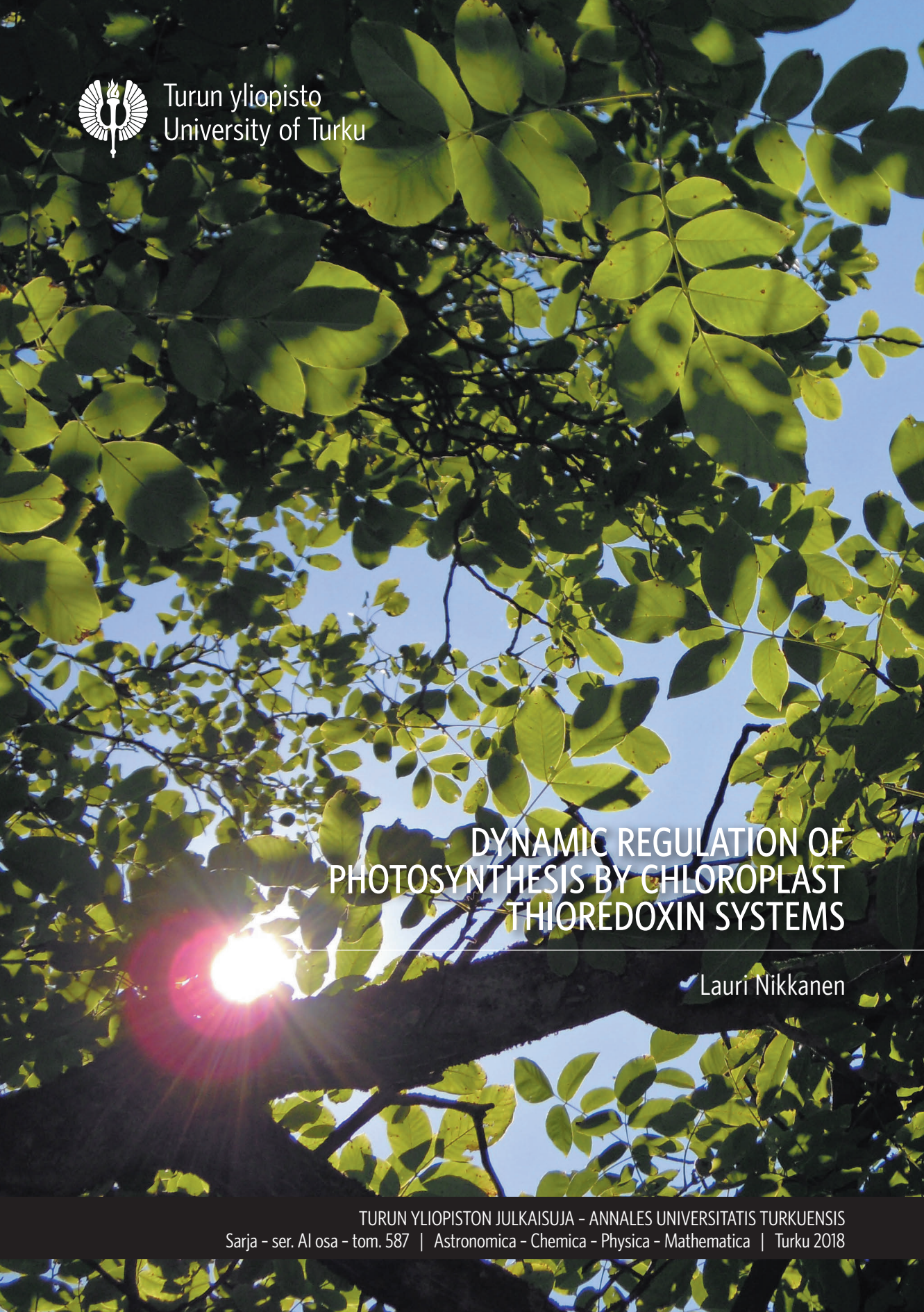

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Lauri Nikkanen

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Nikkanen L, Toivola J, Guinea Diaz M, Rintamäki E (2017) Chloroplast thioredoxin systems: prospects for improving photosynthesis. Philosophical Transactions of the Royal Society B Biological Sciences 372: 20160474.

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ABBREVIATIONS

1-qP	Parameter to estimate excitation pressure at the plastoquinone pool
2-Cys Prx	2-Cysteine peroxiredoxin
ADP	Adesonine diphosphate
AGPase	ADP-glucose-pyrophosphorylase
AMS	4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
Ax	Antheraxanthin
BiFC	Bimolecular fluorescence complementation
CBC	Calvin–Benson cycle
CET	Cyclic electron transfer
$CF_1\gamma$	γ-subunit of the chloroplast ATP synthase
Chl	Chlorophyll
Co-IP	Co-immunoprecipitation
Cys	Cysteine residue
Cyt b_6f	Cytochrome b_6f complex
DTT	Dithiothreitol
ECS	Electrochromic shift
FAD	Flavin adenine dinucleotide
FBPase	Fructose-1,6-bisphosphatase
Fd	Ferredoxin
Fd-TRX	Ferredoxin-dependent thioredoxin system
Fm	Maximal chlorophyll a fluorescence
FNR	Ferredoxin-$NADP^+$ oxidoreductase
FQR	Ferredoxin-plastoquinone reductase
FR	Far-red light
FTR	Ferredoxin-thioredoxin reductase
g_{H^+}	Parameter for proton conductivity of the thylakoid membrane
GLUTR	Glutamyl-tRNA reductase

GRX	Glutaredoxin
GSH	Glutathione
HCF164	High chlorophyll fluorescence 164
HPLC	High-performance liquid chromatography
LET	Linear electron transfer
LHCI/II	Light harvesting complex of PSI and PSII
LTO1	Lumen thiol oxidoreductase 1
MAL-PEG	Methoxypolyethylene glycol maleimide
MS	Mass spectrometry (or Murashige–Skoog medium)
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NDH	NADH dehydrogenase-like complex
NEM	N-ethylmaleimide
NIR	Near-infrared
NPQ	Non-photochemical quenching
NTR	NADPH-thioredoxin reductase
NTRC	Chloroplast NADPH-dependent thioredoxin reductase
OEC	Oxygen-evolving complex
OPPP	Oxidative pentose phosphate pathway
PC	Plastocyanin
PETC	Photosynthetic electron transfer chain
PGR5	Proton gradient regulation 5
PGRL1	PGR5-like 1
P_i	Inorganic phosphate
PIFR	Post-illumination fluorescence rise
pmf	Proton motive force
PQ	Plastoquinone
PQH_2	Plastoquinol
PRK	Phosphoribulokinase
PSI	Photosystem I
PSII	Photosystem II
PTOX	Plastid terminal oxidase

qE	Energy-dependent component of non-photochemical quenching
qH	Lipocalin-dependent component of non-photochemical quenching
qI	Photoinhibition-dependent component of non-photochemical quenching
Q_i	Plastoquinone reduction site in cytochrome *b*$_6$*f*
Q_o	Plastoquinol binding site in cytochrome *b*$_6$*f*
qT	State transition-dependent component of non-photochemical quenching
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SBPase	Seduheptulose-1,7-bisphosphatase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOQ1	Suppressor of quenching 1
TCA	Trichloric acid
TR	Thioredoxin reductase
TRX	Thioredoxin
VDE	Violaxanthin de-epoxidase
Vx	Violaxanthin
WT	Wild-type
Y(I)	Quantum yield of photosystem I
Y(II)	Quantum yield of photosystem II
Y(NA)	Acceptor side limitation of photosystem I
Y(ND)	Donor side limitation of photosystem I
YFP	Yellow fluorescent protein
Y_Z	Tyrosine Z in D1 protein
ZE	Zeaxanthin epoxidase
Zx	Zeaxanthin
ΔpH	pH-component of the proton motive force
$\Delta\psi$	Electric field component of the proton motive force

ABSTRACT

Oxygenic photosynthesis is sunlight-energized conversion of CO_2 into carbohydrates using electrons extracted from water. It occurs in cyanobacteria and in their endosymbiotic evolutionary descendants, the chloroplasts of plants and algae, and enables the existence of most ecosystems on Earth. Electron transfer from water to ferredoxin produces NADPH and generates an electrochemical proton gradient across the thylakoid membrane, which is utilized to power the ATP synthase. In the stroma, the products of the light reactions are then used to assimilate CO_2 into sugar phosphates in the Calvin–Benson cycle. In natural growth conditions, plants experience fast and unpredictable fluctuations in light intensity and other environmental factors. This has necessitated evolution of intricate regulatory mechanisms to prevent damage to the photosynthetic machinery and to avoid energy-expensive futile reactions. An important way to control these mechanisms is through formation and cleavage of disulfide bridges in chloroplast proteins by thioredoxins. Indeed, plant chloroplasts contain a large variety of thioredoxin isoforms, as well as two distinct thioredoxin systems; one dependent on ferredoxin as reductant, the other on NADPH.

In this thesis I have investigated the role of the NADPH-dependent chloroplast thioredoxin system (NTRC) in regulation of photosynthetic processes, as well as the coordination between the NTRC- and ferredoxin-dependent systems. I demonstrate that NTRC forms a crucial regulatory hub in chloroplasts that allows maintenance of redox balance between the photosynthetic electron transfer chain and stromal metabolism, particularly in low light conditions. This is achieved through regulation of the activities of the ATP synthase and enzymes of the Calvin–Benson cycle, as well as non-photochemical quenching, cyclic electron transfer around photosystem I via the NADH dehydrogenase-like complex, and reversible re-distribution of excitation energy between the photosystems. I show that significant crosstalk exists between the thioredoxin systems, which allows dynamic control of photosynthetic processes and photoprotective mechanisms in fluctuating light conditions. Understanding these regulatory mechanisms of photosynthesis is of utmost importance in bioengineering projects aiming to maximize crop yields or biofuel production. Moreover, my results suggest that enhancement of chloroplast thioredoxin activity may provide a simple but effective tool for those purposes.

TIIVISTELMÄ

Happea tuottavassa fotosynteesissä hiilidioksidia muunnetaan sokereiksi auringon valoenergian sekä vedeltä peräisin olevien elektronien avulla. Syanobakteerit sekä niiden evolutiiviset jälkeläiset eli kasvien ja levien viherhiukkaset kykenevät fotosynteettiseen sokereiden tuottoon mahdollistaen lähes kaikkien Maapallon ekosysteemien toiminnan. Fotosynteesin elektroninsiirto vedeltä ferredoksiinille tuottaa NADPH:ta ja johtaa elektrokemiallisen protonigradientin muodostumiseen tylakoidikalvon yli. Protonigradientti toimii ATP-syntaasin käyttövoimana, ja NADPH:ta ja ATP:tä käytetään energialähteenä Calvin–Benson–syklissä tapahtuvassa hiilidioksidin sidonnassa. Luonnonolosuhteissa valon voimakkuus ja muut ympäristötekijät vaihtelevat nopeasti kasvien kasvupaikoilla. Tämä on luonut valintapaineen moninaisten säätelymekanismien kehittymiselle fotosynteesikoneiston energiankeruun turvaamiseksi ja vahingoittumisen välttämiseksi. Tioredoksiinit kuuluvat säätelyproteiineihin, jotka katalysoivat proteiinien rikkisiltojen pelkistystä ja ovat keskeisiä viherhiukkasten toimintaa sääteleviä yhdisteitä. Kasvien viherhiukkasissa on useita tioredoksiini-proteiineja sekä kaksi erillistä tioredoksiinijärjestelmää, joista toinen käyttää ferredoksiinia ja toinen NADPH:ta pelkistyksessä tarvittavien elektronien lähteenä.

Väitöskirjassani olen tutkinut NADPH-riippuvaisen tioredoksiinijärjestelmän (NTRC:n) roolia fotosynteettisten prosessien säätelijänä, sekä NTRC- ja ferredoksiini-riippuvaisen järjestelmän välistä vuorovaikutussuhdetta. Tutkimukseni osoittaa, että NTRC:llä on keskeinen tehtävä hapetus–pelkistys-tasapainon säilyttämisessä fotosynteesin valoreaktioiden ja strooman hiilimetabolian välillä, etenkin heikossa valossa ja valo-olosuhteiden äkillisten muutosten aikana. NTRC säätelee ATP-syntaasin ja Calvin–Benson-syklin entsyymien aktiivisuutta, ylimääräisen viritysenergian hajottamista, syklistä elektronikiertoa, sekä viritysenergian jakautumista valoreaktio II:n ja I:n kesken. Työni osoittaa myös, että NTRC- ja ferredoksiini-riippuvainen tioredoksiinijärjestelmä ovat vuorovaikutuksessa keskenään, mikä mahdollistaa fotosynteettisten reaktioiden ja suojamekanismien dynaamisen säätelyn vaihtelevissa valo-olosuhteissa. Näiden säätelymekanismien ymmärtäminen on hyvin tärkeää kun pyritään bioteknisin keinoin maksimoimaan viljelykasvien tai biopolttoaineen tuottoa. Kloroplastin tioredoksiinijärjestelmien toiminnan tehostaminen yksinkertaisella geenimuokkauksella saattaa olla hyödyllinen työkalu kasvien kasvun ja tuottavuuden parantamiseksi.

1. INTRODUCTION

Primary production in most ecosystems on Earth, including all those sustaining human food production, is based on conversion of the energy contained in a photon of light to energy in chemical bonds in organic compounds by organisms capable of photosynthesis; anoxygenic photosynthetic bacteria and oxygenic cyanobacteria, algae and plants. It is therefore of utmost importance to understand how photosynthesis and its surrounding metabolic infrastructure is regulated in order to ensure the sustainability of Earth's ecosystems and to preserve and enhance food producing crops and biofuel production in an environmentally sustainable manner.

1.1. Evolutionary origin of photosynthesis and chloroplasts

Photosynthesis evolved rather early after the origin of life: the oldest signs of cellular life discovered have been dated 3.95–4.28 billion years (Ga) old (Dodd et al., 2017; Tashiro et al., 2017), while the first photosynthetic organisms emerged perhaps already 3.2–3.5 Ga ago. These organisms were most likely anoxygenic, meaning they neither produced nor tolerated oxygen (Hohmann-Marriott and Blankenship, 2011). Oxygenic photosynthesis based on oxidation of water molecules evolved considerably later, and culminated in the Great Oxygenation Event (GOE) 2.3–2.5 Ga ago in which the Earth's atmosphere was transformed from anoxic to oxygenated due to oxygen production by photosynthetic cyanobacteria (Lyons et al., 2014). Importantly, the GOE ultimately resulted in formation of the ozone layer in Earth's atmosphere and thus enabled the colonization of land by life.

Oxygenic photosynthesis was exclusive to the domain of Bacteria until roughly 1.5 Ga ago, when a photosynthetic cyanobacterium was engulfed by a heterotrophic eukaryote and eventually evolved into an endosymbiotic photosynthetic organelle (Hohmann-Marriott and Blankenship, 2011). This was the second endosymbiotic event in the eukaryotic lineage, as the eukaryotic cell already contained a mitochondrion. The ancient endosymbiont became the ancestor of chloroplasts in all photosynthetic eukaryotes, including land plants, red and green algae and glaucophytes. Primary endosymbiosis has only taken place once in extant evolutionary

lineages of photosynthetic organisms, while secondary and tertiary endosymbioses, events in which a plastid-containing eukaryote is engulfed by another eukaryote, have occurred several times (Hohmann-Marriott and Blankenship, 2011).

1.2. Structure and biogenesis of chloroplasts

Chloroplasts of plants and algae are disc-shaped organelles enveloped by two lipid bilayers, often referred to as the inner and outer membrane. The soluble compartment inside the envelope is called the stroma. A third membranous system within the chloroplast, the thylakoid membrane, encloses a soluble compartment known as the thylakoid lumen and contains the protein complexes of photosynthetic electron transfer reactions (see 1.3). The thylakoids consist of tightly stacked structures known as the grana and unstacked membrane structures connecting the grana known as stroma thylakoids. Plastids are connected to each other by tube-like stroma-filled membrane structures known as stromules (Hanson and Sattarzadeh, 2011).

There can be over 100 chloroplasts in a plant mesophyll cell. In addition to photosynthesis, they perform other vital functions such as biosynthesis of amino acids, fatty acids, nucleotides, pigments and plant hormones, and are involved in assimilation of nitrogen and sulfur (Jarvis and Lopez-Juez, 2013).

