et al., 1981), the ultimate role and possible involvement of LFNR in a specific CET route has remained controversial. Although the NDH-complex and LFNR have been identified from the same protein complex after native PAGE (Guedeney, et al., 1996; Jose Quiles and Cuello, 1998), no further proof for the involvement of LFNR in the NDH-route of CET has been presented. The formation of NDH-PSI supercomplexes has been evidenced, but without the involvement of LFNR (Peng, et al., 2008; Sirpiö, et al., 2009; Suorsa, et al., 2009). Recently, an FD binding site was identified in the NDH-complex (Yamamoto, et al., 2011). This demonstrates that electrons may be transferred directly from FD to the NDH-complex, excluding the need for LFNR in this route. Also, the activity of NDH has been shown to be independent from the activity of LFNR (Teicher and Scheller, 1998)

Several pieces of evidence suggest that LFNR might function in the PGR5-route of CET, and it has been proposed that LFNR could be a component of the as yet uncharacterized FQR enzyme (Cleland and Bendall, 1992). There are reports that LFNR has diaphorase activity *in vitro* (Bojko and Wieckowski, 1995) and that it is able to reduce plastoquinones (Bojko, et al., 2003). LFNR has been purified together with Cyt b_6f (Clark, et al., 1984; Zhang, et al., 2001; Okutani, et al., 2005), and there is *in vitro* evidence showing interaction of LFNR1 and LFNR2 with PGRL1 (DalCorso, et al., 2008). Moreover, a supercomplex containing LFNR has been identified in *C. reinhardtii* (Iwai, et al., 2010), while no such complex has been detected in higher plants. The similarity of *fnr1* and *fnr2* mutant lines observed in our work under standard growth conditions (paper II) indicates that, when environmental conditions are favourable, neither LFNR1 nor LFNR2 has a specific role in LET, CET, or other electron transfer routes.

FD plays an important role in CET, because it directs electrons from PSI to further putative routes of CET. Interestingly, we found that the proportion of *FD1* mRNA within the *FD* gene family increased in relation to *FD2* mRNA in drought-stressed plants, which may indicate that the FD1 isoform is more important for CET, whereas FD2 could be the major isoform for LET. This result is consistent with previously reported observations using Arabidopsis RNAi and knock-out lines of FD1 and FD2 (Hanke and Hase, 2008; Voss, et al., 2008; Liu, et al., 2013). Specificity of FD isoforms has been suggested in maize, in which the FDII isoform may function in CET, whereas the FDI isoform may participate in LET (Majeran, et al., 2005; Kimata-Ariga, et al., 2000). Down-regulation of the FD2 isoform in Arabidopsis has been proposed to function as a protective mechanism under extended high light conditions (Liu, et al.,

2013). Moreover, introduction of the *P. sativum* minor FD isoform in tobacco has been demonstrated to result in enhanced CET (Blanco, et al., 2013). In contrast to previous studies showing prominent down-regulation of FD protein upon various stresses, including water deprivation (Zimmermann, et al., 2004; Tognetti, et al., 2006), our work (Paper I) shows an increase in the total amount of FD in the drought-stressed plants. It should be noted, however, that the accumulation of *FD* mRNA responds strongly to various stresses, with the level of expression changing over time (Liu, et al., 2013), which might explain the discrepancy.

Induction of P_{700}^{+} re-reduction and clear up-regulation of PGRL1 protein suggest increased activity of the PGR5-route in the *fnr1 x fnr2* plants (paper III). It appears that in these plants, even under standard conditions, the reduced amount of LFNR is sufficient enough to push electrons to alternative transfer routes in order to avoid over-reduction of ETC. However, this contradicts the suggestion that the NDH-route would be responsible for protection of the stromal side of the thylakoid from over-reduction (Shikanai, 2007). In contrast, the threshold is apparently not reached in the single *fnr* mutants as the rate of CET is slower as compared to the WT (paper II). Induced CET also increases NPQ via acidification of the lumen and thus excess absorbed energy is removed as heat (Niyogi, 1999). However, the ability of *fnr1 x fnr2* plants to induce CET, despite a clearly reduced amount of LFNR, might indicate that LFNR is not an essential component of the PGR5-route. Similarly to the reduced amount of LFNR in *fnr1 x fnr2* plants, adverse environmental conditions seem to induce CET when the amount of absorbed light energy exceeds the ability to use it via LET. The lack of one of either LFNR isoforms is not limiting enough to induce CET under standard growth conditions, but the combination of LFNR scarcity and low-temperature adaptation causes induction (paper II), which is in line with induced CET in high light adapted *fnr1* mutant plants (Hanke, et al., 2008).

Different environmental conditions lead to the adjustment of ATP/NADPH ratio in the chloroplast enabling a plant to adapt its metabolism (Bendall and Manasse, 1995; Joet, et al., 2002; Bukhov, et al., 2004). Environmental stresses, including drought, are also known to down-regulate carbon assimilation (Golding and Johnson, 2003). Under drought stress, higher plants close their stomata in order to prevent transpiration which impairs carbon assimilation when the internal CO_2 concentration decreases (Golding, et al., 2004; Johnson, 2005). Induction of CET is reported to occur simultaneously (Golding, et al., 2004; Johnson, 2005), which may produce more ATP for photorespiration (Rumeau, et al., 2007) and may also protect photosynthetic machinery by inducing NPQ (Clarke and Johnson, 2001). The NDH-route of CET has been proposed to be essential under drought stress (Horvath, et al., 2000). However, in our study (Paper I), the expression of the components that are known to be specific for the PGR5-route (PGR5, PGRL1a and PGRL1b) was up-regulated both at transcriptional and translational level in plants suffering from drought. Despite this, the expression of NDHH, representative for NDH-route, remained unchanged under drought stress,

potentially indicating that the induced CET in drought-stressed plants takes place via the PGR5-route. In considering these results however, it is important to note that there is no consensus regarding the best methodology to measure CET and so no direct conclusions can be drown about the route of CET by using P_{700}^{+}, F_0 rise or thermoluminescence afterglow measurements. The PGR5-route has also been suggested as being induced under prolonged high light treatment in Arabidopsis mutant plants lacking FD2 (Liu, et al., 2013). Interestingly, no such induction has been seen in WT plants (Liu, et al., 2013). More research is required to clarify the possible roles and routes of CET in C_3 plants, and it will be important to establish whether the other pathways requiring reducing power are induced upon unfavourable growth conditions.

5.2 Both LFNR isoforms are functional and photosynthetically active

Depletion of either isoform of LFNR resulted in slight changes in the phenotype of the mutant plants (paper II), indicating that either LFNR1 or LFNR2 alone is able to secure the photoautotrophic growth of the plants. Although *LFNR1* covers a greater proportion of *LFNR* transcript pool (approx. 70%) (paper I), the lack of LFNR2 induced a more severe phenotype, including a smaller size, decreased Chl content and CO_2 assimilation capacity, as well as chlorotic leaf tips. The location and roles of LFNR isoforms have also been studied in other higher plant species. The genomes of rice (*Oryza sativa*) and wheat (*Triticum aestivum*) encode two LFNR isoforms similar to that of Arabidopsis (Higuchi-Takeuchi, et al., 2011; Gummadova, et al., 2007). Over-expression of rice *LFNR1* has been shown to affect the nitrogen assimilation pathway, whereas over-expression of *LFNR2* has been shown to impair both the LET and the PGR5-route of CET (Higuchi-Takeuchi, et al., 2011). In total, two distinct genes encode eight different LFNR forms in wheat. The forms possess different pIs, resulting from PTMs (Gummadova, et al., 2007; Moolna and Bowsher, 2010). Both *LFNR1* and *LFNR2* are expressed similarly in wheat cells with different developmental stages, but nitrate and dark conditions induce differences in expression (Gummadova, et al., 2007). Maize (*Zea mays*) is an exception among the higher plants, as it has three LFNR encoding genes instead of two (Okutani, et al., 2005). The isoforms possess different membrane binding capacity, LFNR1 is a membrane-bound isoform, LFNR2 exists both at membranes and in the soluble fraction, whilst LFNR3 is only found in the soluble fraction (Okutani, et al., 2005). It has recently been reported that mesophyll cells and bundle sheath cells contain LFNR isoforms in different ratios, and that the ratio of membrane-bound and soluble pools also differ (Twachtmann, et al., 2012).

Up-regulation of nitrate reductase at transcript level, as along with higher levels of nitrite in the leaves of *fnr2* plants (compared to WT) suggests enhanced assimilation of nitrogen and is in agreement with previously reported *fnr1* data (Lintala, et al., 2007; Hanke, et al., 2008). Connection of nitrogen supply and expression level of LFNR isoforms have been reported earlier for Arabidopsis (Hanke, et al., 2005), wheat (Gummadova, et al., 2007), rice (Higuchi-Takeuchi, et al., 2011), and maize (Okutani,

et al., 2005). Both *LFNR1* and *LFNR2* are up-regulated at transcript level in nitrate-grown maize plants (Okutani, et al., 2005). Maize LFNR3 isoform has a suggested heterotrophic role due to up-regulation by both ammonium and nitrate (Okutani, et al., 2005).

