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FERREDOXIN-NADP$^+$ OXIDOREDUCTASES AND PROTEIN ACETYLATION IN THE REGULATION OF PHOTOSYNTHESIS

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ABSTRACT

Photosynthesis sustains life on Earth by converting carbon dioxide into organic compounds, which can be utilized as an energy source by non-photosynthetic organisms. Photosynthetic light reactions produce NADPH and ATP, the energy currencies used for carbon assimilation. The production of NADPH is catalyzed by flavoenzymes known as ferredoxin-$NADP^+$ oxidoreductases (FNRs), which function at the crossroads between the light reactions and carbon assimilation. All plant species contain multiple FNR enzymes each having different substrate binding and catalytic properties. The different FNRs have traditionally been divided into two groups: photosynthetic leaf-type FNRs (LFNRs) and non-photosynthetic root-type FNRs (RFNRs). In addition to L- and RFNRs, plants also have flavoproteins that have not been functionally characterized.

We studied the catalytic properties of an FNR homologue "FNR-LIKE" (FNRL) to investigate whether it can also function in the photoreduction of NADPH. We found that FNRL resembles bacterial-type FNRs both structurally and biochemically. FNRL most likely interacts with one of the uncharacterized ferredoxins or ferredoxin-like proteins thus funneling electrons towards ferredoxin-dependent metabolism.

Arabidopsis thaliana (Arabidopsis) has two photosynthetic FNR isoforms, LFNR1 and LFNR2. Both proteins can be separated into acidic and basic forms with isoelectric focusing, suggesting that LFNRs are regulated via a post-translational modification that alters the isoelectric point of the enzymes. Because LFNRs are central metabolic enzymes, we have investigated the nature of this and other post-translational modifications of Arabidopsis LFNRs. We found that both LFNRs undergo N-terminal and lysine acetylation. N-terminal acetylation was shown to be light responsive and to increase affinity towards ferredoxin. Our findings pave the way for further studies investigating the role of acetylation in the regulation of photosynthesis.

Even though N-terminal and lysine acetylation have emerged as widespread modifications in chloroplasts during the recent years, the chloroplast acetylation machinery has remained mostly uncharacterized. Therefore, we set out to look for putative chloroplast acetyltransferases that could play a role in photosynthesis. We found that knock-out mutants of the acetyltransferase NUCLEAR SHUTTLE INTERACTING (NSI) were defective in state transitions, a regulatory mechanism that ensures excitation energy balance between photosystem I and II in low light. NSI was shown to acetylate lysine residues of several chloroplast proteins, including some involved in photosynthesis, suggesting that one or more of the acetylation sites regulated by NSI might be required for state transitions. Our results add another layer to the complex regulation of light harvesting in plants and will help further studies in unraveling the exact mechanism of state transitions.

TIIVISTELMÄ

Yhteyttäminen ylläpitää elämää maapallolla muuttamalla hiilidioksidia orgaanisiksi yhdisteiksi, joita heterotrofiset eliöt käyttävät energianlähteenä. Hiilidioksidin sidontaan vaadittavat NADPH ja ATP tuotetaan yhteyttämisen valoreaktioiden avulla. Valo- ja hiilensidontareaktioiden välissä toimivat NADPH:n tuotosta vastaavat flavoentsyymit: ferredoksiini-$NADP^+$ -oksidoreduktaasit eli FNR:t. Kaikilla kasveilla on useita eri FNR-isoentsyymejä, jotka eroavat toisistaan katalyyttisiltä ominaisuuksiltaan. Erilaiset FNR-isoentsyymit on perinteisesti jaettu kahteen ryhmään: yhteyttämisessä käytettäviin lehti-tyypin FNR:iin (LFNR:t) sekä heterotrofisessa metaboliassa toimiviin juuri-tyypin FNR:iin (RFNR:t). L- ja RFNR:ien lisäksi kasveilla on monia muita flavoproteiineja, joiden toimintaa ei vielä tunneta.

Väitöskirjassani olen tutkinut aiemmin tuntematonta kasvien flavoproteiinia, joka sekvenssinsä perusteella muistuttaa FNR:iä ("FNR-LIKE", FNRL) selvittääkseni voiko tämä entsyymi toimia NADPH:n pelkityksessä yhteyttämisen valoreaktioissa. Tulostemme perusteella FNRL kuitenkin muistuttaa sekä rakenteeltaan että katalyyttisiltä ominaisuuksiltaan enemmän bakteerien FNR-proteiineja ja siten luultavasti toimii elektroninsiirrossa NADPH:lta heterotrofiseen ferredoksiini-riippuvaiseen aineenvaihduntaan.

Lituruoholla (*Arabidopsis thaliana*) on kaksi LFNR-isoentsyymiä: LFNR1 ja LFNR2. Molemmat proteiinit voidaan erotella isoelektrisellä fokusoinnilla happamaan ja emäksiseen muotoon, mikä viittaa siihen, että LFNR:iä säädellään translaationjälkeisen muokkauksen avulla. Koska LFNR:t ovat keskeisiä kasvien aineenvaihdunnan entsyymejä, on tärkeää selvittää muokkauksen tyyppi ja sen vaikutukset entsyymin toimintaan. Tutkimukseni osoittaa molempien LFNR:ien olevan osittain N-terminaalisesti asetyloituja sekä sisältävän asetyloituja lysiinitähteitä mm. entsyymin aktiivisen keskuksen läheisyydessä. N-terminaalisen asetylaation määrän havaittiin vaihtelevan valon määrän mukaan ja se lisäsi LFNR:ien ja ferredoksiinin välisen vuorovaikutuksen voimakkuutta. Tulostemme perusteella LFNR:ien toimintaa voidaan säädellä asetylaation avulla.

Vaikka N-terminaalista ja lysiinien asetylaatiota on viime vuosien aikana löydetty myös useista muista kloroplastien proteiineista, asetylaatiosta vastaavia kloroplastin entsyymejä, asetyylitransferaaseja, ei vielä tunneta. Tämän vuoksi keskityimme selvittämään onko kloroplastissa asetyylitransferaaesja, jotka voisivat toimia yhteyttämisen säätelyssä. Tutkimuksessani osoitan, että kasveilla, joilta puuttui asetyylitransferaasi NSI (NUCLEAR SHUTTLE INTERACTING), on ongelmia valoenergian jakautumisen säätelyssä valoreaktioiden I ja II välillä. NSI:n havaittiin myös vaikuttavan useiden kloroplastin proteiinien lysiinien asetylaation määrään. Tuloksemme osoittavat suoran yhteyden proteiinien asetylaation ja valoreaktioiden toiminnan välillä ja auttavat tulevaisuudessa selvittämään, kuinka yksittäiset asetylaatiokohdat vaikuttavat valoreaktioiden säätelyyn.

ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BN-PAGE	Blue-native polyacrylamide gel electrophoresis
CET	Cyclic electron transfer
Cyt	Cytochrome
DCPIP	Dichlorophenolindophenol
DM	n-dodecyl β-D-maltoside
DMF	N,N-Dimethylformamide
ETC	Electron transfer chain
FAD	Flavin adenine dinucleotide
Fd	Ferredoxin
Fld	Flavodoxin
FNR	Ferredoxin-$NADP^+$ oxidoreductase
FNRL	FNR-LIKE
KAT	Lysine acetyltransferase
KDAC	Lysine deacetylase
LFNR	Leaf-type FNR
LHC	Light harvesting complex
MS	Mass spectrometry/spectrometer
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NAT	N-terminal acetyltransferase
NPQ	Non-photochemical quenching
NSI	NUCLEAR SHUTTLE INTERACTING
OEC	Oxygen evolving complex
pI	Isoelectric point
PQ	Plastoquinone
PS	Photosystem
PTM	Post-translational modification
RuBisCO	Ribulose-1,5-bisphosphase carboxylase/oxygenase
RFNR	Root-type FNR
RP-HPLC	Reversed-phase high performance liquid chromatography
WT	Wild-type
YFP	Yellow fluorescent protein

1 INTRODUCTION

1.1 Photosynthesis

Oxygen evolving photosynthesis converts carbon dioxide and water into carbohydrates and oxygen with the energy of sunlight (Reaction 1). Photosynthetic organisms use carbohydrates for growth and reproduction, producing biomass which is subsequently used by heterotrophs as an energy source. Consequently, most life on Earth is in one way or another dependent on the energy of sunlight harnessed by photosynthesis. Understanding in detail all the factors that affect the productivity of photosynthetic organisms, starting from the primary light capturing reactions to the utilization of the harvested energy, is therefore arguably one of the most important questions in biology.

Reaction 1. Net reaction of photosynthesis.

$$CO_2 + H_2O + \text{photons} \rightarrow [CH_2O] + O_2$$

Plants perform photosynthesis in chloroplasts (Arnon et al., 1954). Chloroplasts are cell organelles surrounded by two lipid bilayers, the outer and inner envelope membranes (Figure 1). Inside chloroplasts resides a thylakoid membrane network, which is organized into grana stacks and extended membrane double layers called stroma lamellae. Thylakoids enclose a separate soluble compartment, the lumen, from the rest of the soluble space within the chloroplast, the stroma. Photosynthetic light reactions take place at the thylakoids, whereas carbon assimilation occurs in the stroma, where CO_2 is bound into an organic form by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Light reactions consist of the light harvesting antenna system and the electron transfer chain, which together power electron transfer from water to the electron carrier cofactor nicotinamide adenine dinucleotide phosphate ($NADP^+$). Simultaneously with electron transfer, protons are translocated across the thylakoid membrane from the stroma to the lumen. The energy stored in the electrochemical gradient created by proton transfer is used for ATP synthesis. The end products of light reactions, NADPH and ATP, are used to energize CO_2 assimilation by the Calvin cycle, and other metabolic reactions in the chloroplast.

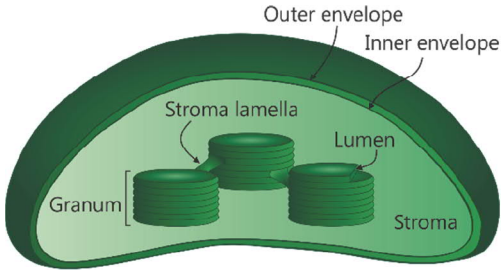

Figure 1. Schematic presentation of a chloroplast. Chloroplasts are enveloped by the outer and inner envelope membranes. The thylakoid membranes inside chloroplasts consists of grana stacks interconnected by stroma lamellae. The soluble space inside a chloroplast is called the stroma and the soluble space within thylakoids the lumen.

1.1.1 Electron transfer reactions

The electron transfer chain (ETC) consist of multiple protein complexes and electron carrier molecules working in series (Figure 2). Upon absorption of a photon, photosystem II (PSII) reaction center chlorophyll P_{680} gets excited and donates an electron to a primary electron acceptor molecule pheophytin, i.e. it undergoes a charge separation (Holzwarth et al., 2006; Kern and Renger, 2007; Miloslavina et al., 2006). The electron is thereafter transferred via plastoquinone Q_A to the final electron acceptor at PSII, plastoquinone Q_B, which becomes reduced to a semiquinone radical $Q_B^{\bullet-}$. The oxidized reaction center $P_{680}^{\bullet+}$ gets reduced back to P_{680} by an electron derived from water splitting at the oxygen evolving complex (OEC) of PSII. Electrons are extracted from water by the OEC manganese cluster (Reaction 2) (Ferreira et al., 2004), which is capable of storing the four electrons derived from water splitting and subsequently donating them one at a time to the reaction center via a tyrosine residue (Tyr_Z) of the PSII reaction center protein D1 (Faller et al., 2001). In addition to providing electrons to the electron transfer chain, water splitting releases protons to the lumen and oxygen to the atmosphere. After a second charge separation and electron transfer to Q_B, the semiquinone $Q_B^{\bullet-}$ becomes fully reduced and protonated to its quinol form Q_BH_2 and dissociates from the reaction center into the thylakoid membrane plastoquinone pool (PQ pool) (Shinkarev, 2006; Velthuys, 1981).

Reaction 2. Water splitting by the OEC.

$$2\ H_2O \rightarrow 4\ e^- + 4\ H^+ + O_2$$

Plastoquinol is oxidized by the cytochrome *b*$_6$*f* (Cyt *b*$_6$*f*) complex. Cyt *b*$_6$*f* has two plastoquinone binding sites: one on the lumenal side that preferentially binds the reduced plastoquinol (PQH_2) and another on the stromal side that has higher affinity for the oxidized quinone form (Stroebel et al., 2003). When PQH_2 binds to the

lumenal pocket of Cyt b_6f, one of its electrons is transferred to the electron carrier protein plastocyanin via a [2Fe-2S] iron-sulphur cluster and Cyt *f*, and the second electron is delivered to the oxidized PQ bound to the stromal pocket of Cyt b_6f via cytochromes b_L, b_H and c_i (Selak and Whitmarsh, 1982; Stroebel et al., 2003). Protons from the PQH_2 are released to the lumen. After another round of PQH_2 oxidation on the lumenal side, the PQ that was bound to the stromal pocket is now fully reduced to PQ^{2-} and becomes protonated to PQH_2 by protons from the stroma. The reduced PQH_2 is subsequently returned to the PQ pool. This cycling of electrons from one PQ to another within the Cyt b_6f is called the Q-cycle, and it results in the transfer of two additional protons from the stroma to the lumen per two electrons transferred along the ETC to plastocyanin (Sacksteder et al., 2000; Selak and Whitmarsh, 1982). Consequently, a total of six protons are translocated to the lumen per water oxidized.