1.2.1. The plastid genome

A vast majority of the genome of the original endosymbiont has over the course of evolution been integrated to the nuclear genome of the eukaryotic host, with genes of cyanobacterial origin comprising roughly 18% of the protein coding genes in the nuclear genome of Arabidopsis (Howe, 2016). Depending on the species, 60–200 genes are, however, still encoded in the plastidial genome (Martin et al., 2002). Transfer of genetic material to the diploid nuclear genome provided improved protection from reactive oxygen species generated in photosynthesis (see 1.5.6), enabled the adaptive benefit from sexual recombination and allowed efficient co-regulation of expression with nuclear genes (Howe, 2016). However, as the proteins encoded by most of the genes transferred to the nucleus still

function in the chloroplast, the gene transfer also necessitated the evolution of protein translocation mechanisms from the cytosol to the chloroplast. In the case of most proteins, this is accomplished through an N-terminal signal peptide that guides a nascent protein through the translocases on the outer and inner chloroplast membranes (TOC and TIC complexes, respectively) (reviewed by Kovacs-Bogdan et al., 2010).

1.2.2. Plastid development

In embryophytes chloroplasts develop from pigmentless precursor forms known as proplastids, which still lack an internal membrane system (Jarvis and Lopez-Juez, 2013). Depending on light conditions, the conversion of proplastids to chloroplasts can happen directly or indirectly via a stage called the etioplast. Etioplasts contain pre-thylakoidal membrane structures called prolamellar bodies, but no chlorophyll (Solymosi and Schoefs, 2010). The development of etioplasts to chloroplasts is triggered by light, which causes activation of NADPH:protochlorophyllide oxidoreductase (POR), a key enzyme in biosynthesis of chlorophyll. Chlorophyll biosynthesis also has a pivotal function in inducing retrograde signaling cascades that activate chloroplast-biogenesis-related nuclear gene expression (Pogson and Albrecht, 2011). This is demonstrated by several *genomes uncoupled* (GUN) mutants, where defects in synthesis of tetrapyrroles result in disturbances in regulation of biogenic nuclear gene expression by retrograde signaling (Susek et al., 1993; Mochizuki et al., 2001; Larkin et al., 2003; Koussevitzky et al., 2007).

In addition to chloroplasts, proplastids are also the ontogenic precursors of the non-green plastid types, namely starch-containing amyloplasts, which are mainly located in seed and root tissues, as well as carotenoid-rich chromoplasts in flowers and fruits, protein-rich proteoplasts and lipid-storing elaioplasts (Jarvis and Lopez-Juez, 2013; Pogson et al., 2015).

Both the plastid envelope and the thylakoid membrane are mainly composed of galactolipids such as mono- and digalactocyldiacylglycerol (MGDG and DGDG respectively), which are synthesized at the plastid membranes (Rast et al., 2015). During chloroplast biogenesis, assembly of the thylakoid membrane is intricately coordinated with the incorporation of pigments, cofactors and protein complexes (Rast et al., 2015).

1.2.3. Plastid division

As a cell grows in size during development, the number of plastids needs to be increased in a strictly regulated manner. Akin to their bacterial ancestors, chloroplasts proliferate by binary fission. Accordingly, the chloroplast division machinery on the inner chloroplast envelope is of the bacterial type that depends on contractile rings consisting of the tubulin-like FtsZ proteins (see recent reviews by Osteryoung and Pyke, 2014 and Yoshida et al., 2016).

When a plant chloroplast starts to divide, FtsZ1 and FtsZ2 proteins oligomerise, forming filaments and eventually ring structures known as Z rings at the centre of the plastid, which function as scaffolds for other divisisome components to bind in. Oligomerization of FtsZ near the poles of the plastid is inhibited by the Min complex, comprising of MinD1, MinE1 and ACCUMULATION AND REPLICATION OF CHLOROPLASTS 3 (ARC3) (comprehensively reviewed by Osteryoung and Pyke, 2014). This regulatory mechanism ensures equal division and uniform distribution of organellar content in daughter plastids. Cells of plants overexpressing or deficient in FtsZ or Min-proteins often have an abnormal number of chloroplasts and/or misshaped chloroplast morphology (Osteryoung and Pyke, 2014). The FtsZ-dependent machinery and a ring structure of unknown composition known as the inner PD (plastid-dividing) ring on the inner chloroplast membrane are connected to eukaryote-derived components on the cytosolic side of the outer membrane. (Osteryoung and Pyke, 2014).

Frequency of plastid division needs to be synchronized with cellular development. The division rate has been shown to be controlled by cytokinin signaling which induces the expression of PLASTID DIVISION 1 and 2 (PDV1 and PDV2) proteins, whose increased amount promotes plastid division (Okazaki et al., 2009). Interestingly, there are major differences in the mechanism of plastid division and regulation of Z-ring placement between chloroplasts and non-green plastids. For instance, the ARC3 protein is essential for equal division of chloroplasts (Shimada et al., 2004; Maple et al., 2007), but redundant in division of non-green plastids (Wang et al., 2013b).

1.3. Photosynthesis

In oxygenic photosynthesis photons of sunlight are harnessed to excite specific reaction center chlorophyll pigments in photosystems II and I in the thylakoid membrane, which allows electrons to be extracted from water molecules, creating oxygen (O_2) and protons (H^+) as byproducts. Electrons are transferred to a series of acceptors of diminishingly negative redox potential in the photosynthetic electron transfer chain (PETC), finally resulting in reduction of $NADP^+$ to NADPH. As the electron transfer chain is coupled to translocation of protons to the lumen, an electrochemical proton motive force (*pmf*) over the thylakoid membrane is established, which is then used to power synthesis of adenosine triphosphate (ATP). Finally, the NADPH and ATP are utilized in stromal fixation of CO_2 to organic carbohydrates in the Calvin–Benson cycle (CBC). A simplified scheme of photosynthetic reactions is shown in Fig. 1.

1.3.1. Structure and function of the photosynthetic electron transfer chain

The linear electron transfer (LET) chain consists of four multi-subunit protein complexes integrated into the thylakoid membrane. To follow the route of an electron passing through the chain, they are Photosystem II (PSII), Cytochrome *b6f* (Cyt *b6f*), and Photosystem I (PSI) (Figure 1). Additionally, the electron transfer chain contains the intersystem electron carrier molecules called plastoquinones that pass electrons from PSII to Cyt *b6f*, and Plastocyanin (PC), a soluble electron carrier protein in the lumen that shuttles electrons from Cyt *b6f* to PSI. From PSI electrons are fed to a stromal acceptor protein Ferredoxin (Fd), which functions as a hub of reducing power distribution. NADPH is produced when Fd is oxidized by Ferredoxin-$NADP^+$-oxidoreductase (FNR). The photosynthetic machinery is not distributed homogenously in the thylakoid membrane. Instead, PSII concentrates in grana cores while PSI and ATP synthase accumulate in stroma lamellae and margins of the grana. This structural arrangement is usually referred to as *lateral heterogeneity* (Andersson and Anderson, 1980; Pribil et al., 2014).

In plant thylakoids PSII functions as a dimeric supercomplex where a monomer comprises at least 20 different protein subunits binding 35

chlorophylls and two pheophytins (Shen, 2015; van Bezouwen et al., 2017). The D1 and D2 subunits in the PSII core bind the cofactors participating in electron transfer, including the four chlorophylls forming the PSII reaction center called P_{680} according to their absorption maxima. When a photon is absorbed, primary charge separation occurs as P_{680} is excited and donates an electron to the first electron carrier pheophytin, from which it is transferred to plastoquinone A (Q_A) and subsequently to plastoquinone B (Q_B). When Q_B has accepted two electrons, it becomes a mobile electron carrier within the thylakoid membrane. Oxidized P_{680} (P_{680}^+) is the strongest oxidant in nature and capable of oxidizing water molecules (H_2O). P_{680}^+ first oxidizes a special tyrosine residue in D1 (Y_Z), which then extracts an electron from the four-manganese cluster (Mn_4CaO_5) in the catalytic center of the oxygen evolving complex (OEC). The plant OEC consists of the proteins PsbO, PsbQ and PsbP and is attached to the luminal side of PSII core via the internal antenna subunits CP43 and CP47. Splitting of two H_2O into four H^+ and one O_2 requires the extraction of four electrons from Mn_4CaO_5 by Y_Z. Cytochrome b_{559}, which consists of the PsbE and PsbF subunits and a heme cofactor, is also part of the PSII core, and likely has a role in photoprotection as well as PSII assembly (Shen, 2015; Nelson and Junge, 2015; van Bezouwen et al., 2017).

In a plant PSII supercomplex each PSII monomer binds three monomeric light harvesting complex II (Lhcb) proteins, one strongly bound (S type) and one or several moderately (M type) and loosely (L-type) bound trimers of Lhcb proteins (LHCII) (Wei, 2016). Lhcb proteins constitute the peripheral antennae that absorb excitation energy and transmit it to the P_{680} chlorophylls in the PSII reaction center, largely via Förster resonance energy transfer (FRET) reactions (Croce and van Amerongen, 2014; Wei, 2016; Su et al., 2017). The monomeric Lhcb proteins CP24 (Lhcb6), CP26 (Lhcb5) and CP29 (Lhcb4) are bound directly to the PSII core proteins CP47 and CP43, and mediate the binding of LHCII trimers consisting of the Lhcb1, Lhcb2 and Lhcb3 proteins, each of which binds fourteen chlorophylls and several carotenoids (van Bezouwen et al., 2017). Nine to thirteen chlorophylls are localized to CP24, CP26 and CP29, which also bind carotenoids (Croce and van Amerongen, 2014; Wei, 2016; van Bezouwen et al., 2017).

From PSII electrons go to the plastoquinone (PQ) pool within the thylakoid membrane and to the Cyt b_6f complex. Cyt b_6f functions as a dimeric multisubunit complex, where each monomer consists of 8 protein subunits, lipids, pigments, hemes and a [2Fe-2S] iron-sulphur cluster. Both the Q_o site on the lumenal side and the PQ reduction site (Q_i) on the stromal side of the complex, which constitute the active sites of the Q cycle, are formed by the Cyt b_6 and suIV subunits (for a recent review see Dumas et al., 2016). Doubly reduced PQ accepts two H^+ from the stroma, becoming plastoquinol (PQH_2), which is a mobile molecule that diffuses within the membrane until it binds in the Q_o site of the Cyt b_6f complex, releasing the two H^+ in the lumen. One of the electrons is transferred onwards to PC, a soluble electron carrier protein in the lumen that reduces PSI, while the other is cycled within Cyt b_6f in what is known as the Q cycle to reduce another PQ molecule. This process therefore increases the ratio of H^+ transferred to the lumen vs electrons transferred in the chain and contributes to the formation of the *pmf* (recently reviewed by Dumas et al., 2016).

While cyanobacterial PSI functions as a trimer, Eukaryotic PSI is a monomeric complex consisting of 16 protein subunits and over 200 prosthetic cofactors, including 156 chlorophylls, carotenoids, lipids and three [4Fe-4S] iron sulfur clusters (Mazor et al., 2017; Caspy and Nelson, 2018). PsaA and PsaB form a heterodimer of 22 transmembrane helices in the PSI core that harbours most of the cofactors necessary for electron transport, including the reaction center chlorophyll pair called P_{700}, more than half of the total internal Chl in PSI as well as a [4Fe-4S] cluster denoted F_X. Binding site for the electron donor PC is located on the lumenal side of PSI and consists of the PsaF and PsaH subunits (Hippler et al., 1996; Caspy and Nelson, 2018). PsaC on the stromal side of PSI contains two more iron sulphur clusters (F_A and F_B) which constitute the terminus of electron transfer within PSI, and forms the ferredoxin binding and reduction site together with PsaD and PsaE. Light-harvesting complex I (LHCI) consists of four Lhca-proteins in the form of an Lhca1-4 and an Lhca2-3 dimer, and is connected to the PSI core via the PsaG subunit (Nelson and Junge, 2015; Mazor et al., 2015; Mazor et al., 2017). Additionally, two minor forms of LHCI, Lhca5 and 6 (Jansson, 1999; Ganeteg et al., 2004) participate in formation of supercomplexes between

PSI and the chloroplast NADH dehydrogenase-like (NDH) complex (see 1.5.4) (Peng et al., 2009).

Ferredoxins are small soluble proteins on the stromal side of the thylakoid membrane and contain a [2Fe-2S] iron sulphur cluster. In the PETC, they are the electron donors to FNR, which reduces $NADP^+$ to NADPH. In Arabidopsis, six Fd isoforms exist, of which two (FD1 and FD2) are involved in photosynthesis and constitute a vast majority of the total Fd content in plant tissues (Hanke et al., 2004; Hanke and Hase, 2008). In addition to FNR, ferredoxins distribute reducing power from PSI to a variety of stromal processes, including the plastidial thioredoxin systems (see 1.4), nitrate and sulphur assimilation, biosynthesis of phytochromes, fatty acids and chlorophyll as well as cyclic electron transfer pathways (see 1.5.4). Coordination of this distribution of reducing power according to environmental and developmental conditions of the cell is a major open question in understanding the regulation of chloroplast metabolism and maintenance of redox homeostasis (Fukuyama, 2004; Hanke and Mulo, 2013).

Photosynthetic Fd-$NADP^+$ oxidoreductase (FNR) contains an FAD cofactor domain that accepts two electrons from two ferredoxins to reduce one molecule of $NADP^+$ to NADPH (Carrillo and Ceccarelli, 2003; Mulo and Medina, 2017). There are two isoforms of chloroplast-targeted leaf-type FNRs, LFNR1 and LFNR2 (Mulo, 2011). FNR can attach peripherally to the thylakoid membrane by forming a complex that requires the presence of LFNR1 (Lintala et al., 2007), Tic62 (Benz et al., 2009) and TROL proteins (Juric et al., 2009), and is dependent on stromal pH (Lintala et al., 2014). Membrane-bound FNR has been shown to possess higher catalytic activity *in vitro* (Forti and Bracale, 1984), but soluble FNR can also function in photosynthetic reduction of $NADP^+$, and Arabidopsis plants lacking membrane-bound FNR do not suffer from a photosynthetically impaired phenotype apart from a decreased NADPH/$NADP^+$ ratio (Lintala et al., 2007; Lintala et al., 2014). Nevertheless, reversible association of FNR to the thylakoids may be an important regulatory element in controlling the redox homeostasis of the chloroplast (Benz et al., 2010; Kozuleva et al., 2017).

The chloroplast ATP synthase is an F_0F_1 type ATPase and homologous to the ATP synthases in bacteria and on mitochondrial respiratory

membranes. The membrane embedded F_0 module consists of 1 a, 2 b and – in plants – 14 c subunits, which form a transmembranous ring-like structure. The peripheral F_1 module in turn comprises a catalytic $\alpha_3\beta_3$ hexamere as well as the γ, δ and ε subunits (Yoshida et al., 1979; Seelert et al., 2000; Yoshida et al., 2001). During catalysis a proton passing through the F_0 module induces the γ subunit to rotate against the $\alpha_3\beta_3$ hexamere. This rotation alters the substrate affinity of the ADP/ATP binding sites in the β subunits in a way that allows ADP binding, ATP synthesis, and release of ATP to the stroma (Abrahams et al., 1994; Sabbert et al., 1996; Noji et al., 1997; Watanabe et al., 2012). It takes 14 H^+ to drive a full 360° rotation of the γ subunit in chloroplasts, which yields three ATP (Seelert et al., 2000; Vollmar et al., 2009).

The ATP synthase catalyzes the production of ATP from ADP and inorganic phosphate (P_i). It utilizes the electrochemical *pmf* generated by water splitting in the OEC, oxidation and reduction of PQH_2 in the Q cycle, and via proton pumping by the NDH complex in cyclic electron flow (see 1.5.4) (Yoshida et al., 2001; Hisabori et al., 2013; Strand et al., 2017). The *pmf* consists of the difference in proton concentration between stroma and lumen, denoted ΔpH, and of the electric field, Δψ, which in addition to the positive charge of protons originates from pumping of positively charged K^+, Ca^{2+} and Mg^{2+} ions out and negatively charged Cl^- ions into the lumen by trans-thylakoid ion transporters (Carraretto et al., 2013; Kunz et al., 2014; Armbruster et al., 2016; Herdean et al., 2016; Armbruster et al., 2017). ΔpH and Δψ are thermodynamically equivalent and contribute equally to the *pmf* that drives ATP synthesis (Hangarter and Good, 1982), but the amount of ΔpH has profound implications for regulation of electron transfer and induction of photoprotective mechanisms (see 1.5) (Avenson et al., 2005; Kanazawa et al., 2017).