During the characterization of LFNR1 and LFNR2 mutant plants, the low-temperature condition enabled identification of a difference in function between the two isoforms, as low-temperature acclimated *fnr2* plants were smaller and contained less Chl when compared to WT and *fnr1* plants (paper II). Induction of photo-oxidative stress and protective mechanisms at many levels implies a specific role for the LFNR2 isoform under unfavourable conditions. Thus, it seems that LFNR1 and LFNR2 are mostly able to compensate the function of each other in plant metabolism, but that either both of the LFNR isoforms, or WT levels of total LFNR, are required for optimal performance of the plants.

It has been assumed that specifically the membrane-bound form of LFNR functions in LET (Forti and Bracale, 1984). However, recent studies have shown that mutant plants lacking either LFNR1 or the FNR membrane anchors, Tic62 and TROL, are not able to accumulate LFNR isoforms at thylakoid membranes, yet are able to maintain normal function of photosystems (Lintala, et al., 2007; Lintala, et al., 2014). This indicates that soluble LFNR is also photosynthetically active and that membrane-binding of LFNR is not critical for photosynthesis, at least in Arabidopsis. It therefore appears that Tic62, TROL (Lintala, et al., 2014) and LFNR1 (Lintala, et al., 2007) are needed for tight binding of LFNRs to membranes. Even though the LFNR1 isoform was observed both as membrane-bound and soluble in mutant plants lacking LFNR2 isoforms, it seems that the existence of LFNR2 is needed for WT level accumulation of the remaining isoform in membrane-bound complexes (paper II). In addition to the tightly membrane-bound and soluble pools of LFNR, a loosely membrane-bound pool might also exist (Matthijs, et al., 1986). This kind of weaker interaction may occur if LFNR interacts, as has been suggested, with NDH (Guedeney, et al., 1996; Jose Quiles and Cuello, 1998), Cyt b_6f (Clark, et al., 1984; Zhang, et al., 2001), or PSI (Andersen, et al., 1992). It might also be that these weaker interactions induce the formation of putative transient supercomplexes during CET, although whether LFNR can be a component of such a complex remains contentious. It is also possible that PTMs, resulting in changes in the interactions between molecules, might be involved in the formation of such transient complexes.

5.3 Induction of photoprotective mechanisms in *fnr* mutant plants

In all *fnr* mutants, more ion leakage was observed from cells compared to cells of WT plants (paper II and III), likely indicating that mutant plants are more prone to oxidative stress. This supports the suggested role of FNR in protection against reactive oxygen species (ROS). In *E. coli*, FNR is reported to be an essential molecule in

oxidative stress tolerance (Krapp, et al., 1997) and over-expression of FNR seems to have a photoprotective role in plant tissue (Rodriguez, et al., 2007). It has been reported that in wheat, the induction of ROS by MV causes dissociation of LFNR from thylakoid membranes without a concomitant increase in accumulation of LFNR (Palatnik, et al., 1997). Generally, photo-oxidative stress arises when the amount of absorbed light energy exceeds the ability of LET to function, and thus harmful oxidizing molecules, ROS, are produced. There are several antioxidative mechanisms in chloroplasts to protect the plant against ROS. Antioxidative mechanisms are however, only a part of all of the photoprotective processes, which operate at many different levels in plants.

When one LFNR isoform was lacking, plants contained decreased amount of pigments and photosynthetic proteins, and there were no clear symptoms of oxidative stress, with the exception of chlorotic spots in the leaves of the *fnr2* plants (paper II). Thus, it seems that the lack of one LFNR isoform under favourable conditions does not lead to an increase in excitation pressure in such a way that the plant would need to efficiently up-regulate protective mechanisms, or that it would result in clear photodamage. It does appear that, when the amount of LFNR is limiting enough, both oxidative stress symptoms and induction of photoprotection at many levels become evident (paper III). Only slightly higher excitation pressure of PSII in *fnr1 x fnr2* mutant plants indicates that, regardless of the limiting amount of LFNR, plants are able to adjust their metabolism to prevent over-excitation of the ETC (paper III), possibly via induction of other electron transfer routes. However, in *fnr1 fnr2* plants, where there is no detectable amount of LFNR, the ETC is extremely over-reduced (paper III).

One level of photoprotection is the regulation of light harvesting. All *fnr* mutant plants contained less pigments and photosynthetic proteins, including LHC proteins, as compared to WT and down-regulation was stronger with decreasing amounts of LFNR (papers II and III). This is in agreement with previous data showing that decreased LFNR correlates with decreased chlorophyll content (Hajirezaei, et al., 2002). In addition, organization of chloroplasts to the anticlinal cell wall in *fnr1 x fnr2* plants reflects adaptation to avoid excess light. This kind of negative phototaxis exists in some algae and plant species (Niyogi, 1999). Another level of photoprotection is to direct electrons to alternative electron transfer routes or to dissipate excess excitation energy as heat (NPQ). As the amount of LFNR in *fnr* mutant plants limits CO_2 assimilation, it can be presumed that the other electron transfer routes are induced. However, this is the case only when the amount of LFNR is limiting enough, as in *fnr1 x fnr2* plants (paper III). Activation of NPQ is dependent on the acidification of the lumen, which is a consequence of an enhanced proton gradient over the thylakoid membrane. Acidification of the lumen activates violaxanthin de-epoxidase (VDE) enzyme, essential in the xanthophyll cycle, and protonates PSBS protein which is proposed to function in sensing pH in the induction of NPQ (Shikanai, 2007; Li, et al., 2002). Indeed, the ability of *fnr1 x fnr2* plants to induce more NPQ in higher light

intensities, as well as the higher de-epoxidation state of xanthophyll pigments, reflect the capability of the plant to dissipate excess excitation energy. Additionally, CET seems to be induced in *fnr1 x fnr2* plants, which also enables enhancement of the proton gradient for NPQ to be activated. One possible alternative route for electrons is the water-water cycle (Asada, 1999). This route includes non-enzymatic and enzymatic antioxidative agents: the superoxide radical (O_2^-) is metabolized to water and oxidized ascorbate by the action of superoxide dismutase and ascorbate peroxidase; oxidized ascorbates are then reduced in reactions involving FD, NAD(P)H and the glutathione cycle (Asada, 1999). In addition to these molecules, carotenoids and tocopherols are also antioxidant molecules. Carotenoids include xanthophyll molecules, which protect against lipid peroxidation and also stabilize membranes (Niyogi, 1999). As an essential antioxidant, α-tocopherol protects against singlet oxygen ($^1O_2$), superoxide radical (O_2^-) and hydroxyl radicals (OH•), and also prevents lipid peroxidation (Havaux, et al., 2005). Under favourable conditions the lack of either LFNR isoform causes no notable induction of antioxidative systems (paper II), but in the *fnr1 x fnr2* plants the amount of xanthophylls and α-tocopherol is markedly increased (paper III). Excess light energy may induce photoinhibition of PSII, where the target of irreversible damage is the D1 protein (Aro, et al., 1993). It has been shown that a decreased content of Chl in plant leaves exposes PSII complexes to photoinhibition (Pätsikkä, et al., 2002), which was evident in *fnr1 x fnr2* plants. Surprisingly, the recovery of PSII complexes was as efficient in *fnr1 x fnr2* plants as in WT plants. This might be due to up-regulation of α-tocopherol in *fnr1 x fnr2* plants, because α-tocopherol quenches $^1O_2$ which is known to inhibit the repair of photoinhibited PSII (Inoue, et al., 2011; Hakala-Yatkin, et al., 2011).

5.4 Deciphering the post-translational modification code in Arabidopsis LFNR isoforms

There are several levels of regulation to adjust metabolism according to environmental cues in plants. Long-term adjustment of the photosynthetic machinery (Walters and Horton, 1995) occurs via transcriptional regulation (Shimizu, et al., 2010), as well as via specific degradation of certain proteins and protein complexes (Yang, et al., 1998). Although PTMs may play a significant role in affecting gene expression and protecting or exposing proteins for degradation, they are also known to change the activity of enzymes, enabling rapid responses to changing conditions. Despite the early stage of research into plant protein PTMs, there is evidence that proteins in the chloroplast contain several PTMs including phosphorylation (Vener, 2007), acetylation (Michel, et al., 1988; Zybailov, et al., 2008), methylation (Trievel, et al., 2003; Mininno, et al., 2012), and N-glycosylation (Nanjo, et al., 2006). It is therefore essential to map the PTM codes of chloroplast proteins and to strive to understand the biological significance behind them.

5.4.1 The effect of post-translational modifications on the localization of LFNR isoforms and interaction with ferredoxin

As LFNR1 and LFNR2 isoforms have been reported to exist as two distinct spots in the membrane-bound pool (Lintala, et al., 2007), the soluble pattern was examined to determine whether the PTMs that cause a shift in the pI are related to membrane binding (paper IV). However, the existence of a similar soluble LFNR pattern indicates that these PTMs are not the sole determinants of localization. Because PTMs often affect interactions with other molecules (Mann and Jensen, 2003), the binding of FD1 and FD2 isoforms to the LFNR isoforms was studied (paper IV). According to our results, the PTMs do not prevent the interaction of LFNR forms with FD1 and FD2 *in vitro*, even though the majority of bound LFNR1 and LFNR2 seem to be the acidic form. However, whether this is the case *in vivo*, or simply an artefact remains to be determined. Additionally, it appears that even if the acetylated K321 / K330 is located in close proximity to the catalytic center of LFNR (Fig. 5), this modification is unlikely to affect interaction with FD.