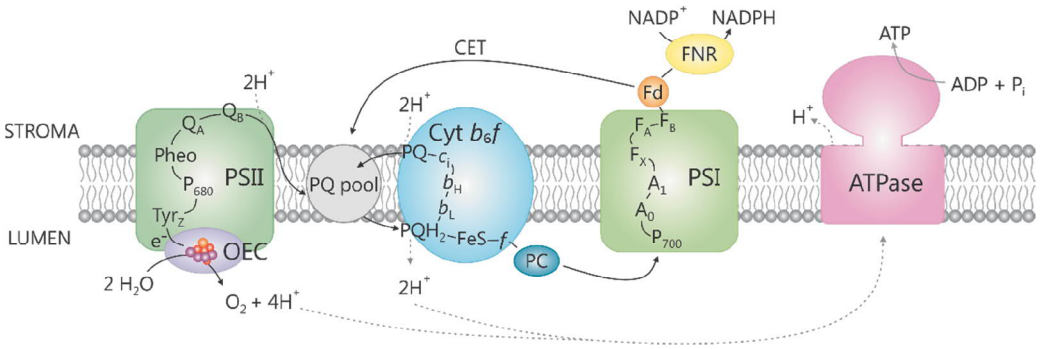

Figure 2. The photosynthetic electron transfer chain (ETC). Electrons are extracted from water at the oxygen evolving complex (OEC; purple) of photosystem II (PSII; green) and transferred via cytochrome b_6f complex (Cyt b_6f; blue) and plastocyanin (PC; dark blue) to photosystem I (PSI; light green). Electrons are transferred from PSI to the electron carrier protein ferredoxin (Fd; orange) and used to reduce $NADP^+$ into NADPH by the ferredoxin-$NADP^+$ oxidoreductase (FNR; yellow). In conjunction with electron transfer, protons are translocated to the lumen creating an electrochemical gradient that is used to generate ATP by the ATP synthase (ATPase; red). Electrons can also be cycled back to the PQ pool via two different cyclic electron transfer (CET) routes increasing the proton gradient and consequently ATP synthesis. The movement of electrons and electron carriers is depicted by black lines, protons by grey dashed lines and ATP synthesis by a grey line.

Plastocyanin is a soluble lumenal Cu-binding protein that transfers an electron from Cyt b_6f to photosystem I (PSI) (Gorman and Levine, 1965). However, PSI can accept the electron only after it has first become oxidized. Like PSII, PSI is also oxidized upon the absorption of a photon which excites an electron in the reaction center

(P_{700}). In the case of PSI, the electron is transferred from the reaction center chlorophyll P_{700} via chlorophyll A_0, phylloqinone A_1 and three [4Fe-4S] iron-sulfur clusters (F_X, F_A, and F_B) to ferredoxin (Fd) on the stromal side of PSI (Amunts et al., 2007; Ben-Shem et al., 2003). Ferredoxin is a soluble [2Fe-2S] protein that functions as a reductant for several different enzymes in the chloroplast (Knaff and Hirasawa, 1991). The majority of photosynthetic electrons however are used for $NADP^+$ reduction by ferredoxin-$NADP^+$ oxidoreductase (FNR) and the generated NADPH is subsequently utilized primarily for CO_2 fixation (Haupt-Herting and Fock, 2002). Two electrons, and thus two reduced Fds, are required for the photoreduction of one $NADP^+$ by FNR (Carrillo and Ceccarelli, 2003).

The proton gradient created by water splitting at the OEC and plastoquinone oxidation at Cyt b_6f is used for ATP synthesis by the thylakoid ATP synthase (ATPase). ATPase consist of multiple thylakoid embedded subunits, which are interconnected via a central stalk to the catalytic stromal subunits (Groth and Pohl, 2001). The thylakoid embedded subunits allow a passage for protons to move down their electrochemical gradient from the lumen to the stroma. The movement of protons makes the central stalk rotate and the motion is used to drive conformational changes in the catalytic subunits thus enabling ATP synthesis (Capaldi and Aggeler, 2002). Approximately four protons are required for the synthesis of one ATP by the chloroplast ATPase (Petersen et al., 2012). Thus, the oxidation of two water molecules and the transfer of four electrons along the ETC produces in total three ATPs and two NADPHs. However, electrons can also be fed back to the ETC from Fd in a process called cyclic electron transfer (CET) (Tagawa et al., 1963). CET functions via two alternative pathways, the NDH-dependent (Shikanai et al., 1998) and PGR5-dependent (DalCorso et al., 2008; Munekage et al., 2002) pathways, which both lead to PQ pool reduction and increased proton pumping to the lumen via the action of the Q-cycle. Consequently, CET can be used to adjust the chloroplast ATP/NADPH ratio to meet the demands of the organelle (Avenson et al., 2005). However, since CET increases the proton concentration in the lumen, it is also important for the induction of pH-dependent photoprotective mechanisms (Munekage et al., 2002) (see 1.1.3).

1.1.2 Reaction centers and light harvesting antennas

Both photosystems, PSI and PSII, are multi-subunit pigment-protein complexes composed of a reaction center core (C) and light harvesting antennas encoded by the

LHC gene family (Jansson, 1994) (Figure 3). The chlorophyll *a* and *b* pigments of the antenna proteins absorb light and funnel the energy to the reaction centers thus providing the reaction centers with a constant stream of energy to drive photosynthesis (Mirkovic et al., 2017).

The PSII core complex contains the reaction center proteins D1 and D2, several small subunits, and the core antenna proteins CP43 and CP47 (Zouni et al., 2001). In plants, the PSII core is present as a dimer surrounded by light harvesting complex II (LHCB) antenna proteins (Boekema et al., 1995; Peter and Thornber, 1991). Each dimer binds two LHCB4, LHCB5 and LHCB6 proteins (also known as CP29, CP26 and CP24, respectively), and a variable amount of LHCII trimers (Boekema et al., 1999). Three types of LHCII trimers are distinguished based on their affinity to the PSII core: S-trimers (tightly bound), M-trimers (moderately bound) and L-trimers (loosely bound) (Boekema et al., 1999). LHCII trimers are composed of LHCB1, 2 and 3 proteins: S- and L-trimers contain mainly LHCB1 and 2 proteins, whereas the M-trimers are composed mostly of LHCB1 and 3 (Galka et al., 2012; Hankamer et al., 1997). PSII is readily isolated as stable $C_2S_2M_{0\text{-}2}$ supercomplexes which are likely associated with heterogenous amounts of L-LHCII trimers *in vivo* (Boekema et al., 1999). The S- and M-trimers are bound to PSII dimers via the monomeric antenna proteins, whereas the molecular interactions of L-trimers are less well defined (Dekker and Boekema, 2005).

The PSI core complex consists of the PSAA and PSAB reaction center proteins and several accessory subunits surrounded by a light harvesting antenna (LHCI) composed of LHCA proteins (Amunts et al., 2007; Ben-Shem et al., 2003). In plants, PSI is a monomer surrounded by four LHCI proteins, LHCA1-4, which are bound side by side to one side of the PSI core (Ben-Shem et al., 2003; Boekema et al., 2001). Under certain light conditions (see 1.1.3), PSI can also have L-LHCII trimers attached to the reaction center via the LHCI antenna or the PSAH subunit on the opposite side of the monomer (Benson et al., 2015; Lunde et al., 2000).

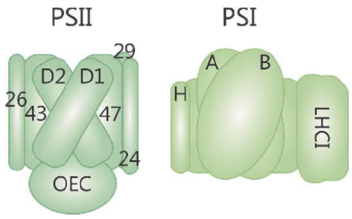

Figure 3. Organization of the photosystem II (PSII) and photosystem I (PSI) reaction center core and antenna proteins. The PSII reaction center core consists of the reaction center proteins D1 and D2, several small subunits (omitted for clarity) and the inner antenna proteins CP43 and CP47. The monomeric antenna proteins CP26, CP24 and CP29 are attached to the core via the inner antenna proteins and the LHCII antenna trimers (not shown) associate with the monomeric antenna proteins. The oxygen evolving complex (OEC) of PSII is also shown. The PSI reaction center core consists of the reaction center proteins PSAA and PSAB and several smaller subunits (omitted for clarity). The light harvesting antenna of PSI (LHCI) consists of four LHCA proteins (LHCA1-4) bound in a crescent formation to one side of PSI. LHCII trimers can bind on top of the LHCI antenna or on the PSAH subunit to increase the antenna size of PSI.

1.1.3 Regulation of light harvesting and thylakoid structure

The quality and quantity of light available to plants in nature varies depending on the growth environment, seasonal changes, prevailing weather conditions and other environmental variables. Hence, the photosynthetic electron transfer chain and light harvesting system need to respond to fluctuations in environmental conditions to, on one hand, ensure maximal energy capture but, on the other hand, protect the photosynthetic machinery from damage. In the heart of the most immediate responses to fluctuating light conditions is the light harvesting antenna LHCII, which plays a dual role by optimizing light harvesting and protecting the ETC from photo-oxidative damage (Horton and Ruban, 2005; Rochaix, 2014).

During a sudden increase in light intensity and at the onset of photosynthesis after dark, the proton concentration in the thylakoid lumen increases. Increased lumenal pH activates mechanisms that increase the capacity of LHCII to dissipate absorbed light energy as heat. Heat dissipation effectively reduces energy transfer from LHCII to PSII and thus protects the ETC from over-reduction and consequent photo-oxidative damage under high light intensities (Ruban et al., 2012). Two different mechanisms are required for the activation of heat dissipation in plants. Thylakoid membranes of vascular plants contain a protein called PSBS, which upon protonation acts as a switch to change LHCII from a light harvesting state into a dissipative one (Li et al., 2000; Li et al., 2004). Lumen acidification also activates the violaxanthin de-epoxidase enzyme, which converts violaxanthin pigments in the LHCII antenna into zeaxanthin leading to increased energy dissipation (Demmig et al., 1987). It has been proposed that these mechanisms aid individual LHCII molecules as well as the

whole antenna system to change into a state that promotes heat dissipation (Duffy and Ruban, 2015; Ruban et al., 2012).

Because PSII and PSI have slightly different absorption spectra (Laisk et al., 2014), different light conditions can lead to the preferential excitation of one or the other. Plants can however adjust the absorption cross-section of the photosystems by altering their antenna size in response to the redox state of the PQ pool. The alterations of the respective antenna sizes of PSII and PSI are called state transitions (Bonaventura and Myers, 1969; Murata, 1969). During state transitions the loosely bound light harvesting antenna L-LHCII changes its association between the two photosystems (Wientjes et al., 2013) depending on its phosphorylation status (Bennett et al., 1980). Under illumination that favors PSII, the PQ pool becomes reduced. The binding of reduced PQ to Cytb_6f activates the thylakoid membrane localized protein kinase STN7, which phosphorylates LHCII antenna trimers (Bellafiore et al., 2005; Depège et al., 2003; Vener et al., 1997). Phosphorylated L-LHCII dissociates from PSII and attaches to PSI inducing state 2. L-LHCII trimers are composed of LHCB1 and LHCB2 subunits (Galka et al., 2012), which are both phosphorylated by STN7, but LHCB2 phosphorylation has been shown to be specifically required for state 1 to 2 transition (Crepin and Caffarri, 2015; Leoni et al., 2013; Pietrzykowska et al., 2014). However, the exact mechanism how phosphorylation causes L-LHCII dissociation from PSII and association with PSI is not known (Allen, 2017). For example, even though the S-LHCII trimers also contain LHCB2 and get phosphorylated, they remain associated with PSII reaction centers in state 2 (Wientjes et al., 2013). Upon illumination that preferentially excites PSI the PQ pool becomes oxidized and STN7 inactivates allowing its counteracting protein phosphatase PPH1/TAP38 to dephosphorylate LHCII (Pribil et al., 2010; Shapiguzov et al., 2010). Unlike STN7, the phosphatase is not regulated by the chloroplast redox state (Silverstein et al., 1993) but is most likely constitutively active (Pribil et al., 2010). Dephosphorylated L-LHCII attaches back to the PSII antenna system leading to state 1. The primary role of state transitions is suggested to be to maximize electron transfer under light limiting conditions (Allen, 2017; Goldschmidt-Clermont and Bassi, 2015; Tikkanen et al., 2006). In accordance with this view, STN7 has been shown to be inactivated in high light by reduced thioredoxins (Rintamäki et al., 2000) connecting light intensity to the regulation of LHCII phosphorylation.

The stacking of thylakoids into grana is a special feature of land plants (Anderson et al., 2008). Interactions between the negative surface charges of thylakoid proteins and cations in the stroma (Barber, 1980), attractive van der Waals interactions between PSII and LHCII proteins of adjacent membrane layers (Chow et al., 1991), and increased entropy of the stroma (Chow, 1999) have been proposed to be central driving forces of grana formation. Consequently, thylakoid protein complexes are not evenly distributed within the membrane system: most of PSII is situated in the grana stacks, whereas PSI and the ATPase are found in the grana margins, end membranes and stroma lamellae (Figure 4) (Andersson and Anderson, 1980) because their stroma exposed parts are simply too big to fit into the tightly packed grana stacks (Dekker and Boekema, 2005). Cytb_6f is thought to be evenly distributed between the thylakoid domains (Anderson, 1982), whereas the curved ends of grana layers are considered to be devoid of photosynthetic protein complexes (Dekker and Boekema, 2005). The lateral heterogeneity of thylakoid membranes poses both challenges and opportunities for light harvesting. For example, during state transitions, the L-LHCII trimers need to move from the crowded grana to meet PSI in the stroma lamellae. Indeed, grana stacks have been shown to undergo dramatic changes upon state transitions: grana stacks become less defined and more loosely structured in state 2 compared to state 1 (Chuartzman et al., 2008). On the other hand, the separation of PSII into grana enables effective light harvesting by shade leaves which grow under light that is enriched in preferably PSI exciting wavelengths (Anderson et al., 1973; Melis and Harvey, 1981). It has been hypothesized that grana have evolved in land plants specifically to enable efficient light harvesting in low light (Mullineaux, 2005). Considering all the implications of grana formation, grana structure and dynamics are clearly important for the regulation of light reactions.

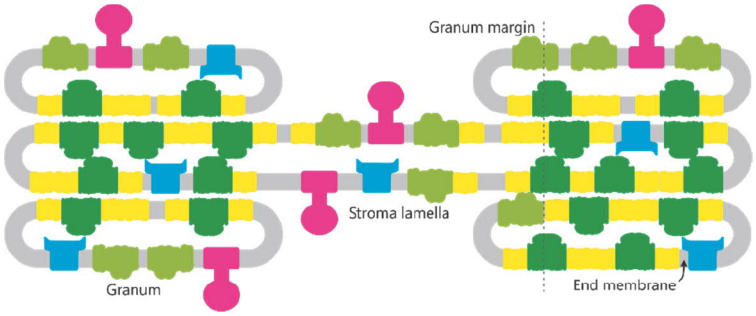

Figure 4. Organization of thylakoid protein complexes within the thylakoid membrane. PSII (green) and LHCII trimers (yellow) are packed within the grana stacks, whereas PSI (light green) and ATP synthase (magenta) are restricted to the end membranes, grana margins and stroma lamellae. Cytochrome b_6f (cyan) is evenly distributed throughout the membrane system. Thylakoid membrane lipid bilayers are depicted in grey. Adapted from Dekker and Boekema (2005).