1.3.2. The Calvin–Benson cycle

In the Calvin–Benson cycle (CBC) inorganic carbon is fixed to carbohydrates in 13 enzymatic reactions catalyzed by 11 enzymes in the chloroplast stroma, powered by the NADPH and ATP produced in photosynthetic electron transfer. First, carboxylation of a 5-carbon acceptor molecule ribulose-1,5-bisphosphate (RuBP) by CO_2 is catalyzed by the

enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which yields two molecules of 3-phosphoclycerate (3-PGA). The key enzyme Rubisco functions as a hetero-oligomer of 8 large and 8 small subunits (Andersson and Backlund, 2008). 3-PGA is then phosphorylated by phosphoglycerate kinase (PGK) to form 1,3-PGA using 1 ATP. 1,3-PGA is the substrate for a tetrameric enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a reaction that oxidizes one NADPH to form glyceraldehyde-3-phosphate (G3P). G3P is then converted to fructose-6-phosphate (F6P) in two reactions catalyzed by aldolase and fructose 1,6 bisphosphatase (FBPase). Alternatively, G3P can be converted first to dihydroxyacetone-3-phosphate (DHAP) by triose phosphate isomerase (TPI), to seduheptulose-1,7-bisphosphate (SDP) by aldolase and to seduheptulose-7-bisphosphate (S7P) seduheptulose-1,7-bisphosphatase (SBPase). Transketolase can convert both F6P and S7P to xylulose-5-phosphate (X5P), two of which are then substrate for ribulose-5-phosphate-3 epimerase (RPE) to produce one ribulose-5-phosphate (RuP). Additional RuP is produced from ribose-5-phosphate in a reaction catalized by ribose-5-phosphate isomerase (RPI). RuP is the substrate in the concluding reaction of the CBC where RuBP is regenerated in an ATP-consuming reaction catalyzed by phosphoribulokinase (PRK). The equation for the fixation of CO_2 in the Calvin-Benson cycle is therefore: $3CO_2 + 9\ ATP + 6\ NADPH + 6\ H_2O \rightarrow$ triose phosphate $+ 9\ ADP + 9\ P_i + 6\ NADP^+ + 3\ H^+$ (Calvin and Benson, 1948).

Due to high concentration of O_2 in chloroplast, Rubisco will intermittently use O_2 as substrate instead of CO_2, which results in production of 2-phosphoglycolate (2-PGA) in addition to one 3-PG. 2-PG is subsequently converted to 3-PGA in a costly enzymatic pathway in peroxisomes and mitochondria in a process termed photorespiration. It has however been argued that photorespiration is in fact not a futile wasteful reaction, but an essential electron sink in low CO_2 concentrations, such as upon stomatal closure during drought (Kirschbaum et al., 1998; Huang et al., 2015; Hagemann et al., 2016).

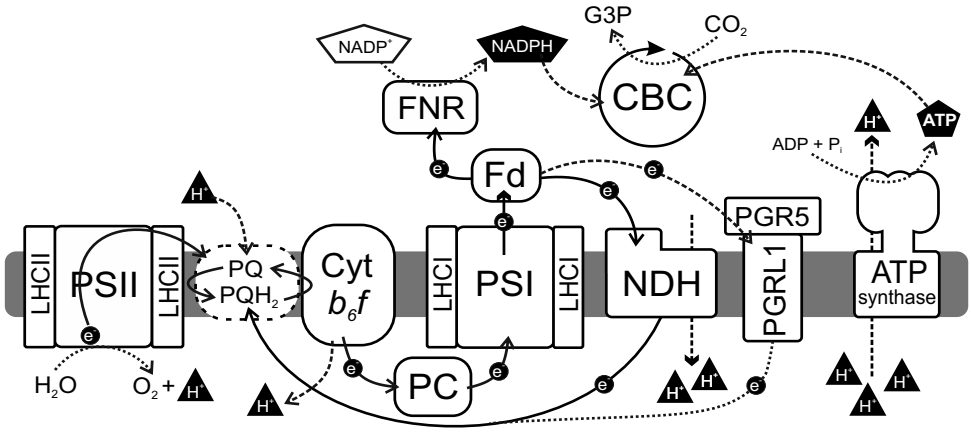

Figure 1. A simplified schematic illustration of photosynthetic reactions in the thylakoid membrane and in the stroma.

LHCII/LHCI = light harvesting complex II/I, PSII = photosystem II, PQ = plastoquinone, PQH_2 = plastoquinol, Cyt *b*$_6$*f* = cytochrome *b*$_6$*f* complex, PC = plastocyanin, PSI = photosystem I, Fd = ferredoxin, FNR = ferredoxin:$NADP^+$ oxidoreductase, NADP/NADPH = oxidized/reduced nicotinamide adenine dinucleotide phosphate, NDH = NADH-dehydrogenase-like complex, PGR5 = proton gradient regulation 5, PGRL1 = PGR5-like 1, CBC = Calvin–Benson cycle, G3P = glyceraldehyde-3-phosphate, ADP = adenosine diphosphate, P_i = inorganic phosphate, ATP = adenosine triphosphate. The cyclic pathway from PGRL1 remains putative and is therefore represented by a dotted line. For details see the text. The arrows from PGRL1 and NDH to the PQ pool have been drawn through the lumen only to clarify the illustration.

1.4. Chloroplast thioredoxin systems

In natural growth habitats of plants the availability, intensity and quality of light is constantly changing. As sessile organisms, plants have evolved several regulatory and photoprotective mechanisms to adjust photosynthetic processes and metabolism to these changes in order to avoid wasteful reactions and damage to the photosynthetic apparatus. Moreover, it is essential that redox homeostasis is maintained between the light reactions on thylakoids and stromal carbon metabolism. Plants, and chloroplasts in particular, possess highly versatile regulatory systems based on disulfide/dithiol exchange reactions in proteins and involving regulatory proteins called thioredoxins. Extensive research over several decades has demonstrated the impact of chloroplast thioredoxins on proper function of chloroplasts under fluctuating environmental conditions.

Covalent but reversible post-translational modification of thiols in side-chains of cysteine residues is a ubiquitous and evolutionarily ancient mechanism of regulating the function, structure, interactions or stability of proteins. The most common thiol modifications are formation of disulfide bonds, S-nitrosylation, and S-glutathionylation. Conversely, cleavage of disulfides is catalyzed by thioredoxins or glutaredoxins, denitrosylation is catalyzed by thioredoxin or glutathione, and deglutathionylation is catalyzed by glutaredoxins (GRXs) (Benhar et al., 2009; Meyer et al., 2012; Couturier et al., 2013; Balsera et al., 2014; Zaffagnini et al., 2016).

Thioredoxins (TRXs) are small dithiol:disulfide oxidoreductases which are present in all extant lineages of organisms. They were most likely crucial regulators of very early biochemistry: TRXs derive from at least the common ancestor of bacteria and archaea that lived circa 4 Ga ago (Ingles-Prieto et al., 2013). All canonical TRXs contain a redox active cysteine pair in a highly conserved amino acid motif WCG/PPC (Balsera et al., 2013; Balsera et al., 2014). The reaction mechanism of TRXs involves a nucleophilic attack on one of the cysteines in a target protein by the catalytic cysteine in the TRX redox active motif. A mixed disulfide is formed between the catalytic Cys of the TRX and a cysteine in the target protein. Subsequently the second, resolving cysteine in the TRX reduces the mixed intermolecular disulfide between the catalytic TRX cysteine and the target, resulting in release of reduced target protein(s) and oxidized TRX (Collet and Messens, 2010; Meyer et al., 2012). Oxidized TRXs need to be reactivated through reduction by a specific enzyme, thioredoxin reductase (TR). A TRX and a corresponding TR constitute a *thioredoxin system*.

In plants maintenance of redox homeostasis is of particular importance because of the generation of reactive oxygen species (ROS) by photosynthesis and the immobile nature of plants, which necessitates both short-term and acclimatory regulation according to fluctuations of light and changes in other environmental conditions (Schürmann and Buchanan, 2008; Serrato et al., 2013; Nikkanen and Rintamäki, 2014; Geigenberger and Fernie, 2014; Balsera et al., 2014; Geigenberger et al., 2017). The diversity and versatility of TRX isoforms in plants reflects this point: whereas in humans and *E. coli*, for example, only two types of classical TRXs exist, over 20 different classical TRX isoforms and over 20 other

TRX-like proteins have been identified in Arabidopsis. TRXs are present in several subcellular compartments, including the cytosol (*h*-type TRXs), nucleus (nucleoredoxins, NRXs), plasma membrane (*h*-type) and mitochondria (*h*- and *o*-type). All cytosolic, nuclear and mitochondrial TRXs in plants are reduced by two isoforms of NADPH:thioredoxin reductase (NTRA and NTRB). Greatest diversity of TRXs, however, exists in the chloroplast: Two *f*-type TRXs (TRX-*f*1 and *f*2), four isoforms of TRX-*m* (*m*1-*m*4), TRX-*x*, TRX-*y*1 and *y*2 as well as TRX-*z* are known to localize in the organelle in *Arabidopsis thatliana*. Additionally, chloroplasts contain an NADPH-dependent thioredoxin reductase (NTRC) as well as several TRX-like proteins (Meyer et al., 2012; Geigenberger et al., 2017).

Two parallel TRX systems co-exist in chloroplasts, with an important functional distinction regarding their sources of reductant. In the *ferredoxin-thioredoxin system* (Fd-TRX), ferredoxin:thioredoxin reductase (FTR) receives reducing equivalents from photosynthetically reduced ferredoxin. Its activity is therefore dependent on the availability of light (Schürmann and Buchanan, 2008; Buchanan, 2016). The NADPH-dependent chloroplast thioredoxin system (NTRC), in turn, is reduced by NADPH (Serrato et al., 2004), which in addition to being produced photosynthetically by FNR is also produced in the oxidative pentose phosphate pathway (OPPP). This at least theoretically enables the NTRC system to maintain activity also in darkness and under low irradiance (Perez-Ruiz et al., 2006).

Proteomic studies have expanded the scope of TRX-regulated plastidial processes since the early biochemical studies on CBC enzymes (see below) (Balmer et al., 2003; Buchanan and Balmer, 2005; Lindahl and Kieselbach, 2009; Montrichard et al., 2009; Hall et al., 2010). It is now understood that TRXs control also chloroplast biogenesis, plastidial transcription, ATP synthesis, photoprotective mechanisms, carbon metabolism beyond the primary photosynthetic reactions, biosynthesis of starch and chlorophyll, nitrogen and sulfur metabolism, the shikimate and oxidative pentose phosphate pathways (see 1.6), as well as oxidative stress responses (for recent reviews see Serrato et al., 2013; Nikkanen and Rintamäki, 2014; Kang and Wang, 2016; and Geigenberger et al., 2017).

1.4.1. The ferredoxin–thioredoxin system

The stromal Fd-TRX system features the canonical low molecular weight chloroplast TRXs and the ferredoxin:thioredoxin reductase (FTR) originally identified and characterized in the 1970's by Bob Buchanan and coworkers. It functions as a light-dependent activator of the CBC enzymes FBPase, SBPase, GAPDH and PRK as well as the ATP synthase and the malate shuttle enzyme NADP-MDH (Schürmann et al., 1976; Wolosiuk and Buchanan, 1977; Wolosiuk et al., 1977; McKinney et al., 1978; Wolosiuk and Buchanan, 1978a; Wolosiuk and Buchanan, 1978b; Wolosiuk et al., 1979; Buchanan, 2016).

FTR receives reducing equivalents from ferredoxin upon illumination and reductively activates *f*, *m*, *x* and *y*-type TRXs, with distinct redox potentials (Bohrer et al., 2012; Yoshida and Hisabori, 2017). FTR is a heterodimeric enzyme that consists of a catalytic subunit (FTR*c*), which includes a [4Fe-4S] iron sulfur cluster and a redox active motif that mediate electron transfer from Fd to TRXs, as well as a variable subunit (FTR*v*) (Dai et al., 2007). Two isoforms of FTR*v* exist in Arabidopsis, but their functional significance is unknown (Keryer et al., 2004). Interestingly, FTR evolutionarily predates photosynthesis and probably originally functioned in regulation of the reverse citric acid cycle in anaerobic bacteria (Balsera et al., 2013).

While the abundance of all TRXs and TRs is low in comparison to their target proteins, TRX-*m*1, *m*2, *m*4 and TRX-*f*1 are the highest-expressed TRX isoforms in leaves (Peltier et al., 2006; Belin et al., 2015), and have been understood to be mainly responsible for the reductive activation of ATP synthesis and stromal carbon metabolism in light (see 1.5.1 and 1.5.5) (Schürmann and Buchanan, 2008; Okegawa and Motohashi, 2015). TRX-*x* and TRX-*y*2 are expressed at slightly lower levels (Belin et al., 2015) and function in oxidative stress response reactions (Broin et al., 2002; Collin et al., 2004).The *f*2, *m*3 and *y*1 isoforms show very low expression in photosynthetic tissues (Belin et al., 2015). TRX-*z* associates with the plastid-encoded RNA polymerase and regulates transcription of plastidial genes (Arsova et al., 2010), but the identity of its reductant remains controversial (Bohrer et al., 2012; Yoshida and Hisabori, 2016).

The Fd-TRX system is essential for plant development and growth, as knockout-mutations of FTRc are lethal, and virus-induced gene silencing of the *FTRB* gene coding for FTRc results in a severe chlorotic phenotype (Wang et al., 2014). Even a substantially decreased FTRc content is however enough to maintain a healthy WT-like phenotype (Yoshida and Hisabori, 2016). Knockout mutants of individual FTR-dependent TRXs seldom exhibit salient phenotypes, and simultaneous mutation of several *m*-type and *f*-type TRXs is required to suppress the apparent within-system redundancy (Wang et al., 2013a; Thormählen et al., 2015; Yoshida et al., 2015; Thormählen et al., 2017).

1.4.2. NTRC system

The NADPH-dependent chloroplast thioredoxin reductase (NTRC) is a complete TRX system in a single polypeptide: it consist of an N-terminal reductase domain (NTRd) containing binding sites for NADPH and two flavine adenine dinucleotide (FAD) cofactors, as well as a C-terminal thioredoxin domain (TRXd) (Serrato et al., 2004; Perez-Ruiz et al., 2006). NTRC functions as a homodimer, where the NTRd of one monomer reduces a disulfide in the redox-active motif in the TRXd of the other monomer (Perez-Ruiz and Cejudo, 2009; Perez-Ruiz et al., 2009). Additionally, it was recently proposed that NTRC can donate reducing equivalents to TRX-*z* (Yoshida and Hisabori, 2016).

Since its discovery, NTRC-mediated regulation has been shown to be involved in scavenging of H_2O_2 (Perez-Ruiz et al., 2006; Kirchsteiger et al., 2009; Pulido et al., 2010; Bernal-Bayard et al., 2014), biosynthesis of starch (Michalska et al., 2009; Lepistö et al., 2013; Skryhan et al., 2015) and chlorophyll (Stenbaek et al., 2008; Richter et al., 2013; Perez-Ruiz et al., 2014), as well as formation of stromules (Brunkard et al., 2015) and lateral roots (Kirchsteiger et al., 2012). Additionally, NTRC has been proposed to have a role in heat tolerance through a function as a molecular chaperone (Chae et al., 2013), and in regulation of translation of the PSII reaction center protein D2 in *Chlamydomonas reinhardtii* (Schwarz et al., 2012).

The *ntrc* knockout line of Arabidopsis has a chlorotic and diminutive phenotype, which is particularly severe in short photoperiods (Lepistö et

al., 2009; Lepistö et al., 2013). Cells of *ntrc* contain a morphologically heterogeneous population of chloroplasts that suffer from varying degrees of impairment in thylakoid development (Lepistö et al., 2009; Lepistö et al., 2012). It is therefore evident that NTRC has an essential role during early leaf and chloroplast development (Lepistö and Rintamäki, 2012).

1.4.3. TRX-like proteins in stroma, thylakoids and the lumen

In addition to the canonical small molecular weight TRXs and NTRC, there are also several less characterized TRX-like proteins in the chloroplast stroma (Meyer et al., 2012). These include four isoforms of Atypical Cys-His-rich Thioredoxin-like proteins (ACHT1-4) that function at least in oxidative inactivation of starch synthesis (Dangoor et al., 2009; Dangoor et al., 2012; Eliyahu et al., 2015), as well as the drought-induced TRX-like protein CDSP32 (Rey et al., 1998).