5.4.2 LFNR isoforms in Arabidopsis are not likely targets of phosphorylation or N-glycosylation

Both LFNR isoforms contain predicted phosphorylation sites which have been shown to be located on the surface of the proteins by structural modelling (Lintala, et al., 2007). Moreover, FNR is reported to be phosphorylated *in vitro*, and phosphorylation has been suggested to affect enzyme activity as well as membrane binding of FNR (Hodges, et al., 1990). Also, conflicting data from phosphoproteome studies concerning the phosphorylation status of LFNR have been reported (Reiland, et al., 2009; Sugiyama, et al., 2008). In wheat, four out of eight forms of LFNR with different pIs are predicted to be phosphorylated. This suggestion, however, is based on calculations and no direct evidence exists (Moolna and Bowsher, 2010). In this work, we have used several methods to study the phosphorylation status of Arabidopsis LFNRs. Although all of our results suggest that LFNR is not a phosphoprotein, the possibility of phosphorylation cannot be totally excluded under some specific environmental conditions. Indeed, various environmental conditions have been shown to change the phosphoproteome (Cloutier and Coulombe, 2013). For example, under high light conditions PSII core proteins are phosphorylated with a concomitant dephosphorylation of the antenna proteins of LHCII (Vener, 2007). These dynamic changes regulate state-transitions and turnover of the D1 protein (Vener, 2007).

The starting point for our investigation of whether LFNR of Arabidopsis is N-glycosylated, was the conserved N-glycosylation site (NxS/T) (Song, et al., 2013), localized on the surface of the protein by structural modelling (paper IV). There are many challenges in studying glycoproteins. Glycosylated peptides and glycans have different chemical properties and the amount of glycosylated proteins in plants is

relatively low (Song, et al., 2013). Based on the results obtained using different methods (immunoblotting with fucose antibody and staining with Pro-Q® Emerald), it seems that LFNR isoforms are not N-glycosylated. Fucose antibody and Pro-Q® Emerald stain gave signals to several membrane-bound proteins, including LFNRs. However, similar signals obtained from *cgl* mutant plants, which lack complex N-linked glycans (von Schaewen, et al., 1993), indicate false positive results. In general, the application of Pro-Q® Emerald Glycoprotein stain appears challenging for 2D-gels. Even though a protein standard is available, its proper amount and the exposure time of the gel limit the possibility of obtaining reliable results.

In the light of present knowledge, the machinery for glycosylation exists only outside of the chloroplast. Notwithstanding, there is evidence of glycosylated proteins situated in the chloroplast stroma (Faye, et al., 1993; Villarejo, et al., 2005). One good example is glycosylation of α-carbonic anhydrase (CAH1), which has been shown to affect folding and export of the protein from the endoplasmic reticulum to the plastid, as well as the enzyme activity (Buren, et al., 2011; Villarejo, et al., 2005). This protein, along with some other chloroplast proteins, contains a predicted signal peptide to ER (Kleffmann, et al., 2004; Friso, et al., 2004). This evokes a question as to how nuclear encoded glycosylated proteins are transferred into chloroplasts (Faye and Daniell, 2006). Generally, nuclear encoded proteins that are targeted to the chloroplast contain a transit peptide in the N-terminus, which guides them through the Tic-Toc translocon complex in chloroplast membrane and is removed when protein is transferred into the chloroplast (Soll, 2002). Nevertheless, the chloroplast proteome also contains proteins which lack predicted transit peptides, which suggests the existence of alternative protein import routes (Kleffmann, et al., 2004; Friso, et al., 2004), such as vesicular transport (Villarejo, et al., 2005).

5.4.3 N-terminal modifications as possible modulators of function

A few examples exist in literature concerning N^{α}-acetylation in plants. N^{α}-acetylation of transit peptide in nuclear-encoded precursor proteins that are targeted to chloroplast is likely to be a co-translational modification (Bienvenut, et al., 2012). Pesaresi *et al*. (2003) characterized an Arabidopsis mutant that was deficient in a cytosolic N-terminal acetyltransferase. This mutant had a clear photosynthetic phenotype reflected by a low quantum yield of PSII, decreased synthesis of PSII proteins D1 and CP47, as well as a decreased amount of thylakoid protein complexes, possibly due to deficient chloroplast protein import. Accumulation of unprocessed N^{α}-acetylated precursor proteins has also been reported in an Arabidopsis mutant lacking a protein translocon complex (Bischof, et al., 2011). N^{α}-acetylation of precursor proteins is proposed to function as a degradation signal for plastid proteins that are not imported to the chloroplast (Bischof, et al., 2011).

N^{α}-acetylation of mature nuclear-encoded chloroplast proteins seems to be a common PTM (Bienvenut, et al., 2012), and indeed certain proteins involved in photosynthesis,

such as: D1, D2 (Michel, et al., 1988); the large subunit of Rubisco (Zybailov, et al., 2008); and the ε subunit of chloroplast ATP synthase (Zybailov, et al., 2008; Hoshiyasu, et al., 2013), contain N^{α}-acetylation. Additionally, both LFNR1 and LFNR2 are N^{α}-acetylated, which results in a more acidic pI (paper IV), similarly to that shown for the ε subunit of chloroplast ATP synthase (Hoshiyasu, et al., 2013). Although the physiological significance of N^{α}-acetylation is still mainly unknown, it has been demonstrated that the amount of the non-N^{α}-acetylated form of ε subunit of chloroplast ATP synthase decreases under drought stress (Hoshiyasu, et al., 2013). *In vitro* analysis demonstrated further, that non-acetylated form is more sensitive to degradation suggesting that N^{α}-acetylation protects the ε subunit (Hoshiyasu, et al., 2013). This supports the historical hypothesis that N^{α}-acetylation protects proteins from proteolytic degradation, a hypothesis that has been lacking experimental evidence (Jornvall, 1975; Mischerikow and Heck, 2011). We also found that in LFNR N^{α}-acetylation responds to environmental cues, especially for the membrane-bound LFNR1 basic form, which becomes more prevalent with increasing light (paper IV, Fig. 5).

In addition to N^{α}-acetylation, LFNR isoforms were shown to possess alternative trimming, and three distinct N-terminal amino acid residues (A, Q, V/I) were documented (paper IV). Two different protein bands of spinach FNR have been suggested to result from proteolytic degradation (Shin, et al., 1990), and more recently two alternative N-termini of LFNR1 and LFNR2 have been reported in wheat (Gummadova, et al., 2007). Previously, in Arabidopsis, the LFNR1 sequence has been reported to start with VTTDTT, and LFNR2 with ITTETD (Hanke, et al., 2005). A stromal processing peptidase cleaves most of the transit peptides after import to chloroplast (Richter and Lamppa, 1998), but the cleavage site is not conserved (Rudhe, et al., 2004). In addition, existence of several chloroplastic aminopeptidases has been reported, which may affect the turnover of proteins via further cleavage of the N-terminus (Walling, 2006). More research is needed to resolve whether the alternative trimming of LFNR N-terminus occurs via unspecific stromal processing, peptidase cleavage, or via function of aminopeptidases (Zybailov, et al., 2008). Interestingly, and in agreement with the data presented in this study, one third of N^{α}-acetylated chloroplast proteins are estimated to contain more than one signal peptide cleavage site (Bienvenut, et al., 2012).

The N-terminus comprises the greatest variation between the LFNR isoforms. The disordered N-terminus in the crystal structure of maize LFNR1 may reflect flexibility (Kurisu, et al., 2001), and it might affect thylakoid interactions (Okutani, et al., 2005), as well as binding of FD (Maeda, et al., 2005). Recently, the N-terminus has been reported to be an essential factor in the membrane association of maize LFNR, and a similar mechanism has been proposed for Arabidopsis (Twachtmann, et al., 2012). The N-terminal structure has also been shown to affect association of LFNR to membrane in wheat (Moolna and Bowsher, 2010). However, alternative trimming of the

Arabidopsis LFNR1 and LFNR2 N-termini does not seem to affect membrane binding, as all of the membrane-bound and soluble LFNR forms contain all three different N-termini (paper IV). LFNR isoforms with different N-termini differ in reaction kinetics with FD (Maeda, et al., 2005; Gummadova, et al., 2007). In particular, the N-terminus of LFNR2 in wheat appears to be essential to interaction with FD (Bowsher, et al., 2011). Further, LFNR forms with different N-termini in wheat are reported to respond differentially to nitrate, oxidative stress and developmental stage of the cell (Moolna and Bowsher, 2010). Taken together, N-terminal modifications seem to be essential for enzyme activity as well as the stability and subcellular localization of proteins. The biological significance of N-terminal cleavage and N^{α}-acetylation of LFNR in Arabidopsis remains to be investigated.