1.1.4 Chlorophyll fluorescence

In addition to photochemistry, excitation energy in the light harvesting antenna system has two alternative fates: it can be dissipated as heat or be emitted as fluorescence (Butler, 1978). Photochemistry and energy dissipation lead to the quenching of chlorophyll fluorescence and are therefore referred to as photochemical and non-photochemical (NPQ) quenching, respectively. Thus, chlorophyll fluorescence can be used as a tool to investigate photosynthesis (Baker, 2008). Several different instruments and parameters have been developed throughout the years to investigate photosynthetic phenomena via chlorophyll fluorescence (Kalaji et al., 2014). Because the oxidized and reduced PSI reaction centers quench fluorescence equally well at room temperature, light-dependent variation in chlorophyll fluorescence arises from PSII and its antenna and can thus be used as a specific probe of PSII function (Duysens and Sweers, 1963). However, it is also possible to obtain information about energy transfer to PSI through chlorophyll fluorescence by measuring the sample in low temperature (-196°C, 77K, the temperature of liquid nitrogen) where PSI fluorescence is also clearly visible and can be separated from fluorescence arising from PSII due to their different emission maxima (Goedheer, 1964).

Even though the term NPQ is often used to refer to the light induced heat dissipation of absorbed energy in the LHCII antenna (also known as "energy-dependent quenching", qE, see 1.1.3), there are in fact two additional mechanisms that can

lower chlorophyll fluorescence and thus contribute to NPQ. Fluorescence can be quenched due to the reorganization of the PSII antenna during state transitions (qT), which is prominent especially under light limiting conditions (Ruban and Johnson, 2009), or due to downregulation of PSII reaction centers and persistent changes in LHCII antenna organization (qI) caused by prolonged high light exposure (Ruban et al., 2012). The different NPQ components can be distinguished by their induction and relaxation kinetics (Baker, 2008). The fast energy-dependent quenching qE is induced and relaxes in seconds and qT in tens of minutes (Ruban and Johnson, 2009), whereas qI is maintained longer (Ruban et al., 2012).

1.1.5 Ferredoxin-NADP$^+$ oxidoreductases

The last step of the photosynthetic ETC is catalyzed by the enzyme ferredoxin-NADP$^+$ oxidoreductase (FNR) (Shin and Arnon, 1965). FNR catalyzes electron transfer between the one-electron-carrying Fd and the two-electron-carrying NADP$^+$ (Reaction 3) (Carrillo and Ceccarelli, 2003). The exchange from one-electron to two-electron "currency" is made possible by the flavin adenine dinucleotide (FAD) prosthetic group of FNR, which is capable of both one and two electron transfer reactions. Since the carbon assimilating reactions require NADPH as a reductant, FNR plays an important role between the electron transfer and carbon fixating reactions of photosynthesis, and even a small decrease in FNR activity leads to impaired carbon assimilation and growth (Hajirezaei et al., 2002). In addition to its role in photosynthetic tissues, FNR also exists in non-photosynthetic plastids, where it catalyzes the reaction to the opposite direction thus feeding electrons towards Fd-dependent heterotrophic metabolism (Morigasaki et al., 1990; Oji et al., 1985), for example nitrogen assimilation (Bowsher et al., 1993).

Reaction 3. Electron transfer catalyzed by FNR.

$$2\ Fd_{red} + NADP^+ + H^+ \rightleftharpoons 2\ Fd_{ox} + NADPH$$

Even though the reaction catalyzed by FNR is reversible, the Fd oxidizing and reducing reactions are usually catalyzed by different FNR types accompanied with the presence of different (photosynthetic and non-photosynthetic) Fds (Hanke et al., 2004; Hanke et al., 2005; Hase et al., 1991; Onda et al., 2000). The photosynthetic leaf-type FNR (LFNR) and Fd are present only in the photosynthetic tissues (Hase et al., 1991; Onda et al., 2000), whereas the heterotrophic root-type FNR (RFNR) and Fd seem to be expressed both in shoots and roots (Hanke et al., 2005; Hase et

al., 1991). The leaf- and root-type FNRs and Fds differ in their redox-potentials, making $NADP^+$ reduction more favorable by the leaf-type proteins, whereas NADPH oxidation is favored by the root-types (Aliverti et al., 2001). The interaction between root-type FNR and Fd is also different compared to that of the leaf-type proteins, which further contributes to the direction of electron flow (Shinohara et al., 2017). In addition to the presence of the photosynthetic and non-photosynthetic FNRs, both *LFNR* and *RFNR* genes are themselves present as multiple copies in many plant species (Hanke et al., 2005). For example, the model plant Arabidopsis has two isoforms of both L- and RFNRs (LFNR1 and 2, and RFNR1 and 2, respectively) (Hanke et al., 2005).

The presence of multiple L- and RFNR isoforms raises the question of their functional specificities. Because FNRs contribute to the ratio of reduced Fd and NADPH, the different FNR isoforms might play a role in regulating the electron flow to different metabolic pathways, and many studies during past decades have aimed at unraveling the specific roles of the different isoenzymes (Goss and Hanke, 2014). All the different FNR isoforms in Arabidopsis have been shown to have tissue specific expression patterns and to respond differentially to various nitrogen regimes (Hanke et al., 2005). In Arabidopsis, *lfnr2* knock-out plants were shown to have enhanced stress responses and downregulation of photosynthetic proteins under cold stress, whereas *lfnr1* mutants were more like wild-type under those conditions (Lintala et al., 2009). However, both LFNR isoforms alone are able to support photoautotrophic growth suggesting that they are at least partially overlapping in function (Lintala et al., 2009). On the other hand, *lfnr1lfnr2* double mutants were shown to be only capable of heterotrophic growth proving that RFNRs and LFNRs are clearly functionally distinct from each other (Lintala et al., 2012).

In most species, from cyanobacteria to flowering plants, LFNR is present as a soluble protein in the stroma as well as bound to the thylakoid membranes (Lintala et al., 2007; Thomas et al., 2006; Twachtmann et al., 2012). The mechanism of membrane attachment and its physiological implications vary between different clades (Goss and Hanke, 2014). In land plants, LFNR is attached to thylakoids via the TIC62 (Küchler et al., 2002) and TROL (Juric et al., 2009) proteins. Both TIC62 and TROL contain proline rich helices where LFNRs bind as homo- or heterodimers forming high molecular mass complexes (Benz et al., 2009; Juric et al., 2009; Küchler et al., 2002). Recently, a small, light regulated protein LIR was shown to govern the

membrane attachment of LFNR (Yang et al., 2016): LFNRs bind to membranes during darkness when LIR is present but are released following light induced degradation of LIR (Benz et al., 2009; Yang et al., 2016). It has been proposed that membrane attachment of LFNR might be important in channeling electrons to the cyclic electron transfer pathway around PSI, and this seems to be the case especially in C_4 plants (Goss and Hanke, 2014). However, in Arabidopsis it was shown that neither LFNR isoform has a preferential effect on CET (Lintala et al., 2009), even though they differ in their membrane binding properties (Lintala et al., 2007; Lintala et al., 2009). Instead, membrane attachment might serve a role in protecting FNR from proteolytic degradation during photosynthetically inactive periods in C_3 plants (Benz et al., 2009).

Proteins with FNR activity are found in all kingdoms of life, but they belong to two evolutionarily non-related families (Ceccarelli et al., 2004). The FNRs of photosynthetic organisms and apicomplexan parasites together with bacterial FNR enzymes belong to the plant-type FNR family (Aliverti et al., 2008; Ceccarelli et al., 2004) (Figure 5), whereas FNRs from mitochondria and some prokaryotes belong to the structurally unrelated glutathione reductase-type family (Aliverti et al., 2008; Ceccarelli et al., 2004; Hanukoglu and Gutfinger, 1989). All plant-type FNRs share a common two domain structure: they consist of an N-terminal FAD binding domain and a C-terminal NADP(H) binding domain (Karplus et al., 1991). They also show high specificity towards NADP(H) over NAD(H) as a substrate (Carrillo and Ceccarelli, 2003) conveyed by amino acids binding to the pyrophosphate backbone and 2'-phosphate of NADP(H) (Hermoso et al., 2002; Ingelman et al., 1997; Tejero et al., 2003) and an aromatic C-terminal residue (Piubelli et al., 2000). The photosynthetic leaf-type FNRs of higher plants are similar to the photosynthetic FNRs from cyanobacteria, whereas the photosynthetic FNRs from green algae resemble more the non-photosynthetic root-type FNRs of vascular plants (Figure 5). FNRs from apicomplexan parasites are related to FNRs from photosynthetic organisms (Figure 5). Together, these proteins form the plastidic-type FNR family, whereas FNRs from heterotrophic bacteria belong to the bacterial-type FNR family (Aliverti et al., 2008; Ceccarelli et al., 2004) (Figure 5).

Despite their similarities and shared evolutionary origin, several important structural and catalytic features separate the plastidic- and bacterial-type FNRs from each other. The most prominent feature separating plastidic and bacterial FNRs is the way

they bind the FAD cofactor. Plastidic-type FNRs contain a beta-hairpin structure, which allows FAD to bind to the enzyme in an extended conformation (Karplus et al., 1991) leading to higher turnover rates and therefore higher catalytic efficiency compared to the bacterial FNRs (Ceccarelli et al., 2004). In contrast, FAD in bacterial-type FNRs is bound in a bent conformation due to the lack of the beta-hairpin (Ingelman et al., 1997). Like RFNRs, bacterial FNRs preferentially catalyze the oxidation of NADP(H) to produce reduced Fd or flavodoxin (Fld) (Aliverti et al., 2008). Flavodoxins are small flavin-adenine mononucleotide containing electron carrier proteins, which can functionally replace Fds in bacteria and unicellular photosynthetic organisms especially under iron limiting conditions (Zurbriggen et al., 2007). The different catalytic rates are thought to reflect the different metabolic roles of the plastidic- and bacterial-type FNRs: plastidic FNRs need to generate NADP(H) at a high rate for carbon assimilation, whereas the bacterial FNRs support more slowly occurring heterotrophic metabolism (Ceccarelli et al., 2004). In heterotrophic bacteria, FNR functions for example in methionine biosynthesis (Fujii and Huennekens, 1974) and protection against oxidative stress (Bianchi et al., 1995).

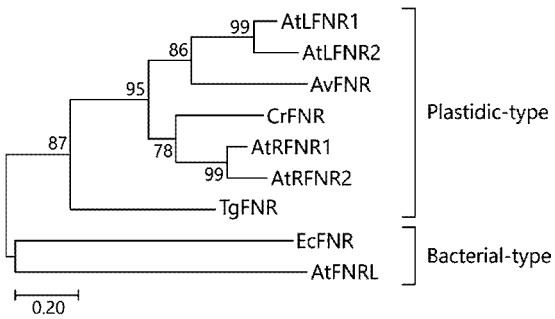

Figure 5. Phylogenetic relationships of the plant-type ferredoxin-NADP$^+$ oxidoreductase (FNR) superfamily. Tree was constructed using the following sequences: *Arabidopsis thaliana* LFNRs (AtLFNR1 and AtLFNR2), RFNRs (AtRFNR1 and AtRFNR2) and FNR-LIKE (AtFNRL); *Anabaena variabilis* FNR (AvFNR); *Chlamydomonas reinhardtii* FNR (CrFNR); *Toxoplasma gondii* FNR (TgFNR) and *Escherichia coli* FNR (EcFNR). The tree was constructed using the Maximum Likelihood method in MEGA7 (Kumar et al., 2016). The tree was bootstrapped (Felsenstein, 1985) a thousand times; confidence values are shown next to the branches. The scale bar shows the number of substitutions per site.

In addition to the well-defined structural domains, the mature forms of plastidic LFNRs have a peculiar feature: they often contain variable N-terminal sequences. For example, the spinach LFNR contains a disordered N-terminal extension (Karplus et al., 1991), which (i) varies in length by few amino acids (Karplus et al., 1984; Shin et al., 1990), (ii) is sensitive to (proteolytic) degradation (Shin et al., 1990) and (iii) occasionally contains an N-terminal pyro-glutamyl residue (a cyclized form of an N-terminal glutamine or glutamate) (Karplus et al., 1984; Shin et al., 1990). Variable

N-termini have also been reported in wheat, where both of the two wheat LFNR isoforms were shown to contain two alternative N-terminal sequences (Gummadova et al., 2007). In contrast, only one N-terminal form was found in both Arabidopsis LFNRs (Hanke et al., 2005). Intriguingly, the different N-termini did not affect the *in vitro* activity of the spinach enzyme (Shin et al., 1990) but had a small effect on the activity and specificity towards different ferredoxins in the wheat proteins (Gummadova et al., 2007). In maize, the differential localization of the three LFNR isoforms was attributed to the N-terminal sequence of the proteins (Twachtmann et al., 2012). In addition to different N-termini, LFNR isoenzymes have also been detected as multiple isoelectric forms in wheat (Moolna and Bowsher, 2010) and Arabidopsis (Lintala et al., 2007). The identity of these forms has not been experimentally verified thus far, although the pI shift was computationally shown to correspond to phosphorylation (Moolna and Bowsher, 2010).

1.2 Post-translational regulation of proteins

Because proteins perform most of the metabolic tasks taking place within a cell, their function needs to be regulated and fine-tuned at many levels. A protein's performance depends on its presence in the right place at the right time in an active form. Protein abundance is ultimately regulated by the rate of its synthesis and degradation, i.e. its turnover. Protein synthesis is however energetically expensive (Russell and Cook, 1995), and protein degradation is irreversible, so other regulatory mechanisms are needed to induce rapid and reversible changes in protein activity. More flexible control of protein function is achieved through post-translational modifications (PTMs) (Prabakaran et al., 2012), which include reversible and irreversible modifications of N-termini and internal amino acid residues with different chemical groups and proteolytic cleavage of peptides. PTMs are widespread in all organisms including plants (Friso and Van Wijk, 2015) and the subcellular organelle chloroplast (Lehtimäki et al., 2015). In chloroplasts, the most prevalent PTMs are phosphorylation and acetylation, which are known to regulate central processes such as photosynthetic light reactions and nucleic acid metabolism (Grabsztunowicz et al., 2017).