Proteomic approaches have established that over 40 % of all lumenal proteins are potentially redox-regulated, but no soluble TRXs have been discovered in the lumen (Hall et al., 2010; Karamoko et al., 2013; Kang and Wang, 2016). Redox signals can however potentially be transmitted from stroma across the thylakoid membrane to the lumen via a pathway comprising the integral thylakoid proteins CcdA and HCF164. CcdA is homologous to a bacterial protein on the cell membrane that can transmit reducing equivalents to the periplasmic space (Page et al., 2004; Motohashi and Hisabori, 2010), while HCF164 is a transmembrane protein in thylakoids that contains a TRX-like redox-active motif on the lumenal side of the membrane (Lennartz et al., 2001; Motohashi and Hisabori, 2006). The CcdA/HCF164 pathway has been suggested to receive stromal electrons via TRX-*m*-mediated reduction of CcdA and to be involved in the biogenesis of Cyt *b6f* (Page et al., 2004; Motohashi and Hisabori, 2010). Another trans-thylakoid protein LUMEN THIOL OXIDOREDUCTASE 1 (LTO1) also has a lumenal TRX-like domain, and has been proposed to be responsible for formation of dilsulfide bonds in PsbO during PSII assembly and in violaxanthin de-epoxidase (VDE, see 1.4.3) upon activation of the enzyme (Karamoko et al., 2011; Yu et al., 2014; Lu et al., 2015). SUPPRESSOR OF QUENCHING 1 (SOQ1) is another more recently

identified integral thylakoid protein which contains a luminal TRX-like domain (Brooks et al., 2013).

1.5. Regulation of photosynthesis during changes in light conditions

Photosynthetic redox balance is maintained through multiple regulatory mechanisms that operate both in the short-term in response to sudden, unpredictable changes in environmental conditions, and via slower processes that allow acclimation to long-lasting changes (for a review see Tikkanen and Aro, 2014). The long-term acclimatory mechanisms in response to changes in environmental conditions that last hours or longer involve changes at the level of gene expression. In particular, the ratio between PSII and PSI can be altered in order to achieve excitation balance between the photosystems during long-lasting changes in light quality (Bailey et al., 2001; Fan et al., 2007a). In shorter time scales, photosynthetic redox balance is maintained by control of ATP synthesis and *pmf* via regulation of the ATP synthase, by dissipation of excessive excitation energy as heat in non-photochemical quenching (NPQ), by phosphorylation-dependent reversible changes in the sizes of PSII and PSI antennae, by cyclic electron transfer around PSI (CET), and by regulation of the activation states of the enzymes of the Calvin–Benson cycle. Moreover, detoxification of reactive oxygen species (ROS) produced by photosynthetic reactions is essential to avoid damage to the photosynthetic apparatus and other biomolecules. In the sections below, several of these regulatory mechanisms are examined more closely, with special emphasis on redox regulation mediated by thioredoxins.

1.5.1. Regulation of the chloroplast ATP synthase and pmf

Activation of the CF_0CF_1 ATP synthase by TRX-mediated reduction of a disulfide in the γ subunit ($CF_1\gamma$) was discovered already more than 30 years ago (McKinney et al., 1978; Nalin and McCarty, 1984; Ketcham et al., 1984). $CF_1\gamma$ is maintained in an inactive oxidized state in darkness to prevent hydrolysis of ATP and futile reverse activity (Hisabori et al., 2013; Kohzuma et al., 2017). Reduction of $CF_1\gamma$ occurs rapidly (in seconds) upon onset of illumination and it lowers the *pmf* threshold required for activation

of the enzyme (Kramer et al., 1990; Konno et al., 2012; Hisabori et al., 2013; Kohzuma et al., 2017). TRX-*f* has been identified by *in vitro* assays to be a more effective reductant of $CF_1\gamma$ than TRX-*m*, and proposed to be primarily responsible for the light-dependent $CF_1\gamma$ reduction (Schwarz et al., 1997). The thiol:disulfide exchange in $CF_1\gamma$ constitutes an on/off switch that controls the activation of the ATP synthase between light and dark. This regulatory mechanism of the ATP synthase is exclusive to chloroplasts and does not occur in mitochondria or in cyanobacteria (Hisabori et al., 2013). Interestingly, also the β subunit of the ATP synthase contains a conserved Cys residue and has been identified as a potential TRX target in a proteomic study (Balmer et al., 2006). The activity of the ATP synthase is also fine-tuned via other, less characterized mechanisms that connect its activity to the metabolic state of the stroma. Very high CO_2 concentrations, for instance, such as occurs when the carbon fixation capacity of the Calvin–Benson cycle is saturated, is known to result in decrease in the activity of the ATP synthase through a mechanism that is independent of the thiol redox state of $CF_1\gamma$ (Kiirats et al., 2009; Kohzuma et al., 2013).

The proton-conductivity of the ATP synthase is a crucial factor in controlling the strength of the *pmf*. In addition to the regulation of ATP synthesis, strict control of the enzyme is also essential for induction of ΔpH-dependent photoprotective mechanisms under excessive illumination in order to avoid over-reduction of the electron transfer chain and PSI photodamage (Tikkanen and Aro, 2014; Kanazawa et al., 2017). Downregulation of the ATP synthase causes acidification of the lumen, which results in induction of a regulatory mechanism referred to as photosynthetic control. Photosynthetic control restricts electron transfer at the Cyt b_6f complex (Joliot and Johnson, 2011; Colombo et al., 2016), likely through inhibition of PQH_2 oxidation in Cyt b_6f in low pH (Tikhonov, 2014). Acidification of the lumen is also required for induction of *non-photochemical quenching* (Demmig-Adams et al., 2012).

1.5.2. Non-photochemical quenching

Non-photochemical quenching (NPQ) refers to controlled thermal dissipation of excess absorbed excitation energy in the light harvesting antenna of PSII that cannot be utilized by the PETC (Niyogi and Truong,

2013; Tian et al., 2017). This dissipation is essential to avoid damage to the photosynthetic machinery particularly in naturally fluctuating light conditions (Külheim et al., 2002).

A distinction is typically made between three components of NPQ: qE, qI and qT (Müller et al., 2001). Firstly, energy dependent quenching (qE) requires acidification of the lumen by proton translocation which then causes protonation of the PsbS protein (CP22), an intrinsic PSII subunit homologous to LHC protein family (Li et al., 2000). Acidification also activates violaxanthin de-epoxidase (VDE), a luminal enzyme that converts violaxanthin (Vx) to zeaxanthin (Zx) via antheraxanthin (Ax) (Niyogi et al., 1998; Jahns et al., 2009). Protonated PsbS and accumulation of Zx induce a rapid and reversible quenching response that can be observed as a decrease in chlorophyll a autofluorescence. The mechanism of qE induction likely involves conformational changes within LHCII and in its pigment composition (Holt et al., 2004; Johnson et al., 2009; Johnson et al., 2011; Ruban, 2016), but specific molecular details have remained elusive.

Shifting of plants to low light or to darkness deceases the Zx content in PSII antenna. The conversion of Zx back to Vx is catalyzed by zeaxanthin epoxidase (ZE), which is localized to the stromal side of the thylakoid membrane. The interconversion of Zx, Ax, and Vx is referred to as the *xanthophyll cycle* (Jahns and Holzwarth, 2012). Both xanthophyll cycle enzymes are thiol-regulated: VDE is deactivated by reduction of several disulfides (Hall et al., 2010; Hallin et al., 2015; Simionato et al., 2015), while *m*-type TRXs were recently reported to regulate ZE oligomerization and activity (Da et al., 2017).

Photoinhibition of PSII decreases Chl a fluorescence and contributes to a slowly reversible component of NPQ (qI). Photoinhibition of PSII is due to irreversibly damaged D1 protein that is degraded, synthesized *de novo* and inserted into PSII reactions centers (Järvi et al., 2015). The third component of NPQ is denoted qT and involves a mechanism that balances the distribution of absorbed excitation energy between the photosystems by controlling the association of a mobile constituent of LHCII to PSII or PSI through phosphorylation and de-phosphorylation of threonine residues in Lhcb1 and Lhcb2 (Bennett et al., 1980; Goldschmidt-Clermont and Bassi, 2015). This mechanism is known as *state transitions*.

1.5.3. Distribution of excitation energy between PSII and PSI

LHCII proteins are phosphorylated in light conditions favouring excitation of PSII over PSI by the thylakoidal STN7 kinase (Bellafiore et al., 2005). When PSII excitation exceeds that of PSI, the PQ pool becomes reduced and PQH_2 mostly occupies the Q_o site of Cyt *b6f*. This allows interaction of STN7 with Cyt *b6f* and subsequent activation of the kinase (Allen et al., 1981; Vener et al., 1997; Zito et al., 1999; Shapiguzov et al., 2016). Phosphorylation of LHCII induces *state II*, where phosphorylated LHCII associates with PSI through interactions with a PSI domain consisting of the PsaH, PsaI and PsaL subunits (Lunde et al., 2000; Zhang and Scheller, 2004; Plöchinger et al., 2016), causing an increase in the size of PSI antenna cross section relative to PSII and restoration of redox balance between the photosystems (recently reviewed by Goldschmidt-Clermont and Bassi, 2015).

In darkness and in light conditions that preferentially excite PSI, such as under light enriched in far red wavelengths, PQ pool is oxidized effectively by PSI. This induces deactivation of STN7 (Allen et al., 1981; Vener et al., 1997; Shapiguzov et al., 2016) with subsequent dephosphorylation of LHCII by the PPH1/TAP38 phosphatase (Pribil et al., 2010; Shapiguzov et al., 2010) and association of de-phosphorylated LHCII with PSII (*state I*). Under high irradiance STN7 is deactivated by the chloroplast thioredoxin systems, possibly through cleavage of a disulfide in the luminal domain of the kinase (Rintamäki et al., 2000; Martinsuo et al., 2003; Shapiguzov et al., 2016). In flowering plants STN7 contains two conserved cysteine pairs, one in the luminal and the other in the stromal part of the kinase (Depege et al., 2003). A disulfide bridge between the luminal cysteines, which unlike the stromal pair are also present in the STN7 Chlamydomonas orthologue Stt7, is essential for the activity of the kinase and for state transition capability both in Chlamydomonas (Lemeille et al., 2009) and in Arabidopsis (Shapiguzov et al., 2016). However, no correlation between the redox state of these cysteines and LHCII phosphorylation or state transitions has been observed in either species (Shapiguzov et al., 2016). The molecular mechanism of redox control of state transitions has therefore still remained elusive.

Phosphorylation of LHCII and PSII core proteins by the STN7 and STN8 kinases (Bonardi et al., 2005) also induce changes in the structure of the thylakoid membrane, affecting the size of grana stacks and mobility of proteins in the membrane (Fristedt et al., 2009; Herbstova et al., 2012; Wood et al., 2018). Specifically, a shift to low light causes a reversible increase in grana stacking within a time scale of minutes, while an increase in light intensity has the reverse effect (Rozak et al., 2002). These changes in membrane topology have been suggested to affect the diffusion distances of the mobile electron carriers PQ and PC in the thylakoid membrane, and to thus provide another mechanism to control the photosynthetic redox poise (Wood et al., 2018).

1.5.4. Cyclic electron transfer

As shown by the equation in chapter 1.3.2, the ATP:NADPH ratio used in the CBC is 3:2. Production of those two NADPH in the PETC takes four electrons and thus coincides with transfer of 12 H^+ to the lumen (4 from OEC and 8 from Q cycle), which is two short of powering a full rotation of the ATP synthase that would yield 3 ATP. The ATP:NADPH ratio produced by the PETC is therefore 2,6:2, and the extra ATP needed to feed the CBC must be produced otherwise (Furbank and Badger, 1983; Allen, 2003). This simplistic arithmetic obviously ignores the effects of all other metabolic as well as biosynthetic processes in the stroma, photorespiration, and potential regulation of the ATP synthase that would alter its kinetic properties (Kramer et al., 2004b).

Cyclic electron transfer (CET) is a process where electrons are recycled from ferredoxin back to the PQ pool instead of stromal acceptors, thereby not producing NADPH, but increasing proton translocation to the lumen to drive the ATP synthase. It has been proposed that the function of CET is to correct the ATP:NADPH imbalance according to environmental conditions and to the needs of the CBC (Kramer et al., 2004b; Yamori and Shikanai, 2016). CET also relieves stromal over-reduction under fluctuating or excessive light conditions (Munekage et al., 2002; Miyake et al., 2004; Suorsa et al., 2012; Yamori et al., 2016) and during early chloroplast development (Allorent et al., 2015; Suorsa, 2015). This allows stromal redox homeostasis to be maintained and PSI photodamage to be avoided,

and provides an additional mechanism to control the *pmf* (Munekage et al., 2004; Wang et al., 2015; Shikanai and Yamamoto, 2017). Furthermore, CET plays a role in balancing excitation between the photosystems during dark to light transitions (Joliot and Joliot, 2002; Joliot and Joliot, 2006; Fan et al., 2007b).

There are two CET pathways in plant chloroplasts. The first route was discovered and found to be sensitive to antimycin A already in the 1950's by Arnon and coworkers (Arnon et al., 1954), and later postulated to involve oxidation of ferredoxin and subsequent reduction of PQ by a hypothetical enzyme ferredoxin-plastoquinone reductase (FQR) (Moss and Bendall, 1984; Bendall and Manasse, 1995). The pathway has since been proposed to comprise the extrinsic membrane protein PROTON GRADIENT REGULATION 5 (PGR5) (Munekage et al., 2002) and the integral thylakoid membrane protein PGR5-LIKE 1 (PGRL1) (DalCorso et al., 2008). PGRL1 has been proposed to function as the long-sought FQR, and to require formation of a heterodimer with PGR5 for oxidation of ferredoxin (Hertle et al., 2013). It has been shown that PGR5/PGRL1-dependent CET is important for generation of *pmf* during dark to light transitions (Munekage et al., 2002; DalCorso et al., 2008; Hertle et al., 2013; Suorsa et al., 2016; Shikanai and Yamamoto, 2017). PGR5-dependent regulation of linear electron transfer at the cyt *b6f* complex in particular has also been proposed to be essential for avoiding photodamage to PSI iron sulfur clusters in fluctuating light conditions (Tikkanen et al., 2010; Suorsa et al., 2012; Tiwari et al., 2016).

The second pathway involves the chloroplast NADH dehydrogenase-like complex (NDH), which functions as a plastoquinone reductase, and is homologous to mitochondrial respiratory complex I. NDH consists of over 30 subunits, 11 of which are encoded in the chloroplast genome (Burrows et al., 1998; Suorsa et al., 2009; Peltier et al., 2016). In contrast to complex I, the electron donor for the chloroplast NDH complex is most likely ferredoxin instead of NADH or NADPH (Yamamoto et al., 2011). The Fd binding and oxidation site is formed on the stromal side of the thylakoid membrane by the NdhS subunit, while NdhJ, NdhU and NdhV are required for stabilization of NdhS in the complex (Yamamoto et al., 2011; Yamamoto and Shikanai, 2013; Fan et al., 2015). The plastid-encoded NdhH, -I, -J and –K subunits are most likely responsible electron transfer

within the complex and for reduction of PQ (Peltier et al., 2016). The NDH complex forms CET supercomplexes with PSI via Lhca5 and 6 at stroma lamellae (Peng et al., 2008; Peng et al., 2009) that may contain up to six PSI complexes bound to a single NDH complex (Yadav et al., 2017). The relatively low content of NDH complexes in thylakoids (Peng et al., 2008) may therefore have crucial physiological significance in regulation of plastidial energy balance. As the plant NDH complex also functions as a proton pump, transferring 2 H^+ from stroma to the lumen per electron (Strand et al., 2017), NDH-mediated CET contributes to lumenal protonation and ATP synthesis relatively more than the PGR5/PGRL1 pathway, where protons are only translocated via the Q cycle.

It has been suggested in several recent studies that the NDH complex has a role in generating *pmf* and maintaining redox homeostasis particularly under low irradiance (Kou et al., 2015; Martin et al., 2015; Yamori et al., 2015), as well as upon accumulation of H_2O_2 (Livingston et al., 2010; Strand et al., 2015), during drought (Horvath et al., 2000) and in low temperature (Yamori et al., 2011). Plants lacking a functional NDH complex do not have visible phenotypes, but double mutants deficient in both pathways of CET suffer from increased impairment of growth when compared to *pgr5* or *pgrl1* mutants, indicating that the two CET pathways can to some extent compensate for each other's absence (Munekage et al., 2004).

The NDH complex has been considered to be responsible for non-photochemical reduction of PQ and maintenance of sufficient ΔpH in darkness (Shikanai et al., 1998; Strand et al., 2017). In absence of light and during excessive excitation of PSII, PLASTID TERMINAL OXIDASE (PTOX) can oxidize PQH_2 to reduce molecular oxygen (O_2) in a process known as *chlororespiration* (Peltier and Cournac, 2002; Krieger-Liszkay and Feilke, 2016).