5.4.4 LFNR isoforms contain acetylated lysines

Recently, a total of 125 proteins have been reported to be N^{ε}-acetylated in Arabidopsis (Finkemeier, et al., 2011; Wu, et al., 2011). Part of these proteins are related to photosynthesis, including the PSII subunits, LHCB proteins, chloroplast ATP-synthase β-subunit, large and small subunit of Rubisco, and three other Calvin cycle enzymes (Finkemeier, et al., 2011; Wu, et al., 2011). Acetylation neutralizes positive charges, and it has recently been shown that LHCB proteins loosely-bound to PSII contained more N^{ε}-acetylation than tightly-bound LHCB proteins. Hence, N^{ε}-acetylation has marked effects on LHCB trimer interactions and thylakoid membrane structure (Wu, et al., 2011). Furthermore, the large subunit of Rubisco contains N^{ε}-acetylation in essential lysine residues in the N-terminal part of the protein, this relates to the formation of its tertiary structure and catalytic activity, and indeed deacetylation has been shown to increase enzyme activity (Finkemeier, et al., 2011). The same lysine residue, lysine-14 of the Rubisco large subunit, has also been suggested to be a target for methylation (Trievel, et al., 2003). However, in Arabidopsis, lysine-14 is not methylated (Mininno, et al., 2012). Instead, isoforms of fructose 1,6-bisphosphate aldolase have been shown to be trimethylated (Mininno, et al., 2012). Together, these results indicate that acetylation and methylation may play an important role in the regulation of carbon metabolism. Our results show that all LFNR forms contain a few acetylated lysines, but methylation could not be reliably confirmed (paper IV). Although one of the acetylated lysine residues is located proximal to the site of FD interaction, it seems plausible that N^{ε}-acetylation does not affect the binding of FAD or $NADP^{+}$, nor the interaction with FD. However, the possibility that this acetylation has an effect on LFNR activity cannot be excluded.

Characterisation of individual modifications is only a small piece of the whole picture. Modifications are often dynamic, and it is the overall view of various modifications in the protein network that creates a particular biological outcome. During metabolic processes there are modifications that have parallel or opposite influence to each other, and certain modifications can take place sequentially. Because PTMs are typically

dynamically regulated and reversible, it is not possible to estimate their existence solely from sequence data, and thus they need to be determined experimentally. In this work, part of PTM code in LFNR isoforms in Arabidopsis was revealed under standard growth conditions. It creates a starting point for further investigation.

6 CONCLUDING REMARKS

In this thesis I have shown that:

- Both LFNR1 and LFNR2 isoforms are photosynthetically competent and neither of them possesses a specific role in CET. Nevertheless, either both isoforms together, or the WT-level of LFNR protein is needed for optimal growth.
- The LFNR2 isoform plays a unique role in electron distribution under unfavourable conditions.
- Drought stress –induced CET is likely to occur via the PGR5-route, and evidence for a specific CET-related role for the FD1 isoform was presented.
- In addition to membrane-bound LFNR isoforms, soluble LFNRs also exist as two forms containing PTMs. All LFNR forms comprise acetylated lysine residues and alternative N-termini, and they are able to bind both FD1 and FD2 isoforms *in vitro*. Acidic LFNR1 and LFNR2 forms are N^{α}-acetylated, and the level of N^{α}-acetylation responds readily to environmental cues. In this thesis the basis for further study concerning PTM code in LFNRs of Arabidopsis was created.

These results emphasize the role of LFNR in regulation of photosynthesis. In future, information especially regarding the determination of the PTM code for LFNRs, as well as for other chloroplast proteins, is essential to meet the challenges of climate change, global problems in the use of fossil fuels as well as effective production of food. Additionally, revealing the mechanisms behind distribution of electrons to various (alternative) downstream reactions under varying environmental conditions is of utmost importance when photosynthetic machinery is harnessed to improve harvesting and utilization of solar energy.

ACKNOWLEDGEMENTS

This work was carried out in the Laboratory of Molecular Plant Biology at the University of Turku. Financial support from the Academy of Finland, the Finnish Centers of Excellence “Integrative Photosynthesis and Bioactive Compound Research at Systems Biology Level” and “Molecular Biology of Primary Producers” as well as the Finnish Doctoral Program in Plant Science is gratefully acknowledged. Professor Eevi Rintamäki is thanked for guiding my studies and helping me with bureaucracy. Professor Eva-Mari Aro is thanked for providing excellent working facilities, and for valuable scientific advice. My supervisor Dr. Paula Mulo is warmly thanked for excellent supervision, positive attitude, patience and encouragement throughout these years, and for enabling scientific growth. Professors Paula Elomaa and Poul Erik Jensen are acknowledged for critically reviewing the thesis. Dr. Fiona Lynch is warmly thanked for proof-reading.

All my co-authors are acknowledged for excellent collaboration. Kurt, Mika, Mirja, Ulla-Maija and Vipu are thanked for providing patient technical support that has made my everyday scientific work possible and smooth. I am enormously grateful for previous and present workmates. These eight years with people from different cultures and backgrounds have created an interesting journey teaching me about other people, and importantly, about myself. Iso-Minna, Pikku-Minna and Ilaf are heartily thanked for being my scientific sisters. I want to thank Saija for Pro Tyrvää moments. Andrea, Anniina, Arjun, Cosmin, Dalton, Gregsu, Hannu, Henna, Heta, Jatta, Laura, Lauri, Luca, Marion, Markus, Marjaana, Masha, Michele, Mikko, Moona, Pasi, Pauli, Pete, Sari, Susa, Tuomas, Veronica, Yagut and all the others: thanks to all for sharing scientific ideas and fun.

I am grateful for so many amazing friends in my life. Anna, Anna-Kaisa, Hanna, Heidi, Laura, Linda, Maija, Martina, Nelly, Sanna, Vesku and Ville: I am so thankful for you being there for me, when I'm sad, angry, disappointed, happy, thrilled and much more. Eve, Hitu and Marja, with your love and support I have survived. I am happy to have godsons, Deniz and Juho, as rays of sunlight in my life. I want to thank Eeva and Martti for your friendship. Äiti, Stina, Saara and Sanna – without you I would not be the woman I am today, kiitos. Jaakko – thank you for being a mirror and for keeping me grounded in reality.

In loving memory of my father.

Aho Mitakuye Oyasin.

Turku May 2014

Ninni

REFERENCES

Ahmad A, Cao X (2012) Plant PRMTs broaden the scope of arginine methylation. J Genet Genomics **39:** 195-208

Albertsson P (2001) A quantitative model of the domain structure of the photosynthetic membrane. Trends Plant Sci **6:** 349-358

Allen JF (2003) Cyclic, pseudocyclic and noncyclic photophosphorylation: new links in the chain. Trends Plant Sci **8:** 15-19

Andersen B, Scheller HV, Moller BL (1992) The PSI-E subunit of photosystem I binds ferredoxin:$NADP^+$ oxidoreductase. FEBS Lett **311:** 169-173

Arakaki AK, Ceccarelli EA, Carrillo N (1997) Plant-type ferredoxin-$NADP^+$ reductases: a basal structural framework and a multiplicity of functions. FASEB J **11:** 133-140

Arnesen T, Van Damme P, Polevoda B, Helsens K, Evjenth R, Colaert N, Varhaug JE, Vandekerckhove J, Lillehaug JR, Sherman F, Gevaert K (2009) Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. Proc Natl Acad Sci U S A **106:** 8157-8162

Arnon DI, Allen MB, Whatley FR (1954) Photosynthesis by isolated chloroplasts. Nature **174:** 394-396

Aro EM, Virgin I, Andersson B (1993) Photoinhibition of Photosystem II. Inactivation, protein damage and turnover. Biochim Biophys Acta **1143:** 113-134

Asada K (1999) The Water-Water Cycle In Chloroplasts: Scavenging of Active Oxygens and Dissipation of Excess Photons. Annu Rev Plant Physiol Plant Mol Biol **50:** 601-639

Balsera M, Stengel A, Soll J, Bolter B (2007) Tic62: a protein family from metabolism to protein translocation. BMC Evol Biol **7:** 43

Barrero-Gil J, Salinas J (2013) Post-translational regulation of cold acclimation response. Plant Sci **205-206:** 48-54

Bause E (1983) Structural requirements of N-glycosylation of proteins. Studies with proline peptides as conformational probes. Biochem J **209:** 331-336

Bendall DS, Manasse RS (1995) Cyclic photophosphorylation and electron transport. Biochimica Biophysica Acta **1229:** 23--38

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

Bienvenut WV, Sumpton D, Martinez A, Lilla S, Espagne C, Meinnel T, Giglione C (2012) Comparative large scale characterization of plant versus mammal proteins reveals similar and idiosyncratic N-alpha-acetylation features. Mol Cell Proteomics **11:** M111.015131

Bischof S, Baerenfaller K, Wildhaber T, Troesch R, Vidi PA, Roschitzki B, Hirsch-Hoffmann M, Hennig L, Kessler F, Gruissem W, Baginsky S (2011) Plastid proteome assembly without Toc159: photosynthetic protein import and accumulation of N-acetylated plastid precursor proteins. Plant Cell **23:** 3911-3928

Blanco NE, Ceccoli RD, Via MV, Voss I, Segretin ME, Bravo-Almonacid FF, Melzer M, Hajirezaei MR, Scheibe R, Hanke GT (2013) Expression of the minor isoform pea ferredoxin in tobacco alters photosynthetic electron partitioning and enhances cyclic electron flow. Plant Physiol **161:** 866-879

Bohme H (1977) On the role of ferredoxin and ferredoxin-$NADP^+$ reductase in cyclic electron transport of spinach chloroplasts. Eur J Biochem **72:** 283-289