1.2.1 Phosphorylation

Phosphorylation is the best studied reversible PTM in eukaryotic cells (Khoury et al., 2011). It is involved in the regulation of multiple cellular functions spanning

from cell cycle control to the fine tuning of metabolic fluxes (Kyriakis, 2014), and it also plays an important role inside chloroplasts (Baginsky, 2016). In chloroplasts, the phosphate group is most commonly attached to the side chain hydroxyl-group of a serine or threonine residue adding a negative charge onto it, while tyrosine phosphorylation seems to be rare (Baginsky, 2016). Phosphorylation is catalyzed by protein kinases which use ATP as a substrate, and the removal of phosphate is catalyzed by protein phosphatases. Up until today 6 kinases and 9 phosphatases have been experimentally verified from chloroplasts but based on genomic data the existence of several more candidates have been proposed (Schliebner et al., 2008). Additionally, over 150 phosphoproteins have been identified from Arabidopsis chloroplasts, representing around 10% of all chloroplast proteins (Reiland et al., 2009).

Photosynthetic light reactions are arguably the best studied example of phosphorylation regulated processes in chloroplasts. The regulation of light harvesting and energy transfer to PSII and PSI was attributed to LHCII phosphorylation already almost 40 years ago (Bennett et al., 1980) (see 1.1.3) and it remains an area of intense investigation even today (Allen, 2017). Other famous examples include the PSII repair cycle, which is regulated by the dynamic phosphorylation/dephosphorylation of PSII core proteins (Baena-González et al., 1999; Rintamäki et al., 1995; Tikkanen et al., 2008), and grana formation, which is likewise affected by PSII core phosphorylation (Fristedt et al., 2009). The kinases responsible for LHCII and PSII core phosphorylation (STN7 and STN8, respectively) and their respective phosphatases (PPH1/TAP38 and PBCP) have been characterized and studied extensively during past decades (Bellafiore et al., 2005; Bonardi et al., 2005; Depège et al., 2003; Pribil et al., 2010; Samol et al., 2012; Shapiguzov et al., 2010; Vainonen et al., 2005). In addition to the well characterized targets, the increased amount of phospho-proteome studies that have revealed tens of novel phosphorylation targets (Durek et al., 2010; Heazlewood et al., 2008; Reiland et al., 2009) provide ample resources for the further elucidation of the physiological consequences of chloroplast protein phosphorylation.

1.2.2 Acetylation

Protein acetylation is a ubiquitous PTM present in all kingdoms of life (Drazic et al., 2016). It can be divided into two distinct types, based on the modified group. N^{α}-acetylation occurs at the α-amino group of the N-terminal amino acid of a

polypeptide chain (Aksnes et al., 2015) (Figure 6A). Unlike most other PTMs, N-terminal acetylation can take place both co- and post-translationally (Aksnes et al., 2015), and it is thought to be irreversible (Drazic et al., 2016). In N^{ε}-acetylation, on the other hand, the acetyl group is attached to the side chain amino group of a lysine (Lys) residue and the reaction is fully reversible. In both acetylation types the acetyl group donor is acetyl coenzyme A (Ac-CoA) and both result in the loss of a positive charge. N-terminal acetylation is catalyzed by N-terminal acetyltransferases (NATs), which belong to the GNAT-family of acetyltransferases (Neuwald and Landsman, 1997; Polevoda and Sherman, 2003). Lys acetylation is catalyzed by four different Lys acetyltransferase (KAT) families (GNAT, MYST, CBP and $TAF_{II}250$) and deacetylation by three different groups of Lys deacetylases (KDACs) (HAD/RPD3, HDT and SRT) (Uhrig et al., 2017). All of the families are conserved between eukaryotes apart from the HDT family, which is present only in land plants (Uhrig et al., 2017).

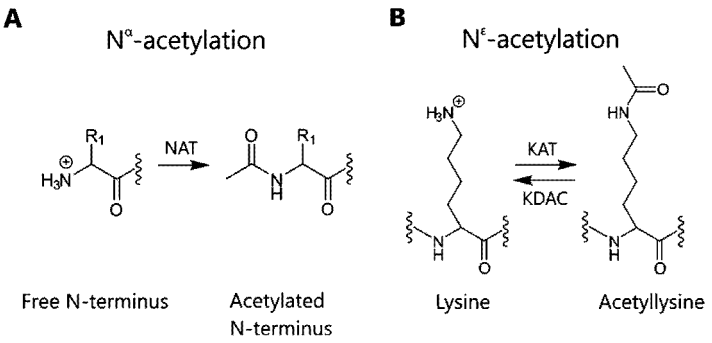

Figure 6. Protein acetylation. **A** Acetylation of the N^{α}-amino group of the N-terminal amino acid residue is catalyzed by N-terminal acetyltransferases (NATs). **B** Acetylation of the N^{ε}-amino group of lysine side chains is catalyzed by lysine acetyltransferases (KATs) and deacetylation by lysine deacetylases (KDACs). Both reactions (A and B) lead to the replacement of a positive charge by a polar group.

Majority of proteins are N-terminally acetylated during translation in the cytosol by different cytosolic N-terminal acetyltransferase (NAT) complexes (Arnesen et al., 2009), making N-terminal acetylation one of the most prevalent PTM in eukaryotes, including plants (Bienvenut et al., 2012). Five different NAT-complexes (NatA-E) are present in plants (Bienvenut et al., 2012). N^{α}-acetylation can affect protein interactions (Scott et al., 2011), localization (Forte et al., 2011), or folding (Holmes et al., 2014), and it seems to play an especially important, albeit conflicting, role in protein stability by either promoting or preventing protein degradation by the

proteasome (Hershko et al., 1984; Hwang et al., 2010). The role of N-terminal acetylation in plants has been less widely studied compared to animals but recently the role of a cytosolic NatA-complex in Arabidopsis was extensively investigated (Linster et al., 2015). NatA was found to be essential for embryo development and its activity was shown to be regulated by the drought response hormone abscisic acid, suggesting that NatA has a role in adaptation to environmental stresses (Linster et al., 2015). Additionally, Arabidopsis mutants with a defective cytosolic NatB complex have been shown to suffer from abnormal development of floral organs and leaf morphology (Ferrández-Ayela et al., 2013). These studies clearly demonstrate that N-terminal acetylation is equally important in plants as it is in animals. In fact, several features of N-terminal acetylation seem to be conserved between different eukaryotic species, including the catalytic subunits of the cytosolic NAT-complexes NatA-E (Bienvenut et al., 2012) and the substrate specificity of the NatA complex (Linster et al., 2015), indicating that the mechanisms how N-terminal acetylation affects protein function might also be conserved.

Because most of the proteins present in chloroplasts are nuclear encoded, cytosolic N-terminal acetylation could also affect the chloroplast proteome. In a study screening for photosynthetic mutants, Pesaresi et al. (2003) found that a mutation in a cytosolic NatC affected the quantum yield of PSII, and the authors hypothesized that N-terminal acetylation might be relevant for the stability or import of chloroplast pre-proteins (Pesaresi et al., 2003). Indeed, N^{α}-acetylation was later shown to be required for the efficient import of chloroplast proteins via the TOC159 mediated import pathway (Bischof et al., 2011). In addition to the N^{α}-acetylation of pre-proteins, N-terminal acetylation is an abundant modification in plastid encoded proteins and in nuclear encoded mature chloroplast proteins, which have had their N-terminal transit peptides removed and have been post-translationally N^{α}-acetylated (Bienvenut et al., 2012; Rowland et al., 2015; Zybailov et al., 2008). The prevalence of N-terminal acetylation in chloroplasts strongly hints that a post-translational N^{α}-acetylation machinery is present in the organelle. As a matter of fact, apart from the cytosolic NATs, Arabidopsis genome encodes several GNAT-family acetyltransferases (Bienvenut et al., 2012), seven of which have been predicted to have a chloroplast targeting peptide (Dinh et al., 2015). Recently, one of these proteins was identified as the first chloroplast N^{α}-acetyltransferase (Dinh et al., 2015), but the physiological consequences of N-terminal acetylation inside chloroplasts have been scarcely studied. In Arabidopsis, N^{α}-acetylation of the

ATPase ε-subunit was shown to increase its stability under drought stress (Hoshiyasu et al., 2013) and in *Chlamydomonas reinhardtii* N^{α}-acetylation was found to be most prevalent in abundant, long-lived stromal proteins (Bienvenut et al., 2011). Together these studies suggest that N-terminal acetylation might serve to increase protein stability within chloroplasts. More studies are however needed to uncover how N^{α}-acetylation affects all the different target proteins.

N^{ε}-acetylation is best-known for its role in the regulation of chromatin structure and gene expression via histone acetylation: acetylation of the Lys rich N-terminal tails of histones is thought to weaken the interaction between histones and the negatively charged DNA backbone thus loosening the chromatin structure and enabling efficient transcription (Grunstein, 1997). However, during the last decade it has become more and more evident that Lys acetylation is a prevalent modification also in non-histone proteins (Choudhary et al., 2009). Studies of Lys acetylation have been made possible by the development of high-precision mass spectrometry devices, which are capable of not only accurately detecting the modification but also pointing out its exact location and providing quantitative information (Olsen and Mann, 2013). The first plant Lys acetylomes were published in 2011 for the model plant Arabidopsis (Finkemeier et al., 2011; Wu et al., 2011), and several studies covering a variety of species have followed since, each identifying tens to hundreds of acetylation sites (Melo-Braga et al., 2012; Meng et al., 2018; Smith-Hammond et al., 2014; Xiong et al., 2016; Xue et al., 2018). In the most recent and comprehensive study of Lys acetylation in Arabidopsis, over two thousand acetylation sites in more than thousand proteins were detected (Hartl et al., 2017). It is noteworthy that in many of the studies chloroplasts were found to be enriched in Lys acetylated proteins (for example half of the acetylation sites identified by Hartl et al. (2017) were in chloroplast proteins) and that many photosynthetic proteins were heavily acetylated (Finkemeier et al., 2011; Hartl et al., 2017; Meng et al., 2018; Wu et al., 2011; Xiong et al., 2016; Xue et al., 2018).

Because the substrate of Lys acetylation, Ac-CoA, is a central cellular metabolite, Lys acetylation is well suited to function as a signal of the cellular energy status (Drazic et al., 2016). In accordance with this idea, Lys acetylation has been shown to govern the rates of several central metabolic pathways in bacteria (Wang et al., 2010) and humans (Zhao et al., 2010) establishing its role as a key PTM regulating diverse cellular functions. It is also interesting that a noticeable fraction of the

acetylation sites are conserved between Arabidopsis and humans (Finkemeier et al., 2011), which suggests that at least some regulatory mechanisms through Lys acetylation might have been conserved during evolution. Due to the abundance of N^{ε}-acetylation in Calvin cycle enzymes it seems likely that Lys acetylation functions as a metabolic sensor in plants, as it does in bacteria and animals (Wang et al., 2010; Zhao et al., 2010). Indeed, it has already been shown that acetylation affects the activities of Calvin cycle enzymes: RuBisCO and phosphoglycerate kinase activities were shown to increase upon deacetylation (Finkemeier et al., 2011), whereas the acetylation of RuBisCO activase at K438 seemed to decrease the sensitivity of RuBisCO activase to ADP inhibition under low light (Hartl et al., 2017). These findings are significant considering the central role of CO_2 assimilation to plant growth.

Lys acetylation has also been shown to be an abundant modification in the ETC, where all of the main complexes (PSII, Cytb_6f, PSI, and ATPase) were found to contain acetylated subunits (Hartl et al., 2017). In addition, the loosely bound LHCII has been shown to contain more N^{ε}-acetylated subunits compared to the more tightly bound antenna (Wu et al., 2011). These results strongly suggest that Lys acetylation can influence light harvesting, but more studies are required to unveil the exact role it plays. To this end, characterization of the acetylation machinery in chloroplasts would be of utmost importance. None of the enzymes responsible for Lys acetylation in chloroplasts are known but considering the abundance of GNAT-family acetyltransferases predicted to be localized in the organelle (Dinh et al., 2015) there are several promising candidates. The first chloroplast Lys deacetylase HDA14 was recently characterized, which was crucial for demonstrating the importance of Lys acetylation to the regulation of RuBisCO activase activity *in vivo*, as described above (Hartl et al., 2017).

2 AIMS

The most commonly used model plant for C_3-plants, and angiosperms in general, is the small mouse-ear cress (*Arabidopsis thaliana*, Arabidopsis), which was also the first plant to have its genome sequenced (The Arabidopsis Genome Initiative, 2000). Due to its well annotated genome, ease of growth and readily available mutant lines, Arabidopsis was chosen as the model plant used in this thesis. This thesis is a compilation of three individual projects, where we have examined novel aspects of plant-type FNR family members and protein acetylation in chloroplasts and their relation to photosynthetic light reactions.

I) Even though the Arabidopsis genome was sequenced almost twenty years ago, only a fraction of all the gene products have been functionally characterized. Because FNRs play such a central role in plastid metabolism, the motivation of the first study was to characterize unknown FNR-family proteins to help build a more comprehensive understanding of electron allocation to different metabolic pathways. Through database search we found that the locus AT1G15140 encodes a putative chloroplast protein whose closest homologue is LFNR2. Hence, we named the protein FNR-LIKE (FNRL). The primary aim of this study was to find out **how FNRL relates to the well-known LFNRs** and **whether it also functions as an electron carrier between ferredoxin and NADP(H) in photosynthesis**.

II) Whether different LFNRs can determine the fate of photosynthetic electrons and how their function is regulated have been long standing questions in the photosynthesis community (Goss and Hanke, 2014). Arabidopsis LFNR1 and LFNR2 have been shown to be present as two forms with different isoelectric points (Lintala et al., 2007), and a similar observation has been made in wheat (Moolna and Bowsher, 2010). The existence of the different isoelectric forms in both Arabidopsis and wheat strongly suggests that LFNRs are regulated via a post-translational modification and that this modification is conserved between species. In the second study we aimed at **revealing the chemical nature of the post-translational modification of LFNRs and** studying **its role in the light-dependent regulation** of LFNR function.