The rate of cyclic electron transfer has been reported to be dependent on stromal redox state (Breyton et al., 2006), and both the PGRL1-and NDH-dependent routes of CET have been proposed to be subject to TRX-mediated redox regulation. Knockout mutants of TRX-*m*4 exhibit increased rates of both NDH- and FQR-dependent CET, and TRX-*m*4 was therefore conjectured to have an inhibitory role in regulation of both pathways (Courteille et al., 2013). Stromal redox state also affects NDH-

CET through formation of hydrogen peroxide (H_2O_2), which increases NDH activity (Strand et al., 2015). No molecular mechanisms of redox regulation of the NDH complex have however been discovered.

PGRL1 contains six conserved Cys residues, which have been proposed to form intermolecular disulfides in PGRL1 homodimers, and potentially between PGRL1 and a single conserved cysteine in PGR5 (Munekage et al., 2002; DalCorso et al., 2008; Petroutsos et al., 2009; Hertle et al., 2013). According to a model postulated by Dario Leister and co-workers, formation of redox-dependent PGRL1-PGR5 heterodimers is required for reception of electrons from ferredoxin, while TRX-*m* mediated cleavage of intermolecular disulfide(s) in PGRL1 homodimers would be a pre-requisite for the potential PQ reductase activity of PGRL1 (Hertle et al., 2013; Leister and Shikanai, 2013). In agreement with the hypothesis, a redox-sensitive component of FQR activation with a midpoint redox potential of -310 mV has recently been identified (Strand et al., 2016). PGRL1 does indeed undergo transient reduction during dark-light transitions, coinciding with an increase in P_{700} oxidation and NPQ induction (Hertle et al., 2013).

1.5.5. Regulation of the Calvin–Benson cycle

Activity of CBC enzymes is regulated by multiple factors that intricately couple carbon fixation to the light reactions. Activation of Rubisco in light requires removal of inhibitory sugar phosphates from the catalytic site by the auxiliary enzyme Rubisco activase (RA) (Parry et al., 2008), as well as carbamylation of a lysine residue by an activator CO_2 (which is distinct from substrate CO_2) (Lorimer et al., 1976; Jensen, 2004). The formation of the carbamate derivative is enhanced by alkalization of the stroma as protons are pumped to the lumen at the onset of illumination (Purczeld et al., 1978). Protonation of the lumen is in turn coupled to release of Mg^{2+} cations to the stroma, and binding of Mg^{2+} stabilizes the carbamate derivative at the catalytic site of Rubisco (Jensen, 2004; Hazra et al., 2015). Two RA isoforms, α and β, are present in Arabidopsis, of which RA-α contains a conserved C-terminal Cys pair that forms a regulatory disulfide. Cleavage of the disulfide by TRX-*f* in light activates RA-α and, consequently, contributes to activation of Rubisco (Zhang et al., 2001; Portis et al., 2008). In darkness Rubisco is inactivated through re-binding

of inhibitors, which prevent interaction with RuBP, and through depletion of Mg^{2+} from the stroma (Andersson, 2008).

Changes in stromal ion concentrations also regulate the activities of CBC enzymes. Free Ca^{2+} is released to the stroma upon the onset of darkness (Sai and Johnson, 2002), which is known to inhibit the catalytic activity of FBPase and SBPase (Charles and Halliwell, 1980; Hochmal et al., 2015). Accumulation of several intermediate metabolites has also been shown to inhibit CBC activity (Gardemann et al., 1983).

Moreover, several CBC enzymes are light-dependently activated by thioredoxin-mediated reduction of disulfides, which further couples CBC activity to that of the light reactions (Buchanan, 2016). Four TRX-regulated CBC enzymes were identified in the 1970's; FBPase, SBPase, GAPDH and PRK. They are in a low-activity oxidized state in the dark and are activated in light by TRX-mediated reduction of an intramolecular disulfide. In addition to this, it has been suggested that all 11 CBC enzymes may be regulated by post-translational thiol modifications; disulfide formation/reduction, S-glutathionylation or S-nitrosylation (reviewed by Michelet et al., 2013). TRX-regulation of PGK has indeed been reported in cyanobacteria (Tsukamoto et al., 2013) and in Chlamydomonas (Morisse et al., 2014).

GAPDH serves as an example of the complexity of the multi-level regulation of CBC enzymes. It was the first CBC enzyme whose activation was discovered to be dependent on photosynthetically produced reductant in light (Ziegler and Ziegler, 1966). Two isoforms of GAPDH exist in land plants, GAPA and GAPB, of which only GAPB contains a C-terminal extension domain (CTE) with a disulfide-forming Cys pair that is the target of TRX-mediated light-dependent activation (Wolosiuk and Buchanan, 1978a; Sparla et al., 2002). TRX-*f* has been shown to be the most effective reductant of the GAPB disulfide (Marri et al., 2009). The active form of the enzyme is a $GAPA_2$-$GAPB_2$ heterotetramer, while oxidation of the GAPB disulfide and a low NADPH:NADH ratio, which causes NADH to bind in the catalytic site instead of NADPH, results in formation of inactive $GAPA_8GAPB_8$ oligomers (Baalmann et al., 1994; Sparla et al., 2002; Trost et al., 2006).

$GAPA_4$ homotetramers also undergo redox regulation via formation of inhibitory ternary complexes in dark with CP12 and PRK ($GAPA_4$-$CP12_4$-PRK_2). CP12, which resembles the CTE of GAPB, contains a regulatory disulfide that is oxidized in the absence of reduced TRX and NADPH, instigating CP12 to bind $GAPA_4$. This binary complex then interacts with oxidized PRK dimers to form the ternary complex where activity of both GAPDH and PRK is inhibited (Wedel et al., 1997; Marri et al., 2005; Marri et al., 2008; Lopez-Calcagno et al., 2017). PRK is homodimeric in eukaryotes, and each PRK monomer contains four conserved Cys residues. Like GAPDH, it is oxidized and inactive in darkness, and activated by TRX-*f*-mediated reduction of an N-terminal disulfide in light (Wolosiuk and Buchanan, 1978b; Brandes et al., 1996; Marri et al., 2009). More recently, it was proposed that formation of a second disulfide between C-terminal Cys residues as well as S-glutathionylation also contribute to PRK inactivation in *Chlamydomonas* (Thieulin-Pardo et al., 2015).

Two FBPase isoforms exist in plant chloroplast, and while both function as homotetramers, only one (FBPase1) is redox-sensitive (Serrato et al., 2009; Gütle et al., 2016). The redox-insensitive FBPaseII is however expressed at a very low level in leaves (Serrato et al., 2009). Oxidized FBPase maintains low activity of 20-30 %, and full activation of the phosphatase requires reduction of a disulfide by TRX-*f* (Wolosiuk and Buchanan, 1977; Jacquot et al., 1978; Jacquot et al., 1997; Gütle et al., 2016). *In vivo*, FBPase is gradually reduced as light intensity increases. However, in apparent contradiction to the *in vitro* results indicating high specificity of TRX-*f* compared to other TRX isoforms (Wolosiuk et al., 1979; Collin et al., 2003; Yoshida et al., 2015; Gütle et al., 2016), TRX-*f* knockout mutants show only moderate impairment of light-dependent FBPase reduction *in vivo* (Yoshida et al., 2015; Thormählen et al., 2015). Very recently, regulation of FBPase activity by S-nitrosylation was also reported (Serrato et al., 2018).

SBPase is structurally similar to FBPase, but functions as a homodimer and is specific to eukaryotes, having evolved from an ancestral archaeal enzyme (Gütle et al., 2016). Like FBPase, its activity is also increased under illumination by TRX-*f*-mediated disulfide reduction, but unlike FBPase, oxidized SBPase is completely inactive (Schürmann and Buchanan, 1975; Breazeale et al., 1978; Gütle et al., 2016).

1.5.6. Regulation of ROS detoxification

Photosynthetic electron transfer produces reactive oxygen species (ROS) via photoreduction of molecular oxygen (O_2) at PSII and PSI. In PSI molecular oxygen is first reduced to superoxide radicals (O_2^-), which are then converted into hydrogen peroxide (H_2O_2) and O_2 either spontaneously or through catalysis by the enzyme superoxide dismutase (SOD) (Asada, 2006). Additionally, excitation of ground state oxygen ($^3O_2$) by triplet-state chlorophyll in PSII produces highly reactive singlet oxygen ($^1O_2$) (Krieger-Liszkay et al., 2008). ROS are produced particularly in conditions where the photosynthetic electron transfer chain becomes over-reduced, such as under high irradiance, resulting in charge recombination within reaction centers. Uncontrolled ROS production causes substantial damage in the chloroplast through oxidation of lipids, nucleic acids and proteins (Asada, 2006; Krieger-Liszkay et al., 2008; Mattila et al., 2015). Importantly however, ROS also function as indicators of redox conditions in the chloroplast and signaling molecules that influence nuclear gene expression and participate in acclimatory responses to changes in environmental conditions (Dietz et al., 2016).

H_2O_2 can be enzymatically scavenged and converted to H_2O by ascorbate and ascorbate peroxidases (APXs) or by peroxiredoxins (Prxs) (Asada, 2006; Kangasjärvi et al., 2008; Awad et al., 2015). As the electrons donated to O_2 are originally derived from water in the OEC, the ascorbate-mediated detoxification process is often referred to as the water-water cycle. Based on proteomic studies, APXs have been proposed to interact with TRXs (Marchand et al., 2004) and cytosolic APX has been shown to be inactivated by TRX-*h in vitro* (Gelhaye et al., 2006). The *in vivo* significance of such interaction and whether similar TRX-mediated inactivation of the thylakoidal or stromal APX isoforms occurs in chloroplasts remains to be investigated.

Four different Prxs exist in Arabidopsis chloroplasts: PrxQ in the lumen (Petersson et al., 2006) and PrxIIE as well as two isoforms of 2-cysteine peroxiredoxins (2-CysPrx) in the stroma (Liebthal et al., 2018). The peroxiredoxin activity of all Prxs depends on N-terminal Cys residues, which upon reacting with H_2O_2 become oxidized and require re-reduction by either TRX, GRX, GSH or cyclophilins to restore activity (Dietz, 2011; Dietz, 2016). NTRC has been identified as the primary reductant of 2-

CysPrx (Perez-Ruiz et al., 2006; Kirchsteiger et al., 2009; Pulido et al., 2010), while TRX-*x* and TRX-*y* are the most efficient electron donors to PrxQ (Collin et al., 2004; Yoshida et al., 2015). How these results with stromal TRXs are compatible with the luminal localization of PrxQ (Petersson et al., 2006) is uncertain.

1.6. Other metabolic pathways in the chloroplast

In addition to photosynthetic carbon fixation, several other metabolic and biosynthetic pathways take place in the chloroplast. These include synthesis and degradation of starch, assimilation of nitrogen and sulfur as well as biosynthesis of chlorophyll and other pigments, heme, amino and fatty acids, plant hormones and purines. For reviews see Herrmann and Weaver (1999), Zrenner et al. (2006), Zeeman et al. (2010), Takahashi et al. (2011), Taniguchi and Miyake (2012), and Wang and Grimm (2015).

The three-carbon CBC intermediate metabolite G3P is a branching point of carbon metabolism: it can be exported from the CBC and transported to the cytosol to be used in synthesis of sucrose, or it can be converted to six-carbon glucose-6-phosphate (G6P), which can either be further converted to glucose-1-phosphate (G1P) to serve as a substrate (with ATP) of ADP-glucose-pyrophosphorylase (AGPase) in the initial key step of starch synthesis (Zeeman et al., 2010). G6P can also enter the *oxidative pentose phosphate pathway* (OPPP) as substrate of glucose-6-phosphate-dehydrogenase (G6PDH) in a reaction that yields NADPH independently of photosynthetic light reactions (i.e. in darkness). Importantly, intermediary metabolites of the OPPP are precursors for several biosynthetic pathways: for instance, ribose-5-phosphate (R5P) is a substrate synthesis of purines, while erythrose-4-phosphate (E4P), which are also produced in the CBC, can be used in the *shikimate pathway*. (Kruger and von Schaewen, 2003). The shikimate pathway, which features the conversion of E4P and phosphoenolpyruvate (PEP) to chorismate in seven enzymatic reactions, is itself another metabolic hub in the chloroplast. Several of its intermediates serve as branch points for other metabolic pathways, including synthesis of aromatic amino acids, the phytohormones salicylic acid and jasmonate, oxylipins, vitamins K and D and galactolipids (Herrmann and Weaver, 1999).

NADPH produced photosynthetically by FNR or in the OPPP powers the exchange of reducing equivalents between the chloroplast and the cytosol through the *malate valve*, which comprises the enzymes NADP-malate dehydrogenase (NADP-MDH) in the stroma and NAD-malate dehydrogenase (NAD-MDH) in the cytosol, as well as the 2-oxoglutarate-malate transporter (OMT) on the chloroplast envelope. In order to export reducing equivalents from the chloroplast to the cytosol, NADP-MDH oxidizes NADPH to reduce stromal oxaloacetate (OAA) to malate, which can be exported to the cytosol through OMT with concomitant import of OAA. Conversely, NAD-MDH oxidizes cytosolic malate to yield OAA and NADH (Taniguchi and Miyake, 2012).

2. AIMS OF THE STUDY

The essential role of chloroplast thioredoxins in light-dependent activation of photosynthesis and several plastidial metabolic processes has been well established and extensively studied over several decades. However, most of these studies have been conducted *in vitro*, and the *in vivo* dynamics of TRX-mediated regulation in varying environmental conditions are poorly understood. Moreover, chloroplasts contain a high number of TRX isoforms and two distinct TRX systems, but their physiological significance and level of redundancy remain poorly characterized. The physiological role of the NTRC system in particular, its molecular targets and its functional interaction with the ferredoxin-TRX system have been little explored. The severe phenotype of the *ntrc* knockout mutant suggests a more pivotal regulatory role for NTRC than is currently appreciated.

Therefore, the objective of my doctoral research was to i) investigate the molecular mechanisms and interconnections of thioredoxin-mediated regulation of photosynthesis and chloroplast metabolism *in planta*, and to examine how it enables maintenance of redox homeostasis in the chloroplast during rapid fluctuations in light conditions; and ii) to identify novel target processes and specific target proteins of NTRC and to study the physiological significance of NTRC-mediated regulation *in vivo*. This information is essential when assessing the potential of using modification of plastidial thiol-regulation systems as a bioengineering tool to increase crop yields or production of biofuel.

3. METHODOLOGICAL ASPECTS

3.1. Plant material and growth conditions

Wild type *Arabidopsis thaliana* plants of the Columbia ecotype (WT Col-0 and WT Col-*gl1*) as well as various T-DNA knockout lines (Alonso et al., 2003) and overexpression lines were used in the experiments (Table 1 and Fig. 2). The *NTRC* overexpression lines were created by cloning the coding sequence of the *NTRC* gene (At2g41680) into the pGWR8 vector where it is expressed under the cauliflower mosaic virus (CaMV) 35S promoter (Rozhon et al., 2010). This construct was then used to transform *ntrc*, *ndho* and *pgr5* plants by *Agrobacterium tumefaciens* -mediated floral dipping (Clough and Bent, 1998). Transgenic plants were selected on Kanamycin-containing 0.5x Murashige–Skoog (MS) plates (Murashige and Skoog, 1962).

To generate the NTRC overexpression lines with an inactivated redox-active motif in either the reductase (NTR) domain (OE-NTRC$_{SAIS}$), TRX domain (OE-NTRC$_{SGPS}$) or both domains (OE-NTRC$_{SAISSGPS}$) used in Papers I and II, point mutations where introduced by site-directed mutagenic PCR to the *NTRC* coding sequence to mutate the cysteine residues in the redox motifs to serines (Fig. 1). Truncated *NTRC* overexpression constructs (OE-NTRd and OE-TRXd) were generated by cloning the reductase domain (amino acids 1–400 of the *NTRC* coding sequence) followed by a stop codon, as well as the TRX domain (amino acids 401–529) with an inserted chloroplast transit sequence and a start codon into the pGWR8 plasmid (Rozhon et al., 2010).

Arabidopsis plants were grown in either a short (8 h light / 16 h dark), intermediate (12 h / 12 h) or long photoperiod (16 h / 8 h) at 23 ^{o}C under 130, 200, 500 or 600 µmol photons m^{-2} s^{-1}, as specified in the papers. For the bimolecular fluorescence complementation tests (see below), wild type tobacco (*Nicotiana benthamiana*) was grown in a 16 h / 8 h photoperiod under 130 µmol photons m^{-2} s^{-1}. Mature leaves of 4–5 week old Arabidopsis plants were used in most experiments. The OE-NTRC *ndho* and OE-NTRC *pgr5* plants used in Paper III were heterozygous T2 generation plants that were first selected on kanamycin-containing plants, then transferred to soil and grown for 4–5 weeks before usage in experiments. As control, WT Col-0, WT Col-*gl1* (genetic background of

the *pgr5* mutant) and OE-NTRC plants were grown on antibiotic-free MS plates for an equivalent time.