Bojko M, Kruk J, Wieckowski S (2003) Plastoquinones are effectively reduced by ferredoxin:$NADP^+$ oxidoreductase in the presence of sodium cholate micelles. Significance for cyclic electron transport and chlororespiration. Phytochemistry **64:** 1055-1060

Bojko M, Wieckowski S (1995) Diaphorase activity of ferredoxin: NADP oxidoreductase in

the presence of dibromothymoquinone. Phytochemistry **40:** 661-665

Bowsher CG, Eyres LM, Gummadova JO, Hothi P, McLean KJ, Munro AW, Scrutton NS, Hanke GT, Sakakibara Y, Hase T (2011) Identification of N-terminal regions of wheat leaf ferredoxin $NADP^+$ oxidoreductase important for interactions with ferredoxin. Biochemistry **50:** 1778-1787

Bukhov NG, Govindachary S, Rajagopal S, Joly D, Carpentier R (2004) Enhanced rates of $P700^{(+)}$ dark-reduction in leaves of *Cucumis sativus* L photoinhibited at chilling temperature. Planta **218:** 852-861

Buren S, Ortega-Villasante C, Blanco-Rivero A, Martinez-Bernardini A, Shutova T, Shevela D, Messinger J, Bako L, Villarejo A, Samuelsson G (2011) Importance of post-translational modifications for functionality of a chloroplast-localized carbonic anhydrase (CAH1) in *Arabidopsis thaliana*. PLoS One **6:** e21021

Carrillo N, Ceccarelli EA (2003) Open questions in ferredoxin-$NADP^+$ reductase catalytic mechanism. Eur J Biochem **270:** 1900-1915

Ceccarelli EA, Arakaki AK, Cortez N, Carrillo N (2004) Functional plasticity and catalytic efficiency in plant and bacterial ferredoxin-NADP(H) reductases. Biochim Biophys Acta **1698:** 155-165

Ceriotti A, Duranti M, Bollini R (1998) Effects of N-glycosylation on the folding and structure of plant proteins. Journal of Experimental Botany **49:** 1091-1103

Chan RL, Ceccarelli EA, Vallejos RH (1987) Immunological studies of the binding protein for chloroplast ferredoxin-$NADP^+$ reductase. Arch Biochem Biophys **253:** 56-61

Clark RD, Hawkesford MJ, Coughlan SJ, Bennett J, Hind G (1984) Association of ferredoxin-$NADP^+$ oxidoreductase with the chloroplast cytochrome b-f complex. FEBS Lett **174:** 137-142

Clarke JE, Johnson GN (2001) In vivo temperature dependence of cyclic and pseudocyclic electron transport in barley. Planta **212:** 808-816

Cleland RE, Bendall DS (1992) Photosystem I cyclic electron transport: Measurement of ferredoxin-plastoquinone reductase activity. Photosynth Res **34:** 409-418

Cloutier P, Coulombe B (2013) Regulation of molecular chaperones through post-translational modifications: decrypting the chaperone code. Biochim Biophys Acta **1829:** 443-454

DalCorso G, Pesaresi P, Masiero S, Aseeva E, Schunemann D, Finazzi G, Joliot P, Barbato R, Leister D (2008) A complex containing PGRL1 and PGR5 is involved in the switch between linear and cyclic electron flow in Arabidopsis. Cell **132:** 273-285

De Pascalis AR, Jelesarov I, Ackermann F, Koppenol WH, Hirasawa M, Knaff DB, Bosshard HR (1993) Binding of ferredoxin to ferredoxin:$NADP^+$ oxidoreductase: the role of carboxyl groups, electrostatic surface potential, and molecular dipole moment. Protein Sci **2:** 1126-1135

Faye L, Daniell H (2006) Novel pathways for glycoprotein import into chloroplasts. Plant Biotechnol J **4:** 275-279

Faye L, Gomord V, Fitchettelaine AC, Chrispeels MJ (1993) Affinity Purification of Antibodies Specific for Asn-Linked Glycans Containing $\alpha 1 \rightarrow 3$ Fucose or $\beta 1 \rightarrow 2$ Xylose. Anal Biochem **209:** 104-108

Finazzi G, Rappaport F, Furia A, Fleischmann M, Rochaix JD, Zito F, Forti G (2002) Involvement of state transitions in the switch between linear and cyclic electron flow in *Chlamydomonas reinhardtii*. EMBO Rep **3:** 280-285

Finkemeier I, Laxa M, Miguet L, Howden AJ, Sweetlove LJ (2011) Proteins of diverse function and subcellular location are lysine acetylated in Arabidopsis. Plant Physiol **155:** 1779-1790

Fischer N, Hippler M, Setif P, Jacquot JP, Rochaix JD (1998) The PsaC subunit of photosystem I provides an essential lysine residue for fast electron transfer to ferredoxin. EMBO J **17:** 849-858

Forti G, Bracale M (1984) Ferredoxin—ferredoxin NADP reductase interaction: Catalytic differences between the soluble and

thylakoid-bound complex. FEBS Lett **166:** 81-84

Foyer C, Rowell J, Walker D (1983) The effect of sucrose on the rate of de novo sucrose biosynthesis in leaf protoplasts from spinach, wheat and barley. Arch Biochem Biophys **220:** 232-238

Fredricks WW, Gehl JM (1982) Kinetics of extraction of ferredoxin-nicotinamide adenine dinucleotide phosphate reductase from spinach chloroplasts. Arch Biochem Biophys **213:** 67-72

Fredricks WW, Gehl JM (1976) Multiple forms of ferredoxin-nicotinamide adenine dinucleotide phosphate reductase from spinach. Arch Biochem Biophys **174:** 666-674

Friso G, Giacomelli L, Ytterberg AJ, Peltier JB, Rudella A, Sun Q, Wijk KJ (2004) In-depth analysis of the thylakoid membrane proteome of *Arabidopsis thaliana* chloroplasts: new proteins, new functions, and a plastid proteome database. Plant Cell **16:** 478-499

Fristedt R, Vener AV (2011) High light induced disassembly of photosystem II supercomplexes in Arabidopsis requires STN7-dependent phosphorylation of CP29. PLoS One **6:** e24565

Glozak MA, Sengupta N, Zhang X, Seto E (2005) Acetylation and deacetylation of non-histone proteins. Gene **363:** 15-23

Gohya T, Zhang X, Yoshida T, Migita CT (2006) Spectroscopic characterization of a higher plant heme oxygenase isoform-1 from Glycine max (soybean) — coordination structure of the heme complex and catabolism of heme. FEBS J **273:** 5384-5399

Golding AJ, Finazzi G, Johnson GN (2004) Reduction of the thylakoid electron transport chain by stromal reductants — evidence for activation of cyclic electron transport upon dark adaptation or under drought. Planta **220:** 356-363

Golding AJ, Johnson GN (2003) Down-regulation of linear and activation of cyclic electron transport during drought. Planta **218:** 107-114

Gorg A (1993) Two-dimensional electrophoresis with immobilized pH gradients: current state. Biochem Soc Trans **21:** 130-132

Grillo MA, Colombatto S (2005) S-adenosylmethionine and protein methylation. Amino Acids **28:** 357-362

Guedeney G, Corneille S, Cuine S, Peltier G (1996) Evidence for an association of *ndh B*, *ndh J* gene products and ferredoxin-NADP-reductase as components of a chloroplastic NAD(P)H dehydrogenase complex. FEBS Lett **378:** 277-280

Gummadova JO, Fletcher GJ, Moolna A, Hanke GT, Hase T, Bowsher CG (2007) Expression of multiple forms of ferredoxin $NADP^+$ oxidoreductase in wheat leaves. Journal of Experimental Botany **58:** 3971-3985

Hajirezaei MR, Peisker M, Tschiersch H, Palatnik JF, Valle EM, Carrillo N, Sonnewald U (2002) Small changes in the activity of chloroplastic $NADP^{(+)}$-dependent ferredoxin oxidoreductase lead to impaired plant growth and restrict photosynthetic activity of transgenic tobacco plants. Plant J **29:** 281-293

Hakala-Yatkin M, Sarvikas P, Paturi P, Mäntysaari M, Mattila H, Tyystjärvi T, Nedbal L, Tyystjärvi E (2011) Magnetic field protects plants against high light by slowing down production of singlet oxygen. Physiol Plant **142:** 26-34

Hanke G, Mulo P (2013) Plant type ferredoxins and ferredoxin-dependent metabolism. Plant Cell Environ **36:** 1071-1084

Hanke GT, Endo T, Satoh F, Hase T (2008) Altered photosynthetic electron channelling into cyclic electron flow and nitrite assimilation in a mutant of ferredoxin:NADP(H) reductase. Plant Cell Environ **31:** 1017-1028

Hanke GT, Hase T (2008) Variable photosynthetic roles of two leaf-type ferredoxins in Arabidopsis, as revealed by RNA interference. Photochem Photobiol **84:** 1302-1309

Hanke GT, Kimata-Ariga Y, Taniguchi I, Hase T (2004) A post genomic characterization of Arabidopsis ferredoxins. Plant Physiol **134:** 255-264