III) In study II we found that LFNRs undergo both N-terminal and Lys acetylation (Lehtimäki et al., 2014). Based on the results obtained from study II, and the fact that acetylation seemed to be widespread in chloroplast proteins (Bienvenut et al., 2011; Finkemeier et al., 2011; Wu et al., 2011;

Zybailov et al., 2008), we decided to look at the effect of protein acetylation on photosynthesis. A commonly used approach to study the physiological impact of a PTM is to study mutant plants which lack the proteins performing the PTM. However, no chloroplast protein acetyltransferases and deacetylases were known at the time. Hence, in the third study we aimed at **characterizing chloroplast acetyltransferase(s) that might have a role in regulating photosynthesis**.

3 METHODOLOGY

3.1 Plant material

3.1.1 Growth conditions

All plants were grown in 2:1 soil:vermiculite mixture and irrigated three times per week. Arabidopsis (*Arabidopsis thaliana*, ecotype Columbia) plants were grown in phytotrons under 100 µmol photons m^{-2} s^{-1}, in short day (8 h light/ 16 h dark), +23°C and 50% relative air humidity. Plants were typically used around five weeks after germination. For flowering and seed production plants were grown in otherwise similar conditions but under long day (16 h light/ 8 h darkness). Before germination seeds were subjected to stratification for three days in +4°C and darkness. Dark treatments were performed by keeping plants in light impermeable boxes (**I-III**), and samples were collected with minimal exposure to light. High light treatment was done under 500-600 µmol photons m^{-2} s^{-1} for two hours; temperature measured under light was +28-30°C (**II**). Red (660 nm) and far red (735 nm) treatments were performed by keeping plants under a LED panel (approximately 50 µmol photons m^{-2} s^{-1}) for one hour (**III**).

3.1.2 Transgenic plant lines

Arabidopsis (WT) and T-DNA insertion lines were ordered from the European Arabidopsis Stock Center (NASC, http://arabidopsis.info/). Plants were screened by PCR for homozygous mutants using DyNAzyme II or Phire Hot Start II polymerases (Thermo Fisher Scientific). Primers for screening were planned with the SALK T-DNA verification primer design tool (http://signal.salk.edu/tdnaprimers.2.html). The absence of mRNA from *nsi* lines was further verified with end-point RT-PCR (**III**). Plants expressing NSI-YFP recombinant protein were generated via Agrobacterium mediated floral transformation (**III**) (Narusaka et al., 2010). All transgenic lines used have been listed in Table 1.

Table 1. Transgenic Arabidopsis lines used in this thesis.

Publication	Line name	T-DNA identifier	Description
I	*fnrl*	GABI_856C02	T-DNA knock-out line of *FNRL* gene
III	*nsi-1*	SALK_033944	T-DNA knock-out line of *NSI* gene
	nsi-2	SALK_020577	T-DNA knock-out line of *NSI* gene
	stn7	SALK_073254	T-DNA knock-out line of *STN7* gene
	NSI-YFP	n/a	Transgenic plants expressing NSI-YFP recombinant protein under 35S-promoter

3.1.3 Chloroplast isolation and fractionation

Chloroplasts were isolated and fractionated to study protein localization between thylakoids and the stroma (see details in **I-III**). Fresh rosettes from five-to-six-week-old plants were ground in a kitchen blender in isotonic buffer and the leaf homogenate was filtered. Chloroplasts were collected by centrifugation and intact chloroplasts were separated from broken ones by centrifugation on Percoll step gradients. For separation of stroma and thylakoid fractions, chloroplasts were broken by freeze-thawing them in hypotonic buffer. Thylakoids were subsequently collected by centrifugation. Samples were frozen in liquid nitrogen and stored in -80°C.

3.1.4 Protein extraction and quantification

For denaturing 2D and 1D electrophoresis experiments (**I-II**), leaf material was collected and frozen in liquid nitrogen until extraction. Frozen leaves were crushed and further homogenized in hypotonic buffer. The homogenate was filtered and fractionated by centrifugation into thylakoid and soluble fractions. Samples were frozen in liquid nitrogen and stored in stored in -80°C. For the Fd binding assay (**II**), Arabidopsis leaf proteins were extracted by homogenizing leaves in low salt buffer with 0.1% Triton X-100 (FNR detaches from membranes to the soluble fraction in these conditions, Hanke et al. (2005)). The homogenate was cleared by centrifugation and desalted prior to the experiment. For blue native gel electrophoresis and immunoblotting of thylakoid proteins (**III**) fresh leaves were gently ground in isotonic buffer and filtered. Chloroplasts were collected by centrifugation and broken in hypotonic buffer. Thylakoids were collected by centrifugation, frozen in liquid nitrogen and stored in -80°C. All sample preparation steps were performed in dark and on ice to protect the samples from degradation. A Pierce Protease Inhibitor Tablet (Thermo Fisher Scientific) was added to the

hypotonic lysis buffer to prevent protein degradation. For phosphorylation studies, 10 mM sodium fluoride was added to all buffers to prevent dephosphorylation. Proteins were quantified with the Bio-Rad Protein Assay using IgG as a standard. The chlorophyll content of thylakoids was quantified according to Porra et al. (1989).

3.1.5 Leaf chlorophyll quantification

Chlorophyll *a* and *b* content was determined spectrometrically after N,N-Dimethylformamide (DMF) extraction (**III**). Leaf discs were cut, weighed and incubated in 1 ml of DMF overnight in darkness and room temperature. Absorbance of the extract was measured at 646.6, 663.6 and 750 nm. Chlorophyll content was calculated according to Inskeep and Bloom (1985).

3.2 Recombinant protein work

3.2.1 Cloning

FNRL coding sequence was cloned to pET-28a(+) (Novagen) to generate the His^6-FNRL recombinant protein for *in vitro* assays (**I**). NSI was cloned to pQE-30 (Qiagen) to generate the His^6-NSI for *in vitro* acetylation assay by Prof. Iris Finkemeier's group at the University of Münster (**III**). NSI was also cloned to pGWR-YFP (Rozhon et al., 2010) to generate the recombinant NSI-YFP expressing Arabidopsis plants (**III**). Coding sequences were amplified from in-house made cDNA and all cloning procedures were done using restriction-ligation cloning using commercial enzymes. Positive colonies were selected on appropriate antibiotic plates and screened for true positives with colony PCR. All constructs were verified by sequencing. Plasmids were transformed into in-house made chemically competent *E. coli* DH5α (plasmid production and storage) or BL21(DE3) (protein expression) with heat shock.

3.2.2 Protein purification

For FNRL *in vitro* assays, His^6-FNRL was produced in *E. coli* BL21(DE3) and purified using gravity-flow metal affinity chromatography and gel filtration with ÄKTA FPLC (GE Healthcare Life Sciences) (**I**). Addition of FAD to lysis buffer was critical for obtaining high yields of folded protein; without added cofactor the majority of the protein aggregated into insoluble form. For Fd affinity chromatography Arabidopsis Fd1, Fd2, FdC1 and maize FNR2 were purified as described in Hanke et al. (2004), Okutani et al. (2005) and Voss et al. (2011) by Dr.

Guy Hanke's group at the Queen Mary University of London (**I** and **II**). His^6-NSI was expressed and purified for acetylation activity measurement using metal affinity chromatography and gel filtration at the University of Münster (**III**).

3.2.3 Enzymatic assays

Steady state kinetics. FNRL substrate specificity was determined by measuring Cyt *c* reduction activity with NADH and NADPH (**I**). Optimal pH and salt concentrations for FNRL were determined by measuring dichlorophenolindophenol (DCPIP) reduction activity using NADPH as substrate (**I**). DCPIP reduction assay was used with increasing NADPH concentration to determine the kinetic parameters of FNRL (**I**).

Stopped-flow pre-steady-state kinetics. The reaction between oxidized FNRL and the substrate NADPH was followed by measuring absorbance change at 400-800 nm in a stopped-flow system under anaerobic conditions (**I**). Kinetic parameters were obtained from deconvoluted absorbance spectra as described in **I**.

Spectroscopic assays. FNRL absorption spectrum was recorded from the purified recombinant protein (**I**). Secondary structure and FAD binding was further studied by circular dichroism spectroscopy (**I**) and $NADP^+$ binding to FNRL was studied by absorbance difference spectroscopy as described in Medina et al. (2001) (**I**). The spectroscopic properties and kinetic parameters of FNRL were determined by Dr. Milagros Medina's group at the University of Zaragoza.

In vitro Lys acetylation activity. Lys acetyltransferase activity of NSI was measured by incubating the purified recombinant protein with an artificial peptide substrate and Ac-CoA (**III**). The peptide substrate was coupled to anthranilic acid to allow its detection at 360 nm. Samples were collected after 1, 3 and 12 h and the acetylated and non-acetylated peptides were separated and analyzed by reversed-phase high performance liquid chromatograph (RP-HPLC) coupled with 360 nm optical detector. The acetylation activity assay was performed at the University of Münster.

3.2.4 Fd affinity chromatography

In study **I**, Fd binding assays were performed by loading recombinant FNRL onto columns containing covalently bound Arabidopsis Fd1 or FdC1. Maize FNR2 was used as a positive control. In study **II**, the binding of different FNR forms to Fd1 and Fd2 was studied by loading Arabidopsis leaf protein extracts to the respective Fd columns. In both studies binding was performed in a salt free buffer at pH 7.5 and

bound proteins were eluted with increasing salt concentration. All Fd affinity assays were performed at the Queen Mary University of London.

3.3 Gene expression

The expression of the *FNRL* gene in leaves during the light-dark cycle was studied with quantitative reverse transcriptase-PCR using standard protocols (**I**).

3.4 Electrophoretic methods

3.4.1 SDS-PAGE

Protein samples were solubilized with Laemmli buffer (Laemmli, 1970) and separated on 12%-15% (w/v) hand-casted acryl-amide gels using standard protocols. The Laemmli buffer and gels were supplemented with 6 M urea when membrane proteins were analyzed. Proteins were visualized with 0.1% Coomassie brilliant blue R-250 in 30% methanol, 5% acetic acid, or the gels were used for immunoblotting. Phos-tag gels were prepared as described in **II**.

3.4.2 2D-PAGE

Isoelectric focusing and SDS-PAGE were performed as described in **II**. In summary, proteins were solubilized in a reducing and highly denaturing buffer and separated based on their isoelectric point (pI) on non-linear immobilized pH gradient strips (pH 3-11). After the isoelectric focusing, proteins on the strips were further reduced and alkylated to prevent reformation of disulfide bonds. Strips were run in second dimension on 14% SDS-PAGE. Proteins were visualized with silver, Coomassie, phospho- or glycoprotein staining, or the gels were used for immunoblotting.

3.4.3 BN-PAGE

Blue native electrophoresis was used for the separation of thylakoid protein complexes as described in Järvi et al. (2011) (**III**). Isolated thylakoids were solubilized in buffer containing 1% digitonin or n-dodecyl β-D-maltoside (DM) as detergent. Digitonin solubilizes the stroma lamella protein complexes while leaving grana stacks mostly intact, whereas DM solubilizes complexes from the whole membrane system but disrupts labile interactions. Solubilizing with digitonin allows the visualization of the PSI-LHCII state transition complex. Protein complexes were separated on 3.5%-12.5% gradient gels at 0°C with increasing voltage during the

run. Cathode buffer was supplemented with 0.01% Serva Blue G for the first half of the run to improve the separation and visualization of the complexes.

3.4.4 Immunoblotting

Proteins were electro-blotted to PVDF membrane using 1 mA/cm^2 multiplied by gel thickness (mm). The used membranes, antibodies and detection systems have been described in detail in the original publications (**I-III**). For Edman sequencing (**II**), membranes were stained with Coomassie instead of immunolabeling prior to cutting the protein spots for analysis.

3.5 Mass spectrometry methods

LFNR PTMs were studied by excising protein spots from silver or coomassie stained 2D gels and subjecting them to in-gel trypsin digestion as described in Shevchenko et al. (2007) (**II**). For N-terminal modification, acetylation and methylation analyses, peptides were fractionated with RP-HPLC and analyzed with an LTQ Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific) at the Turku Centre for Biotechnology. For glycosylation analysis, proteins were de-glycosylated in ^{18}O labeled H_2O, fractionated with RP-HPLC and analyzed with an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) at the University of Münster. Quantitative Lys acetylome analysis of WT and *nsi* plants was performed through stable dimethyl labeling of peptides combined with immunoenrichment of acetylated peptides prior to RP-HPLC fractionation and MS analysis with a Q Exactive HF mass spectrometer (Thermo Fisher) at the University of Münster (**III**).

3.6 Fluorescence measurements

The function of the photosynthetic electron transfer chain in WT and *nsi* plants was studied by measuring chlorophyll fluorescence with pulse amplitude modulated (PAM) fluorometry. Samples (leaves) were illuminated with increasing actinic light intensities and the fluorescence parameters in each condition were determined (**III**). *In vivo* state transition measurements were also performed using PAM fluorometry, but by illuminating the samples (leaves) with state 1 and state 2 lights (**III**). The 77K fluorescence emission spectra were recorded by illuminating isolated thylakoids with blue excitation light under liquid nitrogen (**III**).

3.7 Structural modeling

FNRL sequence was aligned with known FNR structures using VERTAA (Johnson and Lehtonen, 2000), and a structural model was generated with MODELLER (Šali and Blundell, 1993) using *Salmonella typhimurium* FNR structure as a template (**I**). LFNR1 and LFNR2 were modeled in complex with TIC62 peptide using pea FNR-TIC62 complex structure as a template (**II**). Structures were aligned with MALIGN (Johnson et al., 1996) and model generated with MODELLER (Šali and Blundell, 1993). Structural models were constructed by Dr. Tiina Salminen's group at the Åbo Akademi University.

3.8 Microscopy

NSI localization was studied with confocal fluorescence microscopy by Dr. Markus Witz's group at the University of Heidelberg (**III**). Chloroplast structure was studied by transmission electron microscopy at the Max Planck institute (**III**).