Table 1. *Arabidopsis thaliana* lines used in the experiments. OE=overexpression. *: in *ndho*, the lack of the NdhO subunit results in complete absence of the NDH complex (Rumeau et al., 2005). GL1, which is lacking in WT Col-*gl1*, *pgr5* and OE-NTRC *pgr5*, is a trichome differentiation protein whose absence results in a hairless phenotype (Oppenheimer et al., 1991).

Line	**Proteins affected**	**Genetic modification**	**Genetic background**	**Reference**
WT Col-0	-	-		
WT Col-*gl1*	GL1	Mutation (natural polymorphism)	WT Col-0	
ntrc (SALK_096776)	NTRC	T-DNA knockout	WT Col-0	(Lepistö et al., 2009)
ndho (SALK_ 068922)	NdhO*	T-DNA knockout	WT Col-0	(Rumeau et al., 2005)
pgr5	PGR5, GL1	Point mutation	WT Col-*gl1*	(Munekage et al., 2002)
stn7 (SALK_073254)	STN7	T-DNA knockout	WT Col-0	(Bellafiore et al., 2005)
OE-NTRC	NTRC	OE of *NTRC*	*ntrc*	Paper I
OE-NTRC$_{SAIS}$	NTRC	C217S and C220S mutations in the NTR domain, OE	*ntrc*	Paper I
OE-NTRC$_{SGPS}$	NTRC	C454S and C457S mutations in the TRX-domain, OE	*ntrc*	Paper I
OE-NTRC$_{SAISSGPS}$	NTRC	C217S, C220S, C454S and C457S mutations, OE	*ntrc*	Paper II
OE-NTRd	NTRC	OE of *NTRC* reductase domain	*ntrc*	Paper I
OE-TRXd	NTRC	OE of *NTRC* TRX domain	*ntrc*	Paper I
OE-NTRC *ndho*	NTRC, NdhO	OE of *NTRC*	*ndho*	Paper III
OE-NTRC *pgr5*	NTRC, PGR5, GL1	OE of *NTRC*	*pgr5*	Paper III

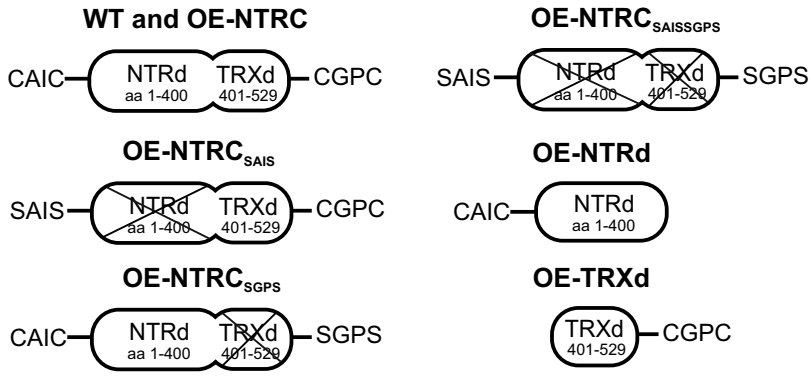

Figure 2. Schematic representation of the genotypes of NTRC overexpression lines (Papers I and II).

CAIC and CGPC denote the amino acid sequences in the redox-active motifs of the reductase and TRX domains, respectively, in the NTRC polypeptide, while SAIS and SGPS denote the mutated forms of those motifs, where the Cys residues have been changed to Ser residues. Crosses represent inactivated domains.

3.2. Analysis of protein content and *in vivo* redox states

Soluble proteins and thylakoid membranes were extracted from leaves as described in Lepistö et al. (2009). Protein content was determined with a Bio-Rad protein assay kit and chlorophyll content according to (Porra et al., 1989).

In papers II and III, the *in vivo* redox states of several chloroplast proteins were analyzed by a method using alkylation of protein thiols with a high-molecular weight agent and subsequent mobility shift assays by gel electrophoresis, similarly to the method described by (Peled-Zehavi et al., 2010). After appropriate light treatments as described in the papers, leaves were frozen in liquid nitrogen and total protein extract was precipitated with 10 % trichloric acid (TCA) and washed with acetone. *In vivo* free thiols were then alkylated with a low-molecular weight agent N-ethylmaleimide (NEM) and *in vivo* disulfides cleaved with 100 mM DTT, after which proteins were TCA-precipitated for a second time. The reduced thiols were then alkylated with 10 mM methoxypolyethylene glycol maleimide M_n 5000 (MAL-PEG, Sigma-Aldrich). Due to the alkylation-induced increase in molecular weight, the *in vivo* oxidized forms of a protein migrate slower than the reduced forms in gel electrophoresis, and the different forms can be detected by a specific antibody. In TRXs the

redox active thiols are located in close proximity to each other, which may hinder the binding of a large molecule such as MAL-PEG and prevent accurate determination of the redox state (Peled-Zehavi et al., 2010). Therefore, in order to analyze the redox states of free TRXs, thiols in TCA-precipitated leaf extracts were alkylated with 10 mM 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS), which increases the molecular mass of a protein by 500 Da (Motohashi et al., 2001). Hence, the *in vivo* reduced form becomes alkylated by AMS and its migration in electrophoresis is slightly slowed down. Accordingly, the different redox forms can be detected by specific antibody.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with specific antibodies was performed as described in Lepistö et al. (2009) and in Papers I–IV.

3.3. Protein interaction assays

3.3.1. Yeast-two-hybrid

A *Saccharomyces cerevisiae* yeast strain CY306, where endogenous yeast genes encoding TRXs have been deleted (Vignols et al., 2005) was used in the yeast-two-hybrid interaction assays in Paper I. The wild type coding sequence of NTRC as well as NTRC sequences with the mutations and truncations described in Paper I were cloned into the pGBK.T7 and pGAD.T7 binary vectors (Clontech), which were then used to transform CY306 cells with a method using lithium acetate as described by Vignols et al. (2005) and Lepistö et al. (2013). Interactions were screened on YNB medium plates lacking tryptophan, leucine and histidine.

3.3.2. Bimolecular fluorescence complementation

The bimolecular fluorescence complementation (BiFC) tests in Papers II and III were conducted by cloning the coding sequences of the proteins to be tested into pSPYNE-35S or pSPYCE-35S binary vectors, in which they form fusion constructs with the N- or C-terminal fragment of the yellow fluorescent protein (YFP), respectively (Walter et al., 2004). These constructs were then used to transform *Agrobacterium tumefaciens* cells

from the GV3101 strain by electroporation, and the transformant GV3101 cells were subsequently infiltrated into 4-weeks-old *N. benthamiana* leaves as described by (Waadt and Kudla, 2008). YFP fluorescence was imaged from the tobacco leaves 3–5 days after infiltration with the Zeiss LSM510 (Paper II) or LSM780 (Paper III) laser scanning confocal microscopes. YFP fragments fused to chloroplast transit peptide sequences and negative results with proteins of similar structure and/or subcellular locations to interacting proteins were used as negative controls (Kudla and Bock, 2016).

3.3.3. Co-immunoprecipitation and mass spectrometry

The Pierce Co-immunoprecipitation (Co-IP) kit (Thermo-Fisher Scientific) with an AminoLink Plus Resin (Thermo Fisher) containing immobilized NTRC antibody (Lepistö et al., 2009) were used to screen for potential NTRC interactors as described in Paper II. Co-IP eluates from total WT Col-0, *ntrc* and OE-NTRC leaf extracts were then separated in SDS-PAGE and immunoblotted with specific antibodies to identify the interactors (Paper II). In Paper III, the Co-IP eluates were digested by in-gel trypsin digestion and the resultant peptides were analyzed with a Q Exactive Hybrid Quadruple-Orbitrap mass spectrometer (Thermo-Fisher Scientific). MS/MS spectra were analyzed with the Mascot (v.2.4) (Matrix Science) search engine and analyzed with Proteome Discoverer (v.1.4) (Thermo-Scientific) as described in (Trotta et al., 2016). To identify potential NTRC interactors, the detected peptides were filtered to include only plastid-localized proteins from which at least two unique peptides were detected in WT and/or OE-NTRC eluates but not in *ntrc* eluates. 100 most abundant proteins detected using these restrictions are listed in Suppl. Table 2 of Paper III.

3.4. Spectrometry

3.4.1. Analysis of Chlorophyll a fluorescence

To determine parameters related to PSII activity and energy dissipation, the Dual-PAM 100 spectrometer (Walz) was used to monitor chlorophyll a fluorescence *in vivo* from detached leaves in Papers I–IV. Specific experimental details are provided in the Papers. The parameters for PSII

quantum yield (Y(II), NPQ and Q_A redox state (1-qL) are outlined in (Bilger and Björkman, 1990; Kramer et al., 2004a; Klughammer and Schreiber, 2008a) and were calculated with the Dual-PAM software.

In Paper IV, determination of state transition capacity (qT), was also performed with the Dual-PAM 100, similarly to the experiment described by Jensen et al. (2000). Dark-adapted leaves were illuminated with 35 µmol photons m^{-2} s^{-1} of blue actinic light for 20 min, after which an Fm´ value in state II (Fm2) was determined by a saturating pulse. In addition to blue actinic light, far red light was then switched on for 20 min, after which Fm´´ in state I (Fm1) was determined. The qT parameter was calculated as (Fm1-Fm2)/Fm2 (Jensen et al., 2000; Ruban and Johnson, 2009).

In Paper III, the Multicolor-PAM fluorometer (Walz) (Schreiber et al., 2012) was used to monitor the post-illumination fluorescence rise (PIFR). To induce a PIFR, dark-adapted detached leaves were illuminated with a white actinic light of 67 µmol photons m^{-2} s^{-1} for 500 s. A measuring beam at the wavelength of 480 nm and an intensity of 0.2 μmol photons m^{-2} s^{-1}, pulsing at a 1000 Hz frequency was used to record fluorescence changes. The traces were normalized with the Fm value obtained by a saturating pulse at the beginning of each measurement. The Multicolor-PAM was also used to record the OJIP transients from dark-adapted detached leaves and leaves pre-illuminated with far red light, in order to estimate the redox state of the PQ pool in darkness. The difference between the F/Fm values at J phase of the transient (3 ms after onset of saturating illumination) in dark-adapted and FR-illuminated leaves provides an estimate of the relative size of the reduced fraction of the PQ pool in darkness (Toth et al., 2007).

The relative sizes of PSII and PSI antenna cross sections were determined by measuring Chl fluorescence emission spectra at 77 K with an Ocean Optics QE Pro Spectrometer. Thylakoid extracts from dark-adapted leaves or light-treated leaves were diluted to 5 µg of Chl ml^{-1}, frozen in liquid N_2 and excited at 440 nm. The acquired spectra were normalized to the fluorescence level at 685 nm.

3.4.2. Analysis P_{700}, PC, and Fd redox changes

In Papers II and III, PSI quantum yield, P_{700} oxidation (Y(ND) and PSI acceptor side limitation (Y(NA)) were measured by monitoring the changes in absorbance difference at 875 and 830 nm simultaneously with chlorophyll a fluorescence with the Dual-PAM 100 according to (Klughammer and Schreiber, 2008b). In papers III and IV, the re-reduction kinetics of P_{700} after FR-induced oxidation and the deconvoluted P700, plastocyanin and ferredoxin redox kinetics were determined by monitoring four absorbance difference signals at near-infrared (NIR) wavelenghts from detached leaves using the Dual/Klas-100 NIR spectrometer (Walz) as described in (Klughammer and Schreiber, 2016; Schreiber, 2017).

3.4.3. Analysis of the electrochromic shift (ECS)

In Papers II, III and IV, the difference in absorbance between 550 and 515 nm, which is known as the electrochromic shift (ECS) (Junge and Witt, 1968), was monitored with the Dual-PAM 100 and its P515/535 accessory module (Walz) (Schreiber and Klughammer, 2008; Klughammer et al., 2013) in order to determine the magnitude of the proton motive force (*pmf*) and proton conductivity of the thylakoid membrane (g_{H+}) *in vivo*. Total *pmf* was estimated as the light-induced difference in the ECS signal in detached leaves normalized with the maximal ECS value obtained by a 20 µs flash of 10 000 µmol photons m^{-2} s^{-1} at the beginning of each measurement. The g_{H+} parameter was determined as the inverse of the time constant of a first order fit to the decay kinetics of the ECS signal upon cessation of illumination (Cruz et al., 2001). The dark-conductivity of the ATP synthase in Paper II was measured by monitoring the kinetics of ECS decay after a non-saturating single turnover flash in dark-adapted leaves in comparison to pre-illuminated leaves (Kramer and Crofts, 1989; Kanazawa and Kramer, 2002). Partitioning of total *pmf* into its ΔpH and Δψ constituents in Papers III and IV was determined by measuring the post-illumination difference between the ECS dark baseline and the magnitude of the ECS signal attributable to electric field inversion (Cruz et al., 2001) using the Dual-PAM 100 as described by (Schreiber and Klughammer, 2008).

In Paper III, the kinetics of *pmf* generation during dark-to-light transitions and increases in light intensity were monitored by administering 400 ms

dark intervals during illumination. First order fits to the ECS decay kinetics during the dark intervals were used to calculate g_{H+} as well as the ECS dark baseline, which was deducted from total ECS to obtain reliable *pmf* values.

3.5. Gas exchange measurements

The photosynthetic CO_2 fixation rate, stomatal conductance and intercellular CO_2 concentrations in Paper II were measured from intact rosettes with a LI-6400 XT portable photosynthesis system (LI-COR). The light response curve of CO_2 fixation was obtained by fitting the results to the model described in (Kaipiainen, 2009). The fit was then used to calculate the maximum gross photosynthetic rate, light compensation point and dark respiration rate according to (Lobo et al., 2014).

3.6. Microscopy

For examination of chloroplast ultrastructure in Paper I, plant leaves were fixed in 3 % glutaraldehyde and subsequently stained as described in (Pätsikkä et al., 2002). The preparates were then imaged with a JEM-1400 Plus TEM transmission electron microscope (JEOL). For the BiFC tests, tobacco leaf discs were examined with a Zeiss LSM510 META (Paper II) or LSM780 (Paper III) laser scanning confocal microscope using a 20x/0.8 objective. YFP and chlorophyll a were simultaneously excited at 514 nm by an argon diode laser, and fluorescence emission was detected through 530–600 nm and 650–710 nm bypass filters, respectively. A pinhole value of 1 AU was used for both channels in all images presented in Papers II and III.

4. OVERVIEW OF THE RESULTS

4.1. The effects of NTRC overexpression and deficiency on plant phenotype

The *ntrc* knockout line of *Arabidopsis* has a severely stunted low-chlorophyll content phenotype due to impaired chlorophyll biosynthesis (Stenbaek et al., 2008; Richter et al., 2013; Perez-Ruiz et al., 2014) and chloroplast development (Paper I) (Lepistö et al., 2009). The phenotype of the mutant is particularly pronounced when grown in a short photoperiod (Papers I and II) (Lepistö et al., 2013). It suffers from decreased rates of photosynthetic electron transfer as well as impaired carbon fixation and starch accumulation (Paper I) (Perez-Ruiz et al., 2006; Lepistö et al., 2009; Michalska et al., 2009; Pulido et al., 2010; Lepistö et al., 2013). These highly pleiotropic effects of *NTRC* knockout reflect the importance of NTRC-mediated regulation for normal functioning of the chloroplast. It also suggests a high number of different target processes and proteins, many of which remain unstudied.

Characterization of *Arabidopsis* plants overexpressing the *NTRC* gene at 15–20-fold levels compared to WT in *ntrc* knockout mutant background (OE-NTRC) revealed that not only was the pleiotropic *ntrc* phenotype complemented, but leaf growth and biomass yield as well as accumulation of starch were enhanced (Paper I). Enhanced leaf growth was enabled by c.a. 20 % higher carbon fixation rate when compared to WT, and increased photosynthetic electron transfer rate in low light conditions (Paper II), which likely allowed continuation of growth of shaded mature leaves. In contrast to the low number and disturbed morphology and ultrastructure of *ntrc* chloroplasts, OE-NTRC mesophyll cells contained morphologically WT-like plastids with intact thylakoid membrane structure (Paper I).