Hanke GT, Okutani S, Satomi Y, Takao T, Suzuki A, Hase T (2005) Multiple iso-proteins of FNR in Arabidopsis: evidence for different contributions to chloroplast function and

nitrogen assimilation. Plant, Cell Environ **28:** 1146-1157

Hase T, Shurmann P, Knaff DB (2006) The interaction of ferredoxin with ferredoxin-dependent enzymes. In JH Golbeck, ed, Photosystem I, The Light-Driven Plastocyanin:Ferredoxin Oxidoreductase Vol 24. Springer Netherlands, Netherlands, pp 477-498

Havaux M, Eymery F, Porfirova S, Rey P, Dormann P (2005) Vitamin E protects against photoinhibition and photooxidative stress in *Arabidopsis thaliana*. Plant Cell **17:** 3451-3469

Havaux M, Rumeau D, Ducruet JM (2005) Probing the FQR and NDH activities involved in cyclic electron transport around Photosystem I by the 'afterglow' luminescence. Biochim Biophys Acta **1709:** 203-213

Haweker H, Rips S, Koiwa H, Salomon S, Saijo Y, Chinchilla D, Robatzek S, von Schaewen A (2010) Pattern recognition receptors require N-glycosylation to mediate plant immunity. J Biol Chem **285:** 4629-4636

Heber U (2002) Irrungen, Wirrungen? The Mehler reaction in relation to cyclic electron transport in C_3 plants. Photosynth Res **73:** 223-231

Heber U, Walker D (1992) Concerning a dual function of coupled cyclic electron transport in leaves. Plant Physiol **100:** 1621-1626

Helenius A, Aebi M (2004) Roles of N-linked glycans in the endoplasmic reticulum. Annu Rev Biochem **73:** 1019-1049

Hertle AP, Blunder T, Wunder T, Pesaresi P, Pribil M, Armbruster U, Leister D (2013) PGRL1 is the elusive ferredoxin-plastoquinone reductase in photosynthetic cyclic electron flow. Mol Cell **49:** 511-523

Higuchi-Takeuchi M, Ichikawa T, Kondou Y, Matsui K, Hasegawa Y, Kawashima M, Sonoike K, Mori M, Hirochika H, Matsui M (2011) Functional analysis of two isoforms of leaf-type ferredoxin-$NADP^{(+)}$-oxidoreductase in rice using the heterologous expression system of Arabidopsis. Plant Physiol **157:** 96-108

Hodges M, Miginiac-Maslow M, Le Marechal P, Remy R (1990) The ATP-dependent post translational modification of ferredoxin: $NADP^+$ oxidoreductase. Biochim Biophys Acta **1052:** 446-452

Hoff T, Truong H-, Caboche M (1994) The use of mutants and transgenic plants to study nitrate assimilation. Plant, Cell Environ **17:** 489-506

Horvath EM, Peter SO, Joet T, Rumeau D, Cournac L, Horvath GV, Kavanagh TA, Schafer C, Peltier G, Medgyesy P (2000) Targeted inactivation of the plastid *ndhB* gene in tobacco results in an enhanced sensitivity of photosynthesis to moderate stomatal closure. Plant Physiol **123:** 1337-1350

Hoshiyasu S, Kohzuma K, Yoshida K, Fujiwara M, Fukao Y, Yokota A, Akashi K (2013) Potential involvement of N-terminal acetylation in the quantitative regulation of the epsilon subunit of chloroplast ATP synthase under drought stress. Biosci Biotechnol Biochem **77:** 998-1007

Inoue S, Ejima K, Iwai E, Hayashi H, Appel J, Tyystjärvi E, Murata N, Nishiyama Y (2011) Protection by alpha-tocopherol of the repair of photosystem II during photoinhibition in Synechocystis sp. PCC 6803. Biochim Biophys Acta **1807:** 236-241

Inskeep WP, Bloom PR (1985) Extinction coefficients of chlorophyll a and B in n,n-dimethylformamide and 80% acetone. Plant Physiol **77:** 483-485

Iwai M, Takizawa K, Tokutsu R, Okamuro A, Takahashi Y, Minagawa J (2010) Isolation of the elusive supercomplex that drives cyclic electron flow in photosynthesis. Nature **464:** 1210-1213

Joet T, Cournac L, Peltier G, Havaux M (2002) Cyclic electron flow around photosystem I in $C_{(3)}$ plants. In vivo control by the redox state of chloroplasts and involvement of the NADH-dehydrogenase complex. Plant Physiol **128:** 760-769

Johnson GN (2011) Physiology of PSI cyclic electron transport in higher plants. Biochim Biophys Acta **1807:** 384-389

Johnson GN (2005) Cyclic electron transport in C_3 plants: fact or artefact? J Exp Bot **56:** 407-416

Joliot P, Johnson GN (2011) Regulation of cyclic and linear electron flow in higher plants. Proc Natl Acad Sci U S A **108:** 13317-13322

Joliot P, Joliot A (2006) Cyclic electron flow in C_3 plants. Biochim Biophys Acta **1757:** 362-368

Joliot P, Joliot A (2002) Cyclic electron transfer in plant leaf. Proc Natl Acad Sci U S A **99:** 10209-10214

Jornvall H (1975) Acetylation of Protein N-terminal amino groups structural observations on alpha-amino acetylated proteins. J Theor Biol **55:** 1-12

Jose Quiles M, Cuello J (1998) Association of ferredoxin-NADP oxidoreductase with the chloroplastic pyridine nucleotide dehydrogenase complex in barley leaves. Plant Physiol **117:** 235-244

Juric S, Hazler-Pilepic K, Tomasic A, Lepedus H, Jelicic B, Puthiyaveetil S, Bionda T, Vojta L, Allen JF, Schleiff E, Fulgosi H (2009) Tethering of ferredoxin:$NADP^+$ oxidoreductase to thylakoid membranes is mediated by novel chloroplast protein TROL. Plant J **60:** 783-794

Kangasjärvi S, Lepistö A, Hännikäinen K, Piippo M, Luomala EM, Aro EM, Rintamäki E (2008) Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses. Biochem J **412:** 275-285

Karplus PA, Daniels MJ, Herriott JR (1991) Atomic structure of ferredoxin-$NADP^+$ reductase: prototype for a structurally novel flavoenzyme family. Science **251:** 60-66

Khoury GA, Baliban RC, Floudas CA (2011) Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. Sci Rep **1:** srep00090

Kimata-Ariga Y, Matsumura T, Kada S, Fujimoto H, Fujita Y, Endo T, Mano J, Sato F, Hase T (2000) Differential electron flow around photosystem I by two $C_{(4)}$-photosynthetic-cell-specific ferredoxins. EMBO J **19:** 5041-5050

Kirchhoff H, Horstmann S, Weis E (2000) Control of the photosynthetic electron transport by PQ diffusion microdomains in thylakoids of higher plants. Biochim Biophys Acta **1459:** 148-168

Kleffmann T, Russenberger D, von Zychlinski A, Christopher W, Sjolander K, Gruissem W, Baginsky S (2004) The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Curr Biol **14:** 354-362

Kramer DM, Avenson TJ, Edwards GE (2004) Dynamic flexibility in the light reactions of photosynthesis governed by both electron and proton transfer reactions. Trends Plant Sci **9:** 349-357

Krapp AR, Tognetti VB, Carrillo N, Acevedo A (1997) The role of ferredoxin-$NADP^+$ reductase in the concerted cell defense against oxidative damage — studies using *Escherichia coli* mutants and cloned plant genes. Eur J Biochem **249:** 556-563

Kuchler M, Decker S, Hormann F, Soll J, Heins L (2002) Protein import into chloroplasts involves redox-regulated proteins. EMBO J **21:** 6136-6145

Kurisu G, Kusunoki M, Katoh E, Yamazaki T, Teshima K, Onda Y, Kimata-Ariga Y, Hase T (2001) Structure of the electron transfer complex between ferredoxin and ferredoxin-$NADP^{(+)}$ reductase. Nat Struct Biol **8:** 117-121

Lehtimäki N, Shunmugam S, Jokela J, Wahlsten M, Carmel D, Keränen M, Sivonen K, Aro EM, Allahverdiyeva Y, Mulo P (2011) Nodularin uptake and induction of oxidative stress in spinach (*Spinachia oleracea*). J Plant Physiol **168:** 594-600

Lehti-Shiu MD, Shiu SH (2012) Diversity, classification and function of the plant protein kinase superfamily. Philos Trans R Soc Lond B Biol Sci **367:** 2619-2639

Li XP, Phippard A, Pasari J, Niyogi KK (2002) Structure–function analysis of photosystem II subunit S (PsbS) *in vivo*. Functional Plant Biology **29:** 1131-1139

Lige B, Ma S, van Huystee RB (2001) The effects of the site-directed removal of N-glycosylation from cationic peanut peroxidase on its function. Arch Biochem Biophys **386:** 17-24

Lintala M, Allahverdiyeva Y, Kidron H, Piippo M, Battchikova N, Suorsa M, Rintamäki E, Salminen TA, Aro EM, Mulo P (2007) Structural and functional characterization of ferredoxin-NADP$^+$-oxidoreductase using knock-out mutants of Arabidopsis. Plant J **49:** 1041-1052

Lintala M, Schuck N, Thormahlen I, Jungfer A, Weber KL, Weber AP, Geigenberger P, Soll J, Bölter B, Mulo P (2014) Arabidopsis *tic62 trol* mutant lacking thylakoid-bound ferredoxin-NADP$^+$ oxidoreductase shows distinct metabolic phenotype. Mol Plant **7:** 45-57