4 RESULTS

4.1 Characterization of a novel FNR-family protein

FNRs can be regarded as key enzymes in directing electron fluxes to important metabolic processes such as carbon fixation in plants (Hajirezaei et al., 2002) and protection against oxidative damage in bacteria (Bianchi et al., 1995). Detailed functional characterization of different FNRs is therefore essential for basic understanding of metabolic fluxes and attempts for metabolic engineering and synthetic biology. Arabidopsis genome encodes a previously uncharacterized flavoprotein (AT1G15140), which shows sequence homology to the LFNR2 isoform and contains a chloroplast targeting peptide. This protein is also conserved in photosynthetic organisms from cyanobacteria to plants (Karpowicz et al., 2011), suggesting an important role in photosynthesis related metabolism. To find out whether this "FNR-LIKE" (FNRL) protein also functions as an oxidoreductase between Fd and NADP(H) during photosynthesis, we studied the structural and catalytic properties of this protein by constructing a structural model *in silico* and expressing the protein *in vitro*. We also investigated the interaction of FNRL with different Fds and followed its expression during the day-night cycle *in planta*.

4.1.1 FNRL structure resembles bacterial-type FNRs

The structure of FNRL was studied by comparing its amino acid sequence to other known FNR-family proteins (**I**: Figure S1). The protein contained the conserved two-domain structure of plant-type FNRs, an N-terminal FAD-binding domain and a C-terminal NADP(H)-binding domain, but had some key differences from the typical plastidic-type FNRs present in photosynthetic organisms. Firstly, FNRL did not have the RX(Y/F)(S/T) motif, which is conserved in plastidic FNRs and involved in FAD binding (Ceccarelli et al., 2004). Secondly, FNRL did not contain the amino acids that form a beta-hairpin structure in plastidic FNRs (Karplus et al., 1991). This hairpin allows plastidic FNRs to bind FAD in an extended conformation, whereas bacterial FNRs, which lack this structure, bind FAD in a bent conformation. These features of the FAD binding domain strongly suggested that FNRL structure resembles bacterial-type FNRs more than plastidic-type FNRs and therefore we constructed a 3D-model of FNRL using *Salmonella typhimurium* FNR structure as a template (**I**: Figure 1). Even though the amino acid identity between the sequences was low, the produced model was shown to be accurate (**I**: Table S2). Our model

supported the notion that FNRL binds FAD in a bent conformation like bacterial FNRs. The binding environment of FAD was predicted to be hydrophobic, whereas electrostatic interactions seemed to be more important for NADP(H) binding.

4.1.2 FNRL oxidizes NADPH with a hydride transfer mechanism

To study the biochemical properties of FNRL, we expressed the protein with an N-terminal His^6-tag in *Escherichia coli*. The protein was successfully purified with a bound FAD-cofactor (**I**: Figure 2A-C, Figure S2). Circular dichroism spectra further supported the presence of the cofactor in the folded protein (**I**: Figure 2D-E) confirming that FNRL is an FAD-binding flavoprotein. To test the substrate specificity of FNRL we performed a Cyt *c* reduction assay using NADH and NADPH as substrates. FNRL was able to reduce Cyt *c* with NADPH but showed no activity with NADH (**I**: Figure S3). To establish optimal reaction conditions for more detailed kinetic measurements we measured the diaphorase activity of FNRL in different physiologically relevant pHs and varying ionic strengths using DCPIP as an electron acceptor. The activity of FNRL seemed to be relatively insensitive to mild changes in pH, with approximately 20-30% higher activity in neutral (pH 7) than in slightly basic (pH 8.2) buffer (**I**: Figure 3A). In contrast, salt concentration was critical to FNRL activity: DCPIP reduction was four times faster in a buffer containing 300 mM NaCl compared to buffer with no added salt (**I**: Figure 3B). The turnover number of FNRL in the optimal conditions (pH 7.0, 300 mM NaCl) was determined as $k_{cat} = 3.2 \pm 0.2\ s^{-1}$ and the Michaelis-constant as $K_M^{NADPH} = 2.0 \pm 0.4\ \mu M^{-1}\ s^{-1}$ (**I**: Figure 3C).

We next studied the substrate ($NADP^+$ and NADPH) binding properties and reaction mechanism of FNRL in more detail. The oxidized FNRL protein was titrated with increasing concentration of oxidized $NADP^+$ and the change in FNRL absorption spectrum was followed simultaneously. Small peaks appeared at 387, 461 and 495 nm with increasing [$NADP^+$] showing that the coenzyme binding altered the structure of the protein around the FAD cofactor (**I**: Figure 3D). However, the intensities of the peaks were relatively small indicating that only a small fraction of FNRL was capable of binding the oxidized $NADP^+$. The reaction of FNRL with reduced NADPH was measured by following FNRL absorption spectrum in anaerobic conditions with a stopped-flow instrument. The change in FNRL spectrum during the first twenty seconds suggested a reaction mechanism where NADPH and FAD first form a charge-transfer complex (CTC) after which NADPH donates two

electrons to FAD in a single hydride transfer (HT) reaction (**I**: Figure 3G-H). The rates of CTC formation and HT were 2.1 ± 0.2 and 1.0 ± 0.1 s^{-1}, respectively.

4.1.3 FNRL expression and interaction with Fds

To gain further insight to the role of FNRL in plants, we first studied the localization of the protein. To this end, we generated a peptide antibody, which showed specific binding to FNRL (**I**: Figure S6). Immunoblotting of Arabidopsis chloroplast fractions showed that the protein is a soluble chloroplast protein (**I**: Figure 4A). We also examined the binding of the recombinant FNRL to Fd with affinity chromatography. We tested binding to Arabidopsis Fd1 and a ferredoxin-like protein FdC1. Fd1 is a chloroplast Fd with high sequence homology and similar redox potential to the major photosynthetic ferredoxin Fd2 (Hanke et al., 2004). FdC1 is a ferredoxin homologue with a C-terminal extension, which is capable of accepting electrons from PSI and donating them to some Fd-dependent enzymes but not to FNR (Guan et al., 2018; Voss et al., 2011). In this assay, FNRL did not show any binding to Fd1 or FdC1, whereas the positive control (photosynthetic FNR from maize) bound readily to Fd1 (**I**: Figure 3I, Figure S4). We also studied the expression of *FNRL* during a 24-h day-night cycle. Even though the gene expression of *FNRL* was shown to increase in the middle of the light period compared to night time, the amount of the protein remained stable throughout the day (**I**: Figure 4B).

4.2 Post-translational modifications of LFNRs

Both photosynthetic LFNRs, LFNR1 and LFNR2, of Arabidopsis have been shown to be present as two forms with different pIs (Lintala et al., 2007) suggesting that Arabidopsis LFNRs might be controlled via post-translational modification(s). A similar pattern of "acidic" and "basic" LFNR forms has also been detected in wheat, where, based on the shift in the pI, the acidic forms were suggested to arise due to phosphorylation (Moolna and Bowsher, 2010). However, experimental evidence on the identity of this modification is still lacking. LFNRs from spinach and wheat have also been shown to contain variable N-termini (Gummadova et al., 2007; Karplus et al., 1984; Shin et al., 1990), but the roles of the various N-termini are not known. Interestingly, only one N-terminal sequence has been reported for both Arabidopsis LFNRs so far (Hanke et al., 2005). To shed light into the post-translational regulation of Arabidopsis LFNRs, we studied the composition of LFNR N-termini and the identity of the putative PTM(s) with a combination of electrophoretic methods and

mass-spectrometry. We also followed the dynamics of the pI altering PTM under various light intensities and its effect on Fd binding to elucidate its regulatory function.

4.2.1 LFNRs contain multiple partially N^{α}-acetylated N-termini

Because FNRs have been shown to contain alternative N-terminal sequences in other species (Gummadova et al., 2007; Karplus et al., 1984; Shin et al., 1990), we investigated the N-terminal sequences of Arabidopsis LFNR1 and LFNR2 via mass spectrometry and Edman sequencing. Arabidopsis thylakoid proteins were separated with 2D electrophoresis to distinguish the acidic and basic LFNR forms and to analyze their N-terminal sequences separately. With mass spectrometry we discovered that both enzymes, LFNR1 and LFNR2, contained three different N-terminal sequences (**II**: Table I). The shorter forms were missing only the first one or two amino acids of the longest form and all N-terminal sequences were present in both the acidic and basic forms. Edman sequencing gave results only from the basic LFNR forms: two of the shorter N-terminal sequences of LFNR1 and one of LFNR2 were detected (**II**: Table II). Unsuccessful sequencing of the acidic LFNR forms indicated that the sequencing reaction might be blocked by an N-terminal modification. N-terminal blocking might also explain why only one N-terminal sequence was obtained in a previous study for Arabidopsis LFNRs, since Edman degradation was used (Hanke et al., 2005).

After analyzing the MS data, we discovered that the acidic LFNR forms contained multiple N-terminally acetylated peptides (**II**: Table III, Supplemental Table S1), whereas only few hits for N^{α}-acetylation were found in the basic spots. We also detected some non-acetylated peptides in the acidic spots but there were less of these than their acetylated counterparts. The cross-detection was probably due to imperfect separation of the proteins during isoelectric focusing and we concluded that the appearance of the acidic FNR forms was down to N^{α}-acetylation. In line with previous observations on spinach LFNR (Karplus et al., 1984; Shin et al., 1990), we also found the presence of N-terminal pyro-glutamate. However, since pyro-glutamate can occur during sample preparation (Friso and Van Wijk, 2015), we could not distinguish whether this modification was biologically relevant and whether it occurred specifically in the acidic and/or basic forms.

4.2.2 LFNRs are Lys acetylated

Even though the existence of the acidic and basic forms was attributed to N-terminal acetylation, this did not exclude the possibility that LFNRs might contain also other PTMs. For example, both LFNRs contain surface exposed serine and threonine residues, which could function as phosphorylation sites (Lintala et al., 2007). We tried to detect the presence of phosphorylated LFNRs with immunoblotting with three different antibodies, with phosphoprotein staining and with phosphatase/phosphatase inhibitor treatments combined with Phos-tag or 2D gel electrophoresis but could not find any evidence of phosphorylation (**II**: Figure S2). LFNRs also contain a putative N-glycosylation site (NXS/T, An et al. (2009)) (**II**: Figure 1, Figure S1). However, we did not find any conclusive evidence of FNR glycosylation with immunoblotting, glycoprotein staining or mass spectrometry (**II**: Figure S3, Supplemental Table S2). We also checked for the existence of acetylated or methylated peptides from our MS data. We discovered two acetylated lysine residues from both LFNR1 and LFNR2 (**II**: Table IV, Figure 1). In order to study the location of the acetylated residues we constructed a 3D model of an LFNR1-LFNR2 heterodimer in complex with the membrane anchoring TIC62 peptide based on the crystal structure from pea (Alte et al., 2010) (**II**: Figure 3). One acetylation site (K321 and K330 of LFNR1 and LFNR2, respectively) was located in a Lys residue that occupies the same position in the 3D structure of both proteins close to the active site. The other acetylation sites (K175 of LFNR1 and K96 of LFNR2) were located on stroma exposed Lys residues.

4.2.3 N-terminal acetylation of LFNRs is light responsive and may affect Fd binding

To study the effect of the most prominent PTM, N^{α}-acetylation, to LFNR function, we tested whether both forms interact similarly with Fd. We subjected Arabidopsis leaf homogenates to Fd-affinity chromatography with two different chloroplast Fds (Fd1 and Fd2) and separated the bound proteins with 2D-electrophoresis. Fd2 is the main Fd isoform functioning in photosynthesis (Hanke et al., 2004). Fd1 is structurally very similar to Fd2 and also present in photosynthetic tissues (Hanke et al., 2004), but might instead be involved in the induction of cyclic electron flow around PSI (Lehtimäki et al., 2010). Surprisingly, both Fds clearly bound the acidic LFNRs more efficiently (**II**: Figure 5).

LFNRs are known to be present both as membrane bound and as soluble proteins. LFNRs bind to the thylakoids in dark and are released to the stroma in light (Benz et al., 2009). To investigate whether N-terminal acetylation affects the light regulated membrane binding of LFNR, we studied the distribution of the acidic and basic forms between the stroma and thylakoids in different light conditions (dark, growth light and high light) with 2D electrophoresis. All FNR forms were present in thylakoids and the stroma in all conditions, but there was an increase in the ratio of basic to acidic (non-acetylated to acetylated) protein with increasing light intensity at the thylakoids (**II**: Figure 6). The most striking difference, however, was the relative amount of LFNR1 compared to LFNR2 in the stroma: soluble LFNR1 was clearly the dominant isoform in dark and LFNR2 in the light.

4.3 Searching for chloroplast acetyltransferases

An important part of unraveling how PTMs regulate the function of their target proteins is identifying the enzymes performing the addition and removal of the modification in question. Arabidopsis genome has been shown to encode several potentially chloroplast localized acetyltransferase proteins (Bienvenut et al., 2012; Dinh et al., 2015). We screened homozygous T-DNA lines of the candidate genes and separated their thylakoid proteins with 2D-electrophoresis in the search for putative NAT mutants with alterations in the accumulation of N-terminally acetylated LFNRs (Koskela et al., unpublished). None of the mutants were clearly lacking the N-terminally acetylated LFNRs, but one line, a mutant of the gene *NUCLEAR SHUTTLE INTERACTING* (*NSI*; AT1G32070), seemed to have some changes in the thylakoid proteome compared to WT (Koskela et al., unpublished). NSI has previously been proposed to be a nuclear Lys acetyltransferase (McGarry et al., 2003), but it has also been suggested to be localized in the chloroplasts where it was suggested to function as a serotonin N-acetyltransferase (Lee et al., 2015). These observations gave us reason to hypothesize that NSI might act as a Lys acetyltransferase in chloroplasts. To investigate the role of NSI in Arabidopsis further, we studied the localization of the protein with the aid of a fluorescently tagged protein and verified its Lys acetyltransferase activity *in vitro*. Thereafter, we identified putative acetylation targets of NSI by comparing the Lys acetylomes between WT and *nsi* knock-out plants and performed photosynthetic characterization of the mutant plants.