4.2. Identification of NTRC target processes and proteins

In order to screen for novel potential target proteins of NTRC, a co-immunoprecipitation assay (Co-IP) was conducted using an antibody raised against NTRC, and eluates from WT, OE-NTRC and *ntrc* total leaf extracts were analyzed by mass spectrometry (Paper III). To exclude false positives, the detected peptides were then filtered to include only those

from plastid-localized proteins that were not eluted from *ntrc* extracts (Suppl. Table 2 in Paper III).

A wide variety of previously unidentified putative NTRC interactors and novel target processes of NTRC were identified by this screening (Fig. 3), as may be expected based on the pleiotropic phenotype of *ntrc*. Components of cyclic electron transfer pathways were strongly represented, as several subunits of the NADH dehydrogenase-like (NDH) complex (NdhH, Ndh-48, NdhS, NdhU) and PGR5 were detected. Several proteins involved in redox regulation, including HCF164, ACHT2, SOQ1, TRX-*f*1 and a recently identified (Fristedt, 2017) but uncharacterized TRX-like thylakoidal protein At5g03880 were also found to co-precipitate with NTRC, suggesting extensive connectivity within the redox regulatory network of the chloroplast.

Photosynthetic electron transfer and light harvesting in thylakoid membranes may also have thiol-regulated components, as the monomeric Lhcb protein CP29 as well as several PSII subunits were detected in WT and OE-NTRC eluates. Similarly, plastidial translation was highlighted as a process potentially regulated by NTRC, as several components of plastidial ribosomes were identified. Several enzymes in starch synthesis and degradation, proteolysis, sulfate assimilation and lipid metabolism pathways also co-precipitated with NTRC (Fig. 3).

Established NTRC interactors such as 2-CysPrx A and B (Perez-Ruiz and Cejudo, 2009; Bernal-Bayard et al., 2014) and the chlorophyll biosynthesis enzymes Mg-protoporphyrin IX methyltransferase (CHLM) and Glutamyl-tRNA reductase (GLUTR) (Richter et al., 2013) were at the top of the list of proteins detected by Co-IP technique, suggesting that the method specifically collects proteins regulated by NTRC. Highly abundant proteins are challenging to conclusively identify with this method, as they often also contaminate the *ntrc* eluates due to their high quantity. Some such proteins, although detected in *ntrc* eluates, were enriched in WT and OE-NTRC eluates, suggesting specific interaction with NTRC (Suppl. Table 3 in Paper III). These proteins included the TRX-regulated CBC enzymes $GAPDH_B$, FBPase and PRK, the shikimate pathway enzyme 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP), and ADP-glucose pyrophosphorylase (APS1), an established NTRC target in the starch biosynthetic pathway (Michalska et al., 2009).

It is also worth noting that it is unlikely that all of the proteins identified by the Co-IP/MS screen would directly be NTRC target proteins. For instance, even though several subunits of the NDH complex co-precipitated with NTRC, most of them likely did so due to reciprocal interactions with a single or a few genuine TRX target protein(s).

The putative interactions between NTRC and NdhS, PGR5, TRX-*f*1, FBPase, and PRK were also confirmed by BiFC tests (Papers II and III).

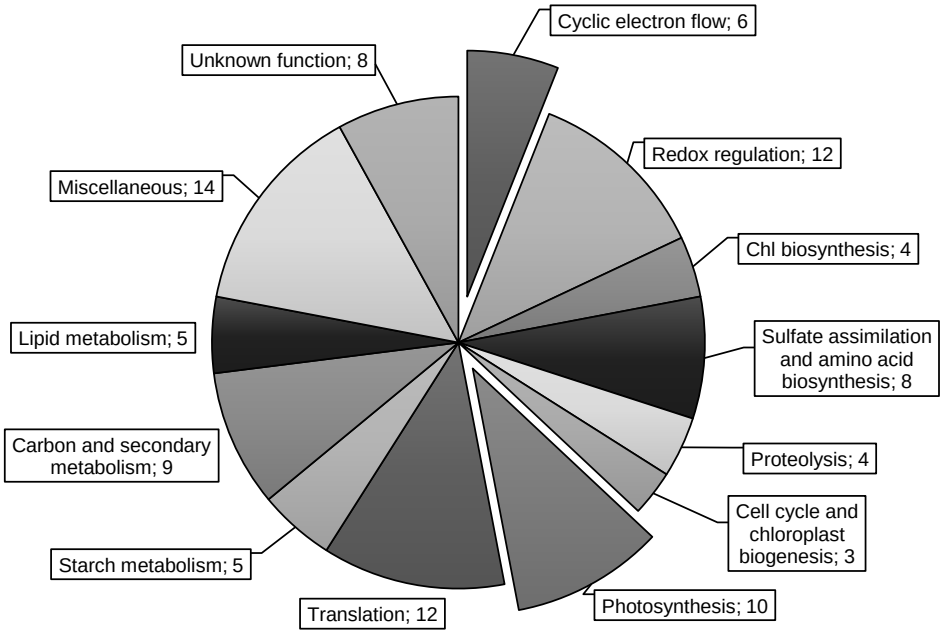

Figure 3. Classification of novel NTRC target candidates based on Co-IP and MS.

One hundred chloroplast proteins present in WT and/or OE-NTRC eluates but absent from *ntrc* eluates are classified in this figure. For a description of experimental procedures see 3.3.3, and for a detailed list of identified potential interactors see Suppl. Table 2 in Paper III.

4.3. Dimeric NTRC is an effective redox regulator in conditions where availability of light limits photosynthesis

In addition to photosynthetically produced NADPH, the OPPP produce NADPH also in darkness and in low light conditions (Perez-Ruiz et al., 2006; Geigenberger et al., 2017), suggesting that NTRC is active also in these conditions. In order to test this hypothesis, I proceeded to investigate

how the *in vivo* redox state of NTRC is affected by the availability and intensity of light. Mobility shift assays indicated that a distinct proportion of the NTRC pool is maintained in a reduced, active state in darkness, low light, growth light and high light conditions, as well as during dark-to-light transitions. Remarkably, this is also true in OE-NTRC, where NTRC content is 15–20-fold higher than in WT (Fig.1 in Paper III). The activation state of the NTRC pool is therefore likely independent of light conditions. TRX-*f*, in contrast, was oxidized (inactive) in darkness, only a small reduced pool is present in low light, while most TRX-*f* proteins are reduced in growth light conditions (Fig. 4B in Paper II). These results indicated that NTRC is a potent reductant of chloroplast TRX targets in darkness, low light and during early photosynthetic induction, while the Fd-TRX system requires at least a moderate intensity of illumination to be fully activated.

Overexpression of NTRC increases the total reducing capacity of the chloroplast TRX network, which overrides the wild-type dynamics of light-dependent redox regulation. Therefore, many TRX-regulated proteins are constantly maintained in an active state in OE-NTRC plants (Paper II). 2-Cysteine peroxiredoxins (2-Cys Prx), whose reduction has been established to primarily depend on NTRC (Kirchsteiger et al., 2009; Pulido et al., 2010), provide a good example of a highly activated protein in OE-NTRC leaves. In WT 2-Cys Prxs were kept reduced in low light by NTRC, but mostly oxidized proteins accumulated under higher irradiance (Paper II). In contrast, mainly active 2-Cys Prxs were detected in all light conditions in OE-NTRC, while in *ntrc* the reduction of 2-Cys Prx was impaired (Fig. 7A in Paper II).

The functional form of NTRC has been suggested to be a homodimer (Perez-Ruiz et al., 2009). I therefore asked whether dimerization, and hence the activation state of NTRC, is dependent of the redox states of the active site cysteines in NTR and/or TRX domains. I observed that transgenic NTRC protein with a mutated redox active site in the NTR ($NTRC_{SAIS}$) or TRX domain ($NTRC_{SGPS}$) dimerized normally *in planta*, but truncated NTRC consisting of only the NTR or TRX domain did not form dimers (Paper I). The ability of NTRC to self-interact was verified by yeast-two-hybrid tests. Self-interaction also occurred between wild-type NTRC and $NTRC_{SGPS}$, but not between NTRC and the truncated forms (Paper I). These observations clearly demonstrated that formation of the functional dimeric

form does not depend on the thiol redox states of the redox active Cys pairs in the NTR or TRX domains, and that a full-length NTRC polypeptide is required for dimerization.

4.4. NTRC regulates electron transfer in thylakoid membranes

One third of the novel putative NTRC target proteins identified by Co-IP/MS are localized to the thylakoid membrane and function in photosynthetic electron transfer and its regulatory mechanisms (Fig. 3 and Suppl. Table 2 in Paper III). Moreover, other reports have in recent years begun to reveal the importance of TRX-mediated redox regulation both in thylakoids and in the lumen (Hall et al., 2010; Motohashi and Hisabori, 2010; Järvi et al., 2013; Kang and Wang, 2016). While thiol-modulation of the activity of the chloroplast ATP synthase has been well-established for decades (Hisabori et al., 2013), non-photochemical quenching (Brooks et al., 2013; Yu et al., 2014; Simionato et al., 2015; Naranjo et al., 2016; Da et al., 2017), LHCII phosphorylation (Rintamäki et al., 2000) as well as cyclic electron transfer (Courteille et al., 2013; Hertle et al., 2013; Strand et al., 2016) have more recently been identified as thiol-regulated processes. Here, I present new evidence for involvement of NTRC in the regulation NPQ, state transitions, the ATP synthase and NDH-dependent cyclic electron transfer.

4.4.1. NTRC is involved in downregulation of non-photochemical quenching

The *ntrc* mutant exhibited elevated NPQ in all light conditions despite having ΔpH similar to WT in growth and high light (Fig. 1 in Paper IV). In low light impaired activation of the ATP synthase and consequent high ΔpH (Papers II and IV), partly explains the elevation of NPQ. Conversely, in OE-NTRC ΔpH was increased in all light conditions but it resulted in elevated NPQ only during the first ~30 s of light-to-dark transitions, after which NPQ was similar to WT in growth light and lower than WT in high light (Papers II–IV, summarized in Fig. 4).

Increased acidification of the lumen induces the energy-dependent qE component of NPQ via protonation of the PsbS protein (Li et al., 2000) as

well as activation of the xanthophyll cycle enzyme VDE and consequent accumulation of zeaxanthin (Zx) (Niyogi et al., 1998; Jahns et al., 2009; Johnson et al., 2009). An increased amount of PsbS was detected in *ntrc* (Fig. 1E in Paper IV), which likely contributes to increased NPQ, as shown previously (Li et al., 2002; Zia et al., 2011). In line with the high NPQ, a significant increase in Zx content was also measured in *ntrc* in both growth and high light (Naranjo et al., 2016) (Paper IV) (Naranjo et al., 2016). A higher level of Zx was observed in high light in OE-NTRC in accordance with the elevated ΔpH, yet NPQ was lower in comparison to WT (figures 1 and 2 in Paper IV). These results suggest existence of a mechanism of NPQ downregulation that is dependent on stromal redox state (or NTRC specifically) but independent of ΔpH (Paper IV). Importantly, the post-illumination relaxation of NPQ in *ntrc* was markedly slower than in WT (Fig. 1 in Paper IV). SOQ1, a TRX-like protein in thylakoid membranes that I identified as a putative NTRC interactor by Co-IP/MS (Paper III), has been shown to function as a suppressor of a sustained NPQ component denoted qH that is independent of PsbS or luminal acidification (Brooks et al., 2013). Therefore, the ΔpH-independent elevation of NPQ in *ntrc* may at least partially be caused by impairment of SOQ1-dependent NPQ suppression, while increased SOQ1 activity may explain the downregulation of NPQ in OE-NTRC.

Low light	**NPQ**	**ΔpH**	**[Zx]**	**[PsbS]**
WT	Low	Low	N.D	Normal
ntrc	High	High	N.D	High
OE-NTRC	Low	Mod.	N.D	Normal

Growth light	**NPQ**	**ΔpH**	**[Zx]**	**[PsbS]**
WT	Mod.	Mod.	Low	Normal
ntrc	High	Mod.	High	High
OE-NTRC	Mod.	High	Mod.	Normal

High light	**NPQ**	**ΔpH**	**[Zx]**	**[PsbS]**
WT	Mod.	Mod.	Mod.	Normal
ntrc	High	Mod.	High	High
OE-NTRC	Lower	High	Higher	Normal

Figure 4. Summary of the factors affecting the induction of qE in WT, *ntrc* and OE-NTRC. Green colour depicts low values or reduced state, orange represents moderate values and red high values or oxidized redox state. Colour assignments are based on measurements of NPQ, ΔpH, Zx content [Zx], and PsbS content [PsbS] in Paper IV. N.D=not determined.

4.4.2. NTRC affects the capacity of state transitions

Reduction of PQ and binding of PQH_2 to Cyt b_6f triggers STN7-mediated phosphorylation of LHCII and contributes to its subsequent association with PSI in state II transitions (Vener et al., 1997; Bellafiore et al., 2005; Shapiguzov et al., 2016). Phosphorylated LHCII proteins accumulate under low light intensity (Rintamäki et al., 1997), whereas the STN7 kinase has been shown to be inactivated by TRXs under higher light intensities (Rintamäki et al., 2000; Lemeille et al., 2009).

Therefore I examined whether modification of chloroplast thiol redox state affects the level of LHCII phosphorylation in NTRC-transgenic lines. NDH-dependent chlororespiration reduces the PQ pool in dark-adapted OE-NTRC leaves (see 4.4.4), which resulted in dark-phosphorylation of LHCII in OE-NTRC thylakoids (Fig. 3A in Paper III). No dark-phosphorylation was detected in WT or *ntrc*. Measurement of chlorophyll fluorescence emission spectra at 77K confirmed that the relative size of the PSI antenna cross section was already increased in dark-adapted OE-NTRC leaves, and similar to illuminated leaves. In WT, as reported earlier (Bennett et al., 1980), an increase in PSI antenna cross section was observed in light (Fig. 3D in Paper IV). Thus, the photosynthetic electron transfer chain in OE-NTRC thylakoids is in state II in darkness. In *ntrc* the relative size of PSI antenna was decreased in both dark-adapted and illuminated leaves in comparison to WT (Fig. 3D in Paper IV). This is likely explained by the observations that the ratio of functional PSI/PSII, as measured by electron paramagnetic resonance (EPR) spectroscopy (Fig. 4 in Paper IV), as well as PSI protein content (Fig. 4 in Paper III), were significantly lowered in *ntrc*. This obviously weakens the mutant's ability to effectively balance absorbed excitation energy between PSII and PSI. Accordingly, the OE-NTRC line had a higher and *ntrc* an impaired capacity of state transitions. OE-NTRC and *ntrc* also showed increased and decreased rapidness of state transitions, respectively (Fig. 3 in Paper IV).

4.4.3. NTRC regulates the activity of the chloroplast ATP synthase

In order to avoid futile reverse reactions, the chloroplast ATP synthase is maintained in an inactive state in darkness through formation of a disulfide bridge in the $CF_1\gamma$ subunit, whose reductive cleavage upon illumination by

TRX contributes to activation of the ATP synthase (Hisabori et al., 2013). I wanted to know if regulation of the ATP synthase is affected by altered NTRC content. To this end, I conducted mobility shift assays with MAL-PEG labelled leaf extracts and an antibody specific to $CF_1\gamma$, which revealed that $CF_1\gamma$ was oxidized in darkness and completely reduced in illuminated WT leaves, but c.a. 50 % of the $CF_1\gamma$ pool was reduced already in darkness in OE-NTRC, while in *ntrc* the reduction of $CF_1\gamma$ was impaired in low light (Fig. 6B in Paper II).

Accordingly, analysis of the post-illumination decay of the electrochromic shift (ECS) signal revealed that the conductivity of the thylakoid membrane to protons (the g_H+ parameter), which primarily depends on the activity the ATP synthase (Kanazawa and Kramer, 2002; Cruz et al., 2005), was impaired under low irradiance in the *ntrc* mutant (Fig. 6A in Paper II and Fig. 7B in Paper III), resulting in dramatically increased *pmf* (Papers III and IV) and consequently, induction of NPQ as discussed above. In growth light or higher intensity light normal g_H+ was observed, in correlation with full reduction of $CF_1\gamma$ in those conditions (Paper II). In contrast, overexpression of NTRC resulted in activation of the ATP synthase also in darkness; high g_H+ was measured following a non-saturating single-turnover flash immediately after dark adaption (Papers II and III).

These results strongly implied that NTRC can directly reduce $CF_1\gamma$. Accordingly, NTRC was found to interact with $CF_1\gamma$ in bimolecular fluorescence complementation (BiFC) tests in *Nicotiana benthamiana* leaves (Fig. 6C in Paper II). $CF_1\gamma$ was also eluted in Co-IP and detected with an antibody (Fig. 5E in Paper II) as well as mass spectrometry (Suppl. Dataset 1 in Paper III). $CF_1\gamma$ also interacted with its canonical reductant TRX-*f*1 in BiFC, suggesting cooperative regulation of ATP synthase activation state by the two plastidial TRX systems.