Liu J, Wang P, Liu B, Feng D, Zhang J, Su J, Zhang Y, Wang JF, Wang HB (2013) A deficiency in chloroplastic ferredoxin 2 facilitates effective photosynthetic capacity during long-term high light acclimation in *Arabidopsis thaliana*. Plant J **76:** 861-874

Lothrop AP, Torres MP, Fuchs SM (2013) Deciphering post-translational modification codes. FEBS Lett **587:** 1247-1257

Maeda M, Lee YH, Ikegami T, Tamura K, Hoshino M, Yamazaki T, Nakayama M, Hase T, Goto Y (2005) Identification of the N- and C-terminal substrate binding segments of ferredoxin-NADP$^+$ reductase by NMR. Biochemistry **44:** 10644-10653

Majeran W, Cai Y, Sun Q, van Wijk KJ (2005) Functional differentiation of bundle sheath and mesophyll maize chloroplasts determined by comparative proteomics. Plant Cell **17:** 3111-3140

Mann M, Jensen ON (2003) Proteomic analysis of post-translational modifications. Nat Biotechnol **21:** 255-261

Matthijs HC, Coughlan SJ, Hind G (1986) Removal of ferredoxin:NADP$^+$ oxidoreductase from thylakoid membranes, rebinding to depleted membranes, and identification of the binding site. J Biol Chem **261:** 12154-12158

Mi H, Endo T, Ogawa T, Asada K (1995) Thylakoid Membrane-Bound, NADPH-Specific Pyridine Nucleotide Dehydrogenase Complex Mediates Cyclic Electron Transport in the Cyanobacterium Synechocystis sp. PCC 6803. Plant and Cell Physiology **36:** 661-668

Michel H, Hunt DF, Shabanowitz J, Bennett J (1988) Tandem mass spectrometry reveals that three photosystem II proteins of spinach chloroplasts contain N-acetyl-O-phosphothreonine at their NH_2 termini. J Biol Chem **263:** 1123-1130

Mininno M, Brugiere S, Pautre V, Gilgen A, Ma S, Ferro M, Tardif M, Alban C, Ravanel S (2012) Characterization of chloroplastic fructose 1,6-bisphosphate aldolases as lysine-methylated proteins in plants. J Biol Chem **287:** 21034-21044

Mischerikow N, Heck AJ (2011) Targeted large-scale analysis of protein acetylation. Proteomics **11:** 571-589

Moolna A, Bowsher CG (2010) The physiological importance of photosynthetic ferredoxin NADP$^+$ oxidoreductase (FNR) isoforms in wheat. J Exp Bot **61:** 2669-2681

Morales R, Charon MH, Hudry-Clergeon G, Petillot Y, Norager S, Medina M, Frey M (2000) Refined X-ray structures of the oxidized, at 1.3 A, and reduced, at 1.17 A, Biochemistry **39:** 2428

Mosammaparast N, Shi Y (2010) Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. Annu Rev Biochem **79:** 155-179

Mulo P (2011) Chloroplast-targeted ferredoxin-NADP$^{(+)}$ oxidoreductase (FNR): structure, function and location. Biochim Biophys Acta **1807:** 927-934

Munekage Y, Hashimoto M, Miyake C, Tomizawa K, Endo T, Tasaka M, Shikanai T (2004) Cyclic electron flow around photosystem I is essential for photosynthesis. Nature **429:** 579-582

Munekage Y, Hojo M, Meurer J, Endo T, Tasaka M, Shikanai T (2002) PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell **110:** 361-371

Nandha B, Finazzi G, Joliot P, Hald S, Johnson GN (2007) The role of PGR5 in the redox poising of photosynthetic electron transport. Biochim Biophys Acta **1767:** 1252-1259

Nanjo Y, Oka H, Ikarashi N, Kaneko K, Kitajima A, Mitsui T, Munoz FJ, Rodriguez-Lopez M, Baroja-Fernandez E, Pozueta-Romero J (2006) Rice plastidial N-glycosylated nucleotide pyrophosphatase/phosphodiesterase is transported from the ER-golgi to the chloroplast through the secretory pathway. Plant Cell **18:** 2582-2592

Niyogi KK (1999) Photoprotection Revisited: Genetic and Molecular Approaches. Annu Rev Plant Physiol Plant Mol Biol **50:** 333-359

Okutani S, Hanke GT, Satomi Y, Takao T, Kurisu G, Suzuki A, Hase T (2005) Three maize leaf ferredoxin:NADPH oxidoreductases vary in subchloroplast location, expression, and interaction with ferredoxin. Plant Physiol **139:** 1451-1459

Onda Y, Hase T (2004) FAD assembly and thylakoid membrane binding of ferredoxin:$NADP^+$ oxidoreductase in chloroplasts. FEBS Lett **564:** 116-120

Osmond CB (1981) Photorespiration and photoinhibition: Some implications for the energetics of photosynthesis. Biochimica et Biophysica Acta (BBA) - Reviews on Bioenergetics **639:** 77-98

Paik WK, Paik DC, Kim S (2007) Historical review: the field of protein methylation. Trends Biochem Sci **32:** 146-152

Palatnik JF, Valle EM, Carrillo N (1997) Oxidative stress causes ferredoxin-$NADP^+$ reductase solubilization from the thylakoid membranes in methyl viologen-treated plants. Plant Physiol **115:** 1721-1727

Pätsikkä E, Kairavuo M, Sersen F, Aro EM, Tyystjarvi E (2002) Excess copper predisposes photosystem II to photoinhibition in vivo by outcompeting iron and causing decrease in leaf chlorophyll. Plant Physiol **129:** 1359-1367

Peltier G, Cournac L (2002) Chlororespiration. Annu Rev Plant Biol **53:** 523-550

Peng L, Shimizu H, Shikanai T (2008) The chloroplast NAD(P)H dehydrogenase complex interacts with photosystem I in Arabidopsis. J Biol Chem **283:** 34873-34879

Persson B, Flinta C, von Heijne G, Jornvall H (1985) Structures of N-terminally acetylated proteins. Eur J Biochem **152:** 523-527

Pesaresi P, Gardner NA, Masiero S, Dietzmann A, Eichacker L, Wickner R, Salamini F, Leister D (2003) Cytoplasmic N-terminal protein acetylation is required for efficient photosynthesis in Arabidopsis. Plant Cell **15:** 1817-1832

Petrescu AJ, Milac AL, Petrescu SM, Dwek RA, Wormald MR (2004) Statistical analysis of the protein environment of N-glycosylation sites: implications for occupancy, structure, and folding. Glycobiology **14:** 103-114

Plaxton W, McManus MT (2008) Control of Primary Metabolism in Plants. **22:** 408

Polevoda B, Sherman F (2002) The diversity of acetylated proteins. Genome Biol **3:** reviews0006

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta (BBA) - Bioenergetics **975:** 384-394

Reiland S, Messerli G, Baerenfaller K, Gerrits B, Endler A, Grossmann J, Gruissem W, Baginsky S (2009) Large-scale Arabidopsis phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks. Plant Physiol **150:** 889-903

Reinbothe C, Bartsch S, Eggink LL, Hoober JK, Brusslan J, Andrade-Paz R, Monnet J, Reinbothe S (2006) A role for chlorophyllide a oxygenase in the regulated import and stabilization of light-harvesting chlorophyll a/b proteins. Proc Natl Acad Sci U S A **103:** 4777-4782

Richter S, Lamppa GK (1998) A chloroplast processing enzyme functions as the general stromal processing peptidase. Proc Natl Acad Sci U S A **95:** 7463-7468

Rodriguez RE, Lodeyro A, Poli HO, Zurbriggen M, Peisker M, Palatnik JF, Tognetti VB, Tschiersch H, Hajirezaei MR,

Valle EM, Carrillo N (2007) Transgenic tobacco plants overexpressing chloroplastic ferredoxin-NADP(H) reductase display normal rates of photosynthesis and increased tolerance to oxidative stress. Plant Physiol **143:** 639-649

Rudhe C, Clifton R, Chew O, Zemam K, Richter S, Lamppa G, Whelan J, Glaser E (2004) Processing of the dual targeted precursor protein of glutathione reductase in mitochondria and chloroplasts. J Mol Biol **343:** 639-647

Rumeau D, Peltier G, Cournac L (2007) Chlororespiration and cyclic electron flow around PSI during photosynthesis and plant stress response. Plant Cell Environ **30:** 1041-1051

Sazanov LA, Burrows P, Nixon PJ (1996) Detection and characterization of a complex I-like NADH-specific dehydrogenase from pea thylakoids. Biochem Soc Trans **24:** 739-743

Schurmann P, Buchanan BB (2008) The ferredoxin/thioredoxin system of oxygenic photosynthesis. Antioxid Redox Signal **10:** 1235-1274

Setif P, Fischer N, Lagoutte B, Bottin H, Rochaix JD (2002) The ferredoxin docking site of photosystem I. Biochim Biophys Acta **1555:** 204-209

Shahak Y, Crowther D, Hind G (1981) The involvement of ferredoxin-$NADP^+$ reductase in cyclic electron transport in chloroplasts. Biochim Biophys Acta **636:** 234-243