4.3.1 NSI is a chloroplast Lys acetyltransferase

To test whether NSI is capable of functioning as a chloroplast Lys acetyltransferase, we first studied its activity and localization. We expressed a His^6-NSI recombinant protein in *E. coli* and incubated the purified enzyme with an artificial peptide substrate. Acetylation of the peptide was followed by separating the substrate and acetylated end product with liquid chromatography. NSI showed clear Lys acetyltransferase activity, while no background reactivity was observed in the assay (**III**: Figure 1C-D). We confirmed the subcellular localization of the protein by following the accumulation of a recombinant NSI-YFP in Arabidopsis protoplasts. The YFP fluorescence signal overlapped with chlorophyll fluorescence, which is strong evidence for chloroplast localization (**III**: Figure 1A). We also isolated intact chloroplasts form NSI-YFP expressing plants and fractionated them into thylakoids and a soluble fraction. Immunoblotting with GFP antibody showed that the protein was mainly localized in the soluble compartment (**III**: Figure 1B). Based on these results we concluded that NSI can function as a chloroplast Lys acetyltransferase.

To uncover the *in vivo* acetylation targets of NSI, we employed quantitative mass spectrometry. We screened two homozygous T-DNA knock-out lines *nsi-1* and *nsi-2* that did not express *NSI* RNA (**III**: Figure 2A-B) and compared their Lys acetylomes to WT. Several chloroplast proteins had altered Lys acetylation levels in the mutants, but a few proteins showed strikingly strong downregulation in the following Lys acetylation sites (**III**: Figure 2C, Supplemental Dataset 1): K168/170 of KEA1/2, K62 of an unknown protein (AT2G05310) and K88 of PSBP. KEA1 and 2 are inner envelope K^+/H^+ antiporters that export K^+ from the chloroplast (Kunz et al., 2014) and PSBP is a subunit of the oxygen evolving complex of PSII (Ido et al., 2014). AT2G05310 is an uncharacterized protein with a predicted chloroplast targeting peptide. Lys acetylation was also downregulated in the light harvesting protein LHCB1.4 (K40), iron binding protein FER1 (K134) and PSI subunit PSAH (K138), although to a lesser extent. There were no major alterations in the protein abundances between the plants (**III**: Figure 2D), demonstrating that the observed changes in acetylation were specific and not due to a change in the abundance of the target proteins. Additionally, some acetylation sites seemed to be upregulated in the mutants (**III**: Figure 2C, Supplemental Dataset 1) suggesting that NSI is not the only Lys acetyltransferase in chloroplasts.

4.3.2 NSI is required for state transitions

To study the effect of NSI to photosynthesis, we further characterized the *nsi* knock-out lines. The mutants grew similarly to WT in our growth conditions (**III**: Figure 2B) but had a slightly lower chlorophyll *a*/*b* ratio (**III**: Table 1). To get a general idea of the function of the ETC in *nsi* plants, we measured chlorophyll fluorescence in different light intensities (**III**: Supplemental Dataset 3). We observed a significant difference in the parameter for photochemical quenching (qL) under low light intensities (<100 μE), where *nsi* plants seemed to have problems keeping the PSII reaction centers oxidized (a low qL is indicative of over-reduced PSII). To investigate the distribution of excitation energy between the photosystems in different light conditions, we isolated thylakoids from dark and growth light adapted plants and measured fluorescence spectra in 77K temperature. In this temperature thylakoids give two fluorescence peaks, one originating from PSII (around 685 nm) and one originating from PSI (around 735 nm), and the intensities of the peaks are proportional to the amount of light energy received by the photosystems. From the result (**III**: Figure 4A) it was clear that in growth light intensities the *nsi* plants had less light energy going to PSI than WT.

State transitions, the movement of L-LHCII antenna trimers between PSII and PSI, are the main mechanism by which plants adjust the distribution of excitation energy between the photosystems under low light intensities. Thus, it seemed conceivable that *nsi* might be impaired in state transitions. To confirm our hypothesis, we studied the functionality of state transitions in *nsi* plants with several different methods. We included a known state transition mutant *stn7* as a control. STN7 is the LHCII kinase that induces transition from state 1 to state 2 under low white light intensities or state 2 light treatment. First, we treated the plants with lights that are specifically known to induce state 1 (far red light) or state 2 (red light) and repeated the 77K experiment. The results were in line with the previous measurements and showed that after state 2 illumination, *nsi* plants, like *stn7*, had less excitation energy going to PSI compared to WT (**III**: Figure 4B). We additionally conducted an *in vivo* fluorescence measurement to follow the state transitions in intact plants and again *nsi* behaved like *stn7* (**III**: Figure 4C). Under state 2 conditions WT plants accumulate a relatively stable complex between PSI and LHCII, which can be visualized with blue native gel electrophoresis. We studied the composition of the thylakoid protein complexes with this method and found that, as expected, both *nsi* and *stn7* were lacking this complex in growth light (**III**: Figure 3B, left panel). To see how this change was

reflected in thylakoid and chloroplast structure we also investigated *nsi* chloroplast structure with transmission electron microscopy. The overall chloroplast morphology was similar between WT and *nsi* (**III**: Figure 3C), but the average height of membrane layers in *nsi* grana was slightly lower (**III**: Figure 3D).

Several factors are known to affect state transitions but the most important requirements for the formation of the PSI-LHCII complex are thought to be LHCII phosphorylation by STN7 and the presence of an intact LHCII docking site at PSI. To exclude the possibility that *nsi* would be impaired in these areas, we studied the LHCII phosphorylation status and PSI subunit composition with immunoblotting. Phosphorylated LHCII can be visualized with a general phospho-threonine antibody from thylakoid extracts. When we blotted the state 1 and 2 light treated thylakoid proteins with the P-Thr antibody, we could clearly see that LHCII was phosphorylated equally in WT and *nsi* in state 2, whereas in the control line *stn7* the phosphorylation was missing (**III**: Figure 5B, left panel). LHCII has been shown to dock to PSI via the PSAH subunit in state 2 (Lunde et al., 2000). However, in the immunoblot analysis this protein seemed to accumulate equally well in *nsi* as in WT (**III**: Figure 5D). The same was true for the PSI antenna protein LHCA4 (**III**: Figure 5D), which has also been suggested to dock LHCII antennas in state 2 (Benson et al., 2015).

5 DISCUSSION

5.1 Functional implications of FNRL structure and activity

In study **I**, we investigated the structural and catalytic properties of Arabidopsis FNRL protein, which is a distant homologue of Arabidopsis LFNR2. Based on amino acid sequence comparison and structural modeling, FNRL was shown to resemble bacterial FNRs more than plastidic FNRs (**I**: Figures 1 and S1; see also Figure 5 of this thesis). One of the most distinguishing features between the two types of FNRs is the way they bind their FAD cofactor. In plastidic FNRs, FAD is bound in an elongated conformation, which is stabilized by a beta-hairpin structure (Karplus et al., 1991). This structure was absent from the FNRL sequence, including another conserved sequence motif RX(Y/F)(S/T), which is important for FAD binding in plastidic FNRs (Ceccarelli et al., 2004). Accordingly, our structural model supported the notion that FNRL binds FAD in a folded conformation, similar to bacterial FNRs (**I**: Figure 1). The binding mode of FAD is related to the catalytic efficiency of FNRs: plastidic FNRs are characterized by high turnover rates of several hundred s^{-1} (Carrillo and Ceccarelli, 2003), whereas bacterial FNRs show rates as low as <1 s^{-1} (Wan and Jarrett, 2002). Another conserved feature of plastidic FNRs is the C-terminal tyrosine moiety, which in FNRL is replaced by phenylalanine (**I**: Figure S1). The presence of C-terminal Tyr is also associated with high catalytic efficiency (Nogués et al., 2004). The measured DCPIP diaphorase activity of FNRL (3.2 ± 0.2 s^{-1}) and reduction by NADPH (1.0 ± 0.1 s^{-1}) were in line with the structural predictions, confirming that FNRL resembles bacterial FNRs.

Like all plant-type FNRs (Ingelman et al., 1997; Tejero et al., 2003), FNRL showed strong preference towards NADP(H) over NAD(H) (**I**: Figure S3). However, FNRL was able to reduce Cyt *c* in the absence of Fd (**I**: Figure S3), which is thought to be required for Cyt *c* reduction by plastidic FNRs in this assay (Arakaki et al., 1997), and it failed to interact with a leaf-type ferredoxin Fd1 (**I**: Figure 3I). Additionally, FNRL showed only a weak interaction with $NADP^+$ (**I**: Figure 3D), but was readily reduced by NADPH (**I**: Figure 3G-H). All in all, the low catalytic efficiency of FNRL and its substrate binding properties strongly suggest that FNRL does not function in electron transfer from photosynthetic Fds towards $NADP^+$. Instead, FNRL more likely catalyzes electron transfer from NADPH to an acceptor. The *in vivo* interaction partner is not likely to be either of the leaf-type ferredoxins (Fd1 or Fd2) because the

redox potentials of these proteins do not favor electron transfer in this direction (Hanke et al., 2004) and because FNRL did not bind to Fd1 (**I**: Figure 3I). The ferredoxin-like protein FdC1 is also an unlikely partner since it failed to interact with FNRL (**I**: Figure S4).

Because plant-type FNRs are only known to interact with Fds or Flds (Ceccarelli et al., 2004), but Flds are not present in vascular plants (Zurbriggen et al., 2007), few possible interactors remain. In addition to the photosynthetic Fds and the FdC1 protein, the Arabidopsis genome encodes two Fds (Fd3 and Fd4) and one ferredoxin-like protein, FdC2 (Hanke et al., 2004). Fd3 is mostly present in roots but also in low amounts in leaves, whereas Fd4 is a low abundance leaf protein (Hanke et al., 2004). Both of these Fds have more positive redox potentials compared to the photosynthetic Fds and could thus function as electron acceptors for FNRL. Fd4 differs from the other Fds in few key aspects, which make it an interesting candidate for being an FNRL interactor. Firstly, Fd4 shows weaker interaction with L- and RFNRs compared to the other Fds and it is unable to function in the photoreduction of $NADP^+$ unlike the other Fds (Hanke et al., 2004). Secondly, Fd4 is only a distant homologue of the other Fds and its strikingly high redox potential resembles that of some bacterial [2Fe-2S] proteins (Hanke et al., 2004). Moreover, the physiological role of Fd4 has not been studied so far. Fd3, on the other hand, is regarded as the *in vivo* redox partner of RFNRs based on its structure, activity and localization (Hanke et al., 2004; Onda et al., 2000). The last possible interactor is the FdC2, which was recently shown to be targeted to chloroplasts in rice (Li et al., 2015), but has otherwise not been functionally studied. In the future, interactions between FNRL and the remaining Fds need to be investigated to establish whether FNRL interacts with Fds or Fd-like proteins or whether it has a non Fd/Fld substrate. Identifying the interaction partner(s) of FNRL will be necessary to establish its physiological function in chloroplasts. It will be interesting to see whether FNRL has similar roles as bacterial FNRs, such as providing tolerance against oxidative stress (Bianchi et al., 1995), or whether its function has diverged.

5.2 N-terminal modifications and Lys acetylation are conserved features of plant LFNRs

In study **II**, we showed that Arabidopsis LFNR1 and 2 contain multiple post-translational modifications. Both proteins were found to contain multiple N-terminal sequences (**II**: Tables I and II), partial N-terminal acetylation (**II**: Table III and

Supplemental Table S1) and acetylated Lys residues (**II**: Table IV). We did not find any evidence of phosphorylation or glycosylation despite using multiple methods (antibody detection, electrophoretic mobility and mass spectrometry) (**II**: Supplemental Figure S2). Some phosphorylated peptides have however been assigned to LFNRs in studies that have focused specifically on phosphoproteomics (Reiland et al., 2009; Roitinger et al., 2015; Schönberg et al., 2017). This discrepancy is most likely due to the fact that phosphorylation in these sites is low in abundance and requires enrichment of the phosphopeptides prior to analysis, which is common for PTMs (Olsen and Mann, 2013). Alternatively, it may be that LFNR phosphorylation is only induced under particular circumstances and simply was not present in our growth conditions. Hence, we concluded that the major PTMs of LFNRs in standard conditions are N-terminal trimming, N-terminal acetylation and Lys acetylation, although our data does not exclude the existence of other modifications. We also concluded that the appearance of the acidic LFNR form after isoelectric focusing was due to N-terminal blocking by acetylation (**II**: Table III, Supplemental Table S1) and not phosphorylation as previously suggested (Moolna and Bowsher, 2010). A similar pattern has previously been reported for the ATPase ε-subunit, where N-terminal acetylation was also shown to lead to a more negative pI (Hoshiyasu et al., 2013).

Since N-terminal trimming of LFNR has been found also in spinach (Karplus et al., 1984; Shin et al., 1990) and wheat (Gummadova et al., 2007), it would seem that it is a conserved feature of LFNRs at least in flowering plants. N-terminal and Lys acetylation of LFNR were detected for the first time in this study, and it will be interesting to see whether they are also conserved. The existence of the similar isoelectric focusing pattern of wheat LFNRs (Moolna and Bowsher, 2010) compared to Arabidopsis (**II**: Figure 2; Lintala et al., 2007) would suggest that this is the case at least for N-terminal acetylation. Interestingly, in a recent study of rice Lys acetylome, K327 of one of the rice LFNR isoforms was shown to be acetylated (Xiong et al., 2016); this residue corresponds to the K321/330 of Arabidopsis LFNR1/2 identified in our study (**II**: Figure 1 and Supplemental Figure 1). This suggests that also Lys acetylation could be a conserved feature of LFNRs. Whether N-terminal and Lys acetylation of LFNRs serve similar purposes in all species and when these modifications have emerged in evolution will be interesting topics for future research.

5.3 Acetylation can alter the interactions between LFNRs and other proteins

Because both N-terminal (Scott et al., 2011) and Lys acetylation (Choudhary et al., 2009) have been shown to affect protein-protein interactions, we investigated the effect of these PTMs on the membrane tethering of LFNRs. N-terminally acetylated and non-acetylated forms were found to be abundant both in the soluble and thylakoid proteomes (**II**: Figure 2) demonstrating that this modification does not dictate membrane binding. However, an increase in the non-acetylated/acetylated ratio with increasing light intensity was detected in the thylakoid bound LFNR pool (**II**: Figure 6), which indicates that N-terminal acetylation may affect at least some of the molecular interactions required for LFNR membrane binding even if it is not a major regulator. All N-terminal sequence variants were also detected in both the membrane bound and soluble LFNR pools (**II**: Supplemental Table S1). N-terminal sequences would therefore seem to have a different role in Arabidopsis as compared to maize, where the N-terminal regions of the different LFNR isoforms have been shown to dictate the membrane binding properties of the different isoforms (Twachtmann et al., 2012).