Sheldon CC, Finnegan EJ, Dennis ES, Peacock WJ (2006) Quantitative effects of vernalization on FLC and SOC1 expression. Plant J **45:** 871-883

Shikanai T (2007) Cyclic electron transport around photosystem I: genetic approaches. Annu Rev Plant Biol **58:** 199-217

Shikanai T, Endo T, Hashimoto T, Yamada Y, Asada K, Yokota A (1998) Directed disruption of the tobacco *ndhB* gene impairs cyclic electron flow around photosystem I. Proc Natl Acad Sci U S A **95:** 9705-9709

Shimizu M, Kato H, Ogawa T, Kurachi A, Nakagawa Y, Kobayashi H (2010) Sigma factor phosphorylation in the photosynthetic control of photosystem stoichiometry. Proc Natl Acad Sci U S A **107:** 10760-10764

Shin M, Tsujita M, Tomizawa H, Sakihama N, Kamei K, Oshino R (1990) Proteolytic degradation of ferredoxin-NADP reductase during purification from spinach. Arch Biochem Biophys **279:** 97-103

Shin M, Ishida H, Nozaki Y (1985) A New Protein Factor, Connectein, as a Constituent of the Large Form of Ferredoxin-NADP Reductase. Plant and Cell Physiology **26:** 559-563

Singh BN, Zhang G, Hwa YL, Li J, Dowdy SC, Jiang SW (2010) Nonhistone protein acetylation as cancer therapy targets. Expert Rev Anticancer Ther **10:** 935-954

Sirpiö S, Allahverdiyeva Y, Holmstrom M, Khrouchtchova A, Haldrup A, Battchikova N, Aro EM (2009) Novel nuclear-encoded subunits of the chloroplast NAD(P)H dehydrogenase complex. J Biol Chem **284:** 905-912

Sirpiö S, Allahverdiyeva Y, Suorsa M, Paakkarinen V, Vainonen J, Battchikova N, Aro EM (2007) TLP18.3, a novel thylakoid lumen protein regulating photosystem II repair cycle. Biochem J **406:** 415-425

Soll J (2002) Protein import into chloroplasts. Curr Opin Plant Biol **5:** 529-535

Song W, Mentink RA, Henquet MG, Cordewener JH, van Dijk AD, Bosch D, America AH, van der Krol AR (2013) N-glycan occupancy of Arabidopsis N-glycoproteins. J Proteomics **93:** 343-355

Spiro RG (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology **12:** 43R-56R

Stengel A, Benz P, Balsera M, Soll J, Bolter B (2008) TIC62 redox-regulated translocon composition and dynamics. J Biol Chem **283:** 6656-6667

Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature **403:** 41-45

Sugiyama N, Nakagami H, Mochida K, Daudi A, Tomita M, Shirasu K, Ishihama Y (2008) Large-scale phosphorylation mapping reveals the extent of tyrosine phosphorylation in Arabidopsis. Mol Syst Biol **4:** 193

Suorsa M, Sirpiö S, Aro EM (2009) Towards characterization of the chloroplast NAD(P)H dehydrogenase complex. Mol Plant **2:** 1127-1140

Tagawa K, Tsujimoto HY, Arnon DI (1963) Role of chloroplast ferredoxin in the energy conversion process of photosynthesis. Proc Natl Acad Sci U S A **49:** 567-572

Takabayashi A, Kishine M, Asada K, Endo T, Sato F (2005) Differential use of two cyclic electron flows around photosystem I for driving CO_2-concentration mechanism in C_4 photosynthesis. Proc Natl Acad Sci U S A **102:** 16898-16903

Tanaka A, Ito H, Tanaka R, Tanaka NK, Yoshida K, Okada K (1998) Chlorophyll a oxygenase (CAO) is involved in chlorophyll b formation from chlorophyll a. Proc Natl Acad Sci U S A **95:** 12719-12723

Teicher B, Scheller V (1998) The NAD(P)H dehydrogenase in barley thylakoids is photoactivatable and uses NADPH as well as NADH. Plant Physiol **117:** 525-532

Tognetti VB, Palatnik JF, Fillat MF, Melzer M, Hajirezaei MR, Valle EM, Carrillo N (2006) Functional replacement of ferredoxin by a cyanobacterial flavodoxin in tobacco confers broad-range stress tolerance. Plant Cell **18:** 2035-2050

Trievel RC, Flynn EM, Houtz RL, Hurley JH (2003) Mechanism of multiple lysine methylation by the SET domain enzyme Rubisco LSMT. Nat Struct Biol **10:** 545-552

Twachtmann M, Altmann B, Muraki N, Voss I, Okutani S, Kurisu G, Hase T, Hanke GT (2012) N-terminal structure of maize ferredoxin:$NADP^+$ reductase determines recruitment into different thylakoid membrane complexes. Plant Cell **24:** 2979-2991

Vallejos RH, Ceccarelli E, Chan R (1984) Evidence for the existence of a thylakoid intrinsic protein that binds ferredoxin-$NADP^+$ oxidoreductase. J Biol Chem **259:** 8048-8051

Vener AV (2007) Environmentally modulated phosphorylation and dynamics of proteins in photosynthetic membranes. Biochim Biophys Acta **1767:** 449-457

Villarejo A, Buren S, Larsson S, Dejardin A, Monne M, Rudhe C, Karlsson J, Jansson S, Lerouge P, Rolland N, von Heijne G, Grebe M, Bako L, Samuelsson G (2005) Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast. Nat Cell Biol **7:** 1224-1231

von Schaewen A, Sturm A, O'Neill J, Chrispeels MJ (1993) Isolation of a mutant Arabidopsis plant that lacks N-acetyl glucosaminyl transferase I and is unable to synthesize Golgi-modified complex N-linked glycans. Plant Physiol **102:** 1109-1118

Voss I, Goss T, Murozuka E, Altmann B, McLean KJ, Rigby SE, Munro AW, Scheibe R, Hase T, Hanke GT (2011) FdC1, a novel ferredoxin protein capable of alternative electron partitioning, increases in conditions of acceptor limitation at photosystem I. J Biol Chem **286:** 50-59

Voss I, Koelmann M, Wojtera J, Holtgrefe S, Kitzmann C, Backhausen JE, Scheibe R (2008) Knockout of major leaf ferredoxin reveals new redox-regulatory adaptations in *Arabidopsis thaliana*. Physiol Plant **133:** 584-598

Wada H, Schmidt H, Heinz E, Murata N (1993) *In vitro* ferredoxin-dependent desaturation of fatty acids in cyanobacterial thylakoid membranes. J Bacteriol **175:** 544-547

Waegemann K, Soll J (1996) Phosphorylation of the transit sequence of chloroplast precursor proteins. J Biol Chem **271:** 6545-6554

Walling LL (2006) Recycling or regulation? The role of amino-terminal modifying enzymes. Curr Opin Plant Biol **9:** 227-233

Walters RG, Horton P (1995) Acclimation of *Arabidopsis thaliana* to the light environment: regulation of chloroplast composition. Planta **197:** 475-481

Whitmarsh AJ, Davis RJ (2000) Regulation of transcription factor function by phosphorylation. Cell Mol Life Sci **57:** 1172-1183

Williams-Smith DL, Cammack R (1977) Oxidation-reduction potentials of cytochromes P-450 and ferredoxin in the bovine adrenal. Their modification by substrates and inhibitors. Biochim Biophys Acta **499:** 432-442

Wu X, Oh MH, Schwarz EM, Larue CT, Sivaguru M, Imai BS, Yau PM, Ort DR, Huber SC (2011) Lysine acetylation is a widespread protein modification for diverse proteins in Arabidopsis. Plant Physiol **155:** 1769-1778

Xing S, Poirier Y (2012) The protein acetylome and the regulation of metabolism. Trends Plant Sci **17:** 423-430

Yamamoto H, Peng L, Fukao Y, Shikanai T (2011) An Src homology 3 domain-like fold protein forms a ferredoxin binding site for the chloroplast NADH dehydrogenase-like complex in Arabidopsis. Plant Cell **23:** 1480-1493

Yamamoto H, Shikanai T (2013) In planta mutagenesis of Src homology 3 domain-like fold of NdhS, a ferredoxin-binding subunit of the chloroplast NADH dehydrogenase-like complex in Arabidopsis: a conserved Arg-193 plays a critical role in ferredoxin binding. J Biol Chem **288:** 36328-36337

Yang DH, Webster J, Adam Z, Lindahl M, Andersson B (1998) Induction of acclimative proteolysis of the light-harvesting chlorophyll a/b protein of photosystem II in response to elevated light intensities. Plant Physiol **118:** 827-834

Zanetti G, Arosio P (1980) Solubilization from spinach thylakoids of a higher molecular weight form of ferredoxin-$NADP^+$ reductase. FEBS Lett **111:** 373-376

Zhang H, Whitelegge JP, Cramer WA (2001) Ferredoxin:$NADP^+$ oxidoreductase is a subunit of the chloroplast cytochrome b6f complex. J Biol Chem **276:** 38159-38165

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol **136:** 2621-2632

Zybailov B, Rutschow H, Friso G, Rudella A, Emanuelsson O, Sun Q, van Wijk KJ (2008) Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. PLoS One **3:** e1994