In order to investigate the structural position of the found Lys acetylation sites, we constructed a 3D model of LFNR1 and LFNR2 bound to the TIC62 peptide, which functions as the membrane binding site for LFNRs (Küchler et al., 2002) (**II**: Figure 3). None of the acetylation sites collided with the LFNR-TIC62 interaction surface or the LFNR-LFNR interface suggesting that Lys acetylation does not directly regulate LFNR membrane binding. It is however possible that the surface exposed acetylation sites might affect the binding of other proteins present in the membrane bound LFNR complexes such as the recently discovered LIR protein (Yang et al., 2016). According to the current view, the degradation of LIR in the light leads to the release of LFNR from the thylakoid membranes (Yang et al., 2016) but whether the detachment of LIR from LFNR is required prior to its degradation and how the interaction between the two proteins is regulated is not known. Unfortunately, LFNR abundance in the soluble proteome was too low to detect PTMs reliably in our set-up, so we were not able to assess the relative abundances of this modification between the thylakoid bound and soluble proteomes. This would require for example the enrichment or purification of LFNR from the soluble fraction or making site directed mutagenesis to this residue and investigating its effect on the accumulation of the thylakoid bound complexes.

Fd is the electron transfer partner of LFNRs *in vivo*, and thus the regulation of this interaction could have significant impact on electron allocation in chloroplasts. Hence, we investigated how N^{α}- and N^{ε}-acetylation affect Fd binding of LFNRs. To our surprise, the acidic (N^{α}-acetylated) LFNR forms bound significantly more to both Fd1 and Fd2 (**II**: Figure 5). Because N-terminal acetylation is regarded irreversible (Drazic et al., 2016), it would mean that part of LFNR in the leaves is present in a "low affinity" state (i. e. non-N^{α}-acetylated) and could be activated by N-terminal acetylation into a "high affinity" Fd-binding state. On the other hand, it is possible that N-terminal acetylation promotes Fd binding in our experimental conditions (binding was performed in low ionic strength), which are significantly different from physiological conditions. It is also possible that the non-acetylated protein is less stable during the assay, which would lead to an apparent increase in the affinity of the acetylated form due to an increase in its relative abundance. Additional experiments will be required to clarify this significant point. The effect of Lys acetylation to Fd binding was examined with the help of the constructed 3D model (**II**: Figure 3). Interestingly, one of the acetylation sites, K321 in LFNR1 and K330 in LFNR2, was found to occupy a homologous position in the 3D structures close to the Fd binding site. Even though the site was not directly on the LFNR-Fd interaction surface, acetylation of this residue could still alter the relative orientation of important amino acid residues at the active site and thus affect the Fd binding environment or influence the electron transfer rate. Importantly, since acetylation of this residue was shown to be conserved also in rice (Xiong et al., 2016), it seems likely that this residue could serve a regulatory role. It will be interesting to see whether acetylation of this site can affect the Fd reduction rate and Fd binding affinity of LFNRs.

5.4 NSI has diverse acetylation targets

In study **III**, we have shown that NSI is an active chloroplast Lys acetyltransferase (**III**: Figure 1) which targets several chloroplast proteins (**III**: Figure 2C, Supplemental Dataset 1). By studying the Lys acetylomes of two *nsi* knock-out lines compared to WT we found three proteins with strikingly downregulated Lys acetylation sites: K168/170 of the inner envelope K^{+}/H^{+} antiporters KEA1/2, respectively (both share an identical peptide at the acetylation site making differentiation between the proteins impossible), K62 of an unknown chloroplast protein (AT2G05310) and K88 of the oxygen evolving complex subunit PSBP (**III**:

Figure 2C, Supplemental Dataset 1). In the lack of structural data on KEA1/2 and the unknown protein it is not possible to make functional predictions of the effect of the acetylation. In the case of PSBP, however, the affected acetylation site (K88 of the pre-protein, K11 of the mature protein) is situated in a Lys residue which has been shown to be crucial for ion binding and hence to the function of the OEC (Ifuku et al., 2008). Since this residue is functionally so important and highly conserved from green algae to plants (Ifuku et al., 2008), acetylation of the site is likely to have functional implications. Acetylation could, for example, directly affect the ion binding properties of PSBP due to removing the positive charge from the Lys residue. It is intriguing that PSBP is a lumenal protein, whereas NSI does not contain a lumen targeting sequence and hence most likely resides in the stroma. This would suggest that PSBP gets acetylated before its import to the lumen and assembly to the OEC. Hence, it is unlikely that this acetylation site is regulated dynamically, but it is more likely to be a static modification in a subpopulation of PSBP.

5.5 Why is NSI required for state transitions?

Because PSBP is essential for the function of PSII, we expected to find alterations in the function of PSII. Interestingly, the maximum capacity of PSII was unaffected in *nsi* mutants (**III**: Table 1). Instead, we found that the plants were defective in state transitions and permanently locked in state 1 regardless of light conditions (**III**: Figure 3B, Figure 4 and Table 1). LHCII phosphorylation by STN7 kinase has been shown to be the major regulator of state 1 to state 2 transition (Bellafiore et al., 2005; Bennett et al., 1980). However, based on phosphorylation analysis of thylakoid proteins, LHCII phosphorylation in *nsi* was not lowered but even slightly upregulated (**III**: Figure 5A-C) ruling out a possible defect in STN7 function in *nsi*. During state 2 L-LHCII interacts with PSI via the PSAH subunit or the LHCI antenna, which are situated on the opposite sides of PSI (Benson et al., 2015; Lunde et al., 2000). *nsi* plants contained equal amounts of PSAH and LHCA4 compared to WT (**III**: Figure 5D) showing that the accumulation of the docking site proteins was not affected. However, we did observe a slight decrease in the acetylation of K138 of PSAH (**III**: Figure 2C, Supplemental Dataset 1). If this residue is important for the interaction between L-LHCII and PSAH, the decreased acetylation of this site could affect state transitions. Interestingly, state transitions in *nsi* seem to be affected almost to a similar extent as in the *psah* cosuppression line: the relative fluorescence change F_r was around 20% of the WT value in *psah* (Lunde et al., 2000) whereas in

nsi it was 24% (**III**: Table 1). In contrast, Benson et al. 2015 reported the state transition quenching parameter qT to be around 30% of the WT value in the LHCI mutant *lhca4*, which was the most affected one in state transitions out of the *lhca1-4* single mutants, (Benson et al., 2015) while in *nsi* qT was only 10% of WT (**III**: Table 1). Thus, the extent to which state transitions are reduced in *nsi* would quantitatively fit to an impaired interaction between L-LHCII and PSAH. This hypothesis is further supported by the fact that *nsi* specifically lacked the PSI-LHCII supercomplex (**III**: Figure 3B) which consists of one L-LHCII attached to PSI via the PSAH docking site (Galka et al., 2012).

In addition to LHCII phosphorylation and PSI docking site, an intact LHCII antenna seems to be crucial for optimal state transitions. For example, the mutant *ch1-2*, which has reduced chlorophyll *b* accumulation compared to WT (Espineda et al., 1999), lacks state transitions (Wang and Grimm, 2016). However, due to the lack of chlorophyll *b* these mutants also accumulate less LHCII compared to LHCI (Espineda et al., 1999; Wang and Grimm, 2016). Consequently, the PQ pool in these mutants remains constantly oxidized preventing the activation of STN7, which requires reduced PQ (Horton and Black, 1980; Vener et al., 1997), and no LHCII phosphorylation occurs (Wang and Grimm, 2016). These mutants are in fact permanently in a state 2 resembling state, where PSI absorbs more light and keeps the PQ pool oxidized and are thus different from *nsi*, which are locked in state 1. Another Arabidopsis mutant, a cpSRP43 lacking line called *chaos* (Klimyuk et al., 1999), has also been identified as lacking state transitions despite having clearly observable LHCII phosphorylation (Wang and Grimm, 2016). In these mutants, the amounts of both LHCI and LHCII are reduced by more than half compared to WT and, like *nsi*, they are also locked in state 1 (Wang and Grimm, 2016). It was hypothesized that the reduced LHCII amount resulted in the specific loss of the mobile L-LHCII pool (Wang and Grimm, 2016), which is required for state transitions to occur (Galka et al., 2012; Wientjes et al., 2013). However, LHCII accumulation in *nsi* was no different from WT: the total amount of LHCII was unchanged when whole thylakoids were solubilized with DM and run on BN-PAGE (**III**: Figure 3A) and no changes in LHCII subunit amounts were detected in our MS analysis (**III**: Supplemental Dataset 2). The chlorophyll *a*/*b* ratio in *nsi* plants was also lowered only marginally (**III**: Table 1) suggesting that the total pigment-protein accumulation was similar between *nsi* and WT. However, the immunoblots with LHCB1 and LHCB2 antibodies gave lower signals from *nsi* than WT (**III**: Figure

5C). It is possible that the LHCB1 and 2 antibodies bind less efficiently to the phosphorylated proteins and therefore give lower signal in *nsi* where both proteins are hyperphosphorylated (**III**: Figure 5C). LHCB1 and 2 hyperphosphorylation on the other hand is probably a consequence of increased STN7 activity in *nsi* plants due to their overreduced PQ pool as seen from the low value of qL (**III**: Supplemental Dataset 3). In conclusion, the observed state transition phenotype of *nsi* is not likely due to problems in the overall accumulation of LHCII antenna trimers.

Even though LHCII phosphorylation has been well documented to regulate state transitions, the exact mechanism how phosphorylation causes the detachment of L-LHCII from PSII and its attachment to PSI is not known (Allen, 2017). According to the "molecular recognition model" (Allen, 2017; Allen and Forsberg, 2001), phosphorylation regulates the interaction between LHCII and the photosystems directly. A mechanism has been proposed where the phosphorylation of the threonine residue close to the N-terminus screens the positive charges of the nearby positive Lys and Arg residues allowing the N-terminal region to assume an alpha helical conformation (Allen, 2017). The structural ordering of the N-termini would thereafter either enhance the interaction between LHCII and PSI or detach LHCII from PSII (Allen, 2017). Interestingly, we observed a decrease in the acetylation of K40 of the LHCB1.4 protein (**III**: Figure 2C, Supplemental Dataset 1), which is a subunit of the L-LHCII trimers (Galka et al., 2012). This residue is the fifth amino acid of the mature protein in Arabidopsis. Even more intriguing is the fact that LHCB1.4 is the only LHCB1 protein that lacks the T3 moiety that undergoes phosphorylation upon state transitions (Leoni et al., 2013). Therefore, acetylation of K5 might serve the same function in LHCB1.4 as phosphorylation does in the other isoforms: enabling a conformational shift between unordered and ordered N-terminus by removing some of the closely spaced positive charges. On the other hand, phosphorylation of LHCB2 has been shown to be the primary modification required for state transitions (Crepin and Caffarri, 2015; Leoni et al., 2013; Pietrzykowska et al., 2014) and it has been suggested to directly mediate the interaction between LHCII and PSAH (Crepin and Caffarri, 2015) arguing against the role of LHCB1.4. In addition, LHCB1.4 is probably not present in all L-LHCII trimers, so it is hard to reconcile how the lack of LHCB1.4 acetylation could lead to such a severe impairment of state transitions. Regardless, the impact of LHCB1.4 acetylation on LHCII structure and localization deserves future attention.

In addition to the possible role that phosphorylation has in determining the local structure of LHCII trimers, LHCII phosphorylation also has a global effect on the surface charge of thylakoid membranes (Barber, 1980). Because attractive Van der Waals interactions between LHCII trimers across the stromal gap (Chow et al., 1991) and electrostatic screening of the negative charges of thylakoid proteins (Barber, 1980) are major driving forces of grana formation, the extensive phosphorylation may alter the ultrastructure of the whole thylakoid membrane network. Indeed, grana stacks undergo structural changes in less than 10 minutes upon changes in illumination, concomitant with state transitions (Chuartzman et al., 2008; Rozak et al., 2002). Therefore, it seems reasonable to assume that at least some of the changes could be driven by LHCII phosphorylation (Barber, 1980; Rozak et al., 2002). However, since *nsi* and *psah* undergo full LHCII phosphorylation (**III**: Figure 5A-C; Lunde et al., 2000) but are still incapable of state transitions, the possible changes in grana stacking caused by phosphorylation are clearly not enough to induce state transitions. Indeed, according to the "electrostatic screening model" (Barber, 1980), ions also play a significant role in thylakoid stacking and the lateral segregation of PSII from PSI by altering the extent of electrostatic repulsion between the negatively charged proteins and membrane surfaces. Intriguingly, one of the most downregulated acetylation sites was found in KEA1/2 (**III**: Figure 2C, Supplemental Dataset 1). KEAs have been shown to be important for chloroplast ion balance and the formation of proton motive force (Kunz et al., 2014) and could therefore contribute to the electrostatic screening and stacking of thylakoid membranes. We also found that the acetylation of PSBP was heavily downregulated in *nsi* (**III**: Figure 2C, Supplemental Dataset 1). PSBP is also associated with ion binding, although on the lumenal side, and has in fact been shown to be required for PSII stability and normal thylakoid stacking (Yi et al., 2009). Considering the above, it is possible that state transitions in *nsi* are hampered by one or several factors that prevent proper thylakoid dynamics needed for state transitions to occur. Accordingly, we found that the thylakoid stacks in *nsi* were more tightly packed than in WT (**III**: Figure 3C-D).

In conclusion, several plausible explanations exist for the lack of state transition in *nsi* mutants and regardless of whether state transitions are driven according to the "molecular recognition model" (Allen, 2017; Allen and Forsberg, 2001) or the "electrostatic screening model" (Barber, 1980), or a combination of the two, it is clear that NSI is required for the process. In addition to the discussed sites and mechanisms, one of the most downregulated acetylation sites was situated in an

uncharacterized chloroplast protein (**III**: Figure 2C, Supplemental Dataset 1). Thus, the possibility remains that this protein has a yet undiscovered role in state transitions. It is also possible that the defect in state transitions in *nsi* results from a cumulative effect of all the downregulated acetylation sites or that NSI has additional acetyltransferase activities besides Lys acetylation that could affect state transitions. Investigating how acetylation affects the function of each of the target proteins will be essential to unraveling the link between NSI and state transitions and might shed more light into the mechanism of state transitions.

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