Apart from activation via reduction of $CF_1\gamma$, TRXs may control the activity of the ATP synthase by an additional mechanism in high light. In WT plants, thylakoid proton conductivity is decreased upon transitions to high irradiance, indicating downregulation of the ATP synthase (Fig. 7B in Paper III). As reported previously (Avenson et al., 2005; Suorsa et al., 2012), the *pgr5* mutant is unable to control the conductivity of the thylakoid membrane to protons when shifted from low to high light, which results in loss of *pmf* and coincides with impairment of the ability to induce NPQ or

photosynthetic control at Cyt b_6f (Figures 7D–E, 8B, and 9B in Paper III). In contrast, the decrease in thylakoid conductivity to protons was stronger in OE-NTRC in comparison to WT, but not in a line overexpressing *NTRC* in *pgr5* background (Fig. 7 in Paper III). Downregulation of ATP synthase activity was not mediated by oxidation of $CF_1\gamma$, as it remained fully reduced during low-to-high light transition (Suppl. Fig. 3B in Paper III). These results support the hypothesis that the PGR5 protein is required for inhibition of ATP synthase activity in high light (Avenson et al., 2005; Tikkanen et al., 2015; Kanazawa et al., 2017; Armbruster et al., 2017), and importantly, that this function of PGR5 is regulated through stromal TRX systems. In agreement with this view, PGR5 co-precipitated with NTRC (Suppl. Table 2 in Paper III) and interacted with NTRC in BiFC (Fig. 10 in Paper III), while PGR5 protein content was decreased in *ntrc* (Fig. 4 in Paper III).

Interestingly, despite elevated activity of the ATP synthase in OE-NTRC *pgr5*, overexpression of NTRC in *pgr5* background allowed maintenance of the *pmf* at wild type-levels in high light (Fig. 7C in Paper III), most likely through enhanced activation of NDH-dependent cyclic electron transfer (see below). However, this was not sufficient to induce photosynthetic control at Cyt *b6f* in OE-NTRC *pgr5*, as electron transfer to PSI could not be limited (Fig. 9B in Paper III). As a consequence, fluctuating light conditions were only marginally less damaging to PSI activity in OE-NTRC *pgr5* than in *pgr5* (Fig. 9A in Paper III). This result suggests that the PGR5 protein is directly required to induce photosynthetic control.

4.4.4. Regulation of NDH-dependent cyclic electron transfer and the proton motive force by NTRC

As several components of the chloroplast NDH complex as well as PGR5 were identified as putative NTRC interactors by Co-IP/MS (Paper III), I investigated if the capacity to perform cyclic electron transfer around PSI was altered in NTRC overexpression or knockout lines. By measurement of the post-illumination Chl a fluorescence rise (PIFR), estimation of the redox state of the PQ pool in darkness, measurement of the kinetics of *pmf* generation, Chl fluorescence, and P700 oxidation during changes in light intensity, as well as protein–protein interaction assays, I showed in Paper

III that NTRC regulates the activity of the NDH-dependent pathway of cyclic electron transfer. The molecular mechanism likely involves a TRX target in vicinity of the ferredoxin binding and oxidation site on the stromal side of the NDH complex (Yamamoto et al., 2011; Peltier et al., 2016), and possibly involves the NdhS subunit.

The PIFR has been proposed to derive from non-photochemical reduction of the PQ pool via the NDH complex (Burrows et al., 1998; Gotoh et al., 2010). NTRC overexpression caused significant increase in the PIFR, while in *ntrc* the induction of PIFR was retarded (Fig. 2 in Paper III). No PIFR was measured from *ndho* knockout plants, which lack a functional NDH complex (Rumeau et al., 2005), or from plants overexpressing NTRC in *ndho* background (OE-NTRC *ndho*) (Fig. 2 in Paper III). These results suggested that NTRC contributes to activation of NDH-mediated cyclic electron transfer / chlororespiration.

To investigate the hypothesis further, I estimated whether the redox state of the PQ pool in darkness is altered in OE-NTRC and *ntrc* by comparing the level of Chl a fluorescence in dark-adapted and FR-pre-illuminated leaves at the J phase (3 ms) of the OJIP transient (Toth et al., 2007). The measurements revealed that a significantly larger proportion of the PQ pool is reduced in both OE-NTRC and *ntrc* than in WT. A more oxidized PQ pool in darkness was detected in *ndho* and OE-NTRC *ndho*, clearly indicating that the increased non-photochemical reduction of PQ in dark in OE-NTRC depends on the NDH-complex (Fig. 3 in Paper III).

Since it has been demonstrated that the NDH complex acts as a proton pump with a 2 H^+/1 e^- stoichiometry (Strand et al., 2017), I analyzed the electrochromic shift (ECS) signal and its post-illumination decay kinetics to examine if regulation of the transthylakoid proton motive force (*pmf*) is affected by NTRC overexpression or deficiency (Figures 5 and 7 in Paper III and Fig. 5). NTRC overexpression significantly increased *pmf* in all steady state light conditions and during dark-to-light transitions. The increase in *pmf* did not derive from downregulation of the ATP synthase (Fig. 5B in Paper III) or from increased linear electron transfer rates (Fig. 6A in Paper III). These results strongly suggested that the *pmf* increase resulted from enhanced CET, except for an initial *pmf* peak. The initinal *pmf* peak was likely caused by faster induction of linear electron transfer, which was observed in OE-NTRC and persisted both in OE-NTRC *ndho*

and OE-NTRC *pgr5* (Fig. 5). The *ntrc* mutant showed unaltered *pmf* in steady state growth light, but was characterized by impaired transient *pmf* increases during photosynthetic induction (Fig. 5).

In comparison to OE-NTRC, overexpression of NTRC in *ndho* background resulted in a *pmf* decrease during a specific time period of c.a. 10–40 s into dark-to-growth light transitions, while at steady state *pmf* was mostly unaffected (Fig. 5). P_{700} oxidation was significantly lowered during this time in comparison to WT, while limitation at the PSI acceptor side was increased (Fig. 6 in Paper III). In contrast, NTRC overexpression in *pgr5* background (OE-NTRC *pgr5*) resulted in similar kinetics of *pmf* formation during dark-to-light transitions as in the OE-NTRC line, but the magnitude of *pmf* was impaired and similar to the *pgr5* mutant at steady state, due to drastically increased thylakoid proton conductivity (Fig. 7 in Paper III). These results demonstrate that the *pmf* is intricately regulated by multiple redox-dependent mechanisms in a way that allows rapid adjustment of the *pmf* according to the physiological state of the chloroplast and environmental conditions. The results indicate that NDH-mediated CET maintains photosynthetic redox balance specifically during an early phase of photosynthetic induction, and that this function is enhanced by elevation of stromal thiol redox state in OE-NTRC plants.

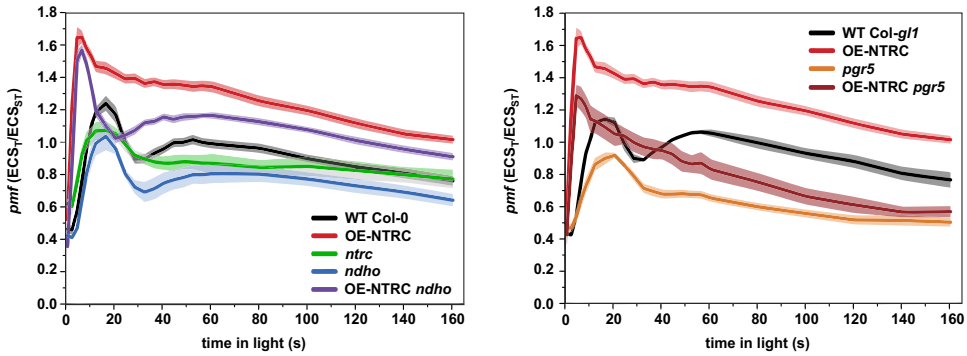

Figure 5. Proton motive force (*pmf*) during dark-to-growth light transitions. Frequent dark intervals were administered during illumination to determine the light-induced change in the ECS signal in dark-adapted leaves of WT, OE-NTRC, *ntrc*, *ndho*, OE-NTRC *ndho*, *pgr5* and OE-NTRC *pgr5*. The values were normalized with the ECS amplitude during a 20 µs saturating single turnover flash before onset of illumination (ECS_{ST}). The *pmf* values are averages from 4–16 replicate measurements ±SE. Adapted from Paper III.

In order to determine the subunit(s) of the NDH complex that could constitute the molecular target of NTRC-mediated redox regulation, I analyzed the amino acid sequences of the NDH subunits identified as putative NTRC interactors by Co-IP/MS. Subunits located on stromal side of the complex (Peltier et al., 2016), namely NdhH, Ndh-48, and NdhS, contain conserved Cys residues that may be subject to regulatory post-translational thiol modifications (Supplemental Data in Paper III). Moreover, NTRC interacted with NdhS in BiFC assays (Fig. 10 in Paper III). Mobility shift assays confirmed that NdhS contains Cys residue(s) that can undergo disulfide:dithiol exchange, and that the NdhS pool is highly oxidized in *ntrc* (Paper III). As NdhS participates in formation of the ferredoxin binding and oxidation site of the NDH complex (Yamamoto et al., 2011), it constitutes a potential and functionally logical target of thiol regulation.

4.5. NTRC is essential for redox-activation of Calvin–Benson cycle enzymes in low light

Impaired and enhanced carbon fixation rates of *ntrc* and OE-NTRC, respectively (Fig. 3 in Paper II), suggested that the activation states of Calvin–Benson cycle (CBC) enzymes may either directly or indirectly be affected by deficiency or overexpression of NTRC. Indeed, reduction of the CBC enzymes FBPase and PRK was impaired in low light conditions in *ntrc*, as revealed by MAL-PEG mobility shift assays. In OE-NTRC the *in vivo* amount of reduced FBPase was higher than in WT in all light conditions, while the amount of reduced PRK enzymes was even more dramatically increased, even in darkness (Fig. 7A in Paper II). As both NTRC and TRX-*f*1 interacted with FBPase and PRK in BiFC (Fig. 7B in Paper II) as well as in Co-IP (Fig. 5 in Paper II), it is likely that at least these CBC enzymes are directly and cooperatively regulated by both TRX systems.

Moreover, the quantum yield of PSI was decreased in low light and during dark-to-light transitions in *ntrc*, and enhanced in OE-NTRC (Fig. 2 in Paper II and figures 6 and 9 in Paper III). This was due to increased and decreased limitation of electron transfer at the acceptor-side of PSI in *ntrc* and OE-NTRC, respectively (Fig. 2 in Paper II). These results indicate that stromal

electron sinks, most importantly the CBC, failed to consume the reducing equivalents produced by the PETC in *ntrc*, while a high capacity to pull electrons downstream of PSI implied an enhanced activation state of the CBC in OE-NTRC. Oxidation of P_{700} by far red light in dark-adapted OE-NTRC was substantially faster and its re-reduction in darkness slower when compared to WT, indicating improved capacities of stromal electron sinks. The reverse was observed in *ntrc* (Fig. 5 in Paper IV). The FR-induced P_{700} oxidation kinetics in OE-NTRC closely resembled those measured from pre-illuminated WT leaves (Fig. 6 in Paper IV).

4.6. Evidence of crosstalk and partial redundancy between the NTRC and Fd-TRX systems

In order to elucidate the roles of the redox active 2-Cys motifs in the NTR and TRX domains of NTRC, we generated *Arabidopsis* lines overexpressing mutated forms of the *NTRC* gene, where both redox-active Cys residues in either the NTR domain (OE-$NTRC_{SAIS}$), TRX domain (OE-$NTRC_{SGPS}$) or in both domains (OE-$NTRC_{SAISSGPS}$) were mutated to serines. Interestingly, the OE-$NTRC_{SAIS}$ and especially the OE-$NTRC_{SGPS}$ plants showed partial recovery of the *ntrc* phenotype in terms of leaf growth, starch accumulation, photosynthetic electron transfer and carbon fixation. No phenotypic recovery was observed in OE-$NTRC_{SAISSGPS}$ plants (Fig. 6) (Papers I and II). These results strongly indicated existence of previously unknown crosstalk and redundancy between the NTRC and Fd-TRX systems; the intact reductase domain in OE-$NTRC_{SGPS}$ may be able to reduce free TRXs whose enhanced activity would partially compensate for NTRC deficiency, while the intact TRX domain in OE-$NTRC_{SAIS}$ may be reduced by FTR and thus maintain a residual reducing capacity.

In order to identify the specific TRX(s) that interact with NTRC, the electrostatic surface charge structures of the NTR and TRX domains as well 10 free plastidial TRXs were modelled *in silico*. The models revealed that the closest similarity to the strongly positive charge structure as well as to the amino acid sequence in the NTR-interacting site of the TRX domain of NTRC is found in TRX-*f*1, followed by the *f*2 and *m*1 isoforms (Fig. 11 in Paper I). Based on this analysis, we proposed TRX-*f*1 to be the most likely interactor with NTRC. Thereby, the remaining functional NTR

domain in NTRC$_{SGPS}$ line could, at least when overexpressed, enhance reduction of TRX-*f*1 and thus explain the recovery of the *ntrc* phenotype in the OE-NTRC$_{SGPS}$ line.

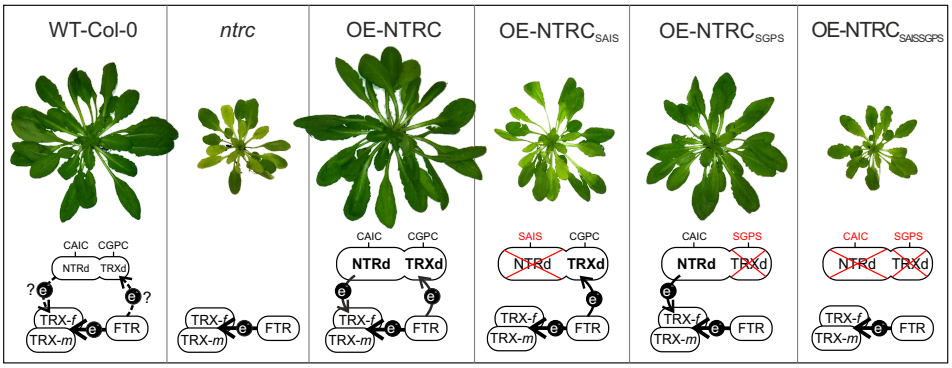

Figure 6. Phenotypes of NTRC-transgenic lines, and schematic illustrations of exchange of reducing equivalents between the Fd-TRX and NTRC systems in each of the lines (for details, see the text). Arrows indicate direction of electron flow. Plant were grown for 6 weeks in an 8 h photoperiod under 500 µmol photons m^{-2} s^{-1}. Adapted from Paper II.

In agreement with our proposal, NTRC was found to interact with TRX-*f*1 as well as TRX-*m*1 and the catalytic subunit of FTR (FTRc) in BiFC. Additionally, NTRC also interacted with TRX-*m*3, TRX-*y*1 and TRX-*x* (Fig. 4 in Paper II). Moreover, NTRC overexpression resulted in an increase in accumulation of reduced TRX-*f* in both low light and growth light (Paper II). Similar enhancement, albeit to lesser extent, was also observed in the OE-NTRC$_{SGPS}$ but not in the OE-NTRC$_{SAIS}$ line, while in *ntrc* TRX-*f* redox state was indistinguishable from WT (Fig. 4 in Paper II). In fact, TRX-*f* redox state was more oxidized in OE-NTRC$_{SAIS}$ than in WT, suggesting that the overexpressed but non-functional TRX domain in NTRC$_{SAIS}$ may compete with TRX-*f* for binding with FTR. Taken together, these results supported the hypothesis that the NTR domain of NTRC is capable of reductive activation of TRX-*f*1, and possibly other Fd-dependent TRXs (Fig. 6).

High excitation pressure at the PQ pool (1-qP), NPQ and acceptor side limitation of PSI (Y(NA)) during dark-to-low light transitions in *ntrc* and OE-NTRC$_{SAISSGPS}$ were partially alleviated by NTRC$_{SGPS}$ but not by NTRC$_{SAIS}$ overexpression (Fig. 2 in Paper II). This indicates that availability of NTR reductase activity, rather than target specificity of

TRXs, is more important for photosynthetic induction in light-limiting conditions. Interestingly however, reduction of the CBC enzymes FBPase and PRK was not recovered in either OE-NTRC$_{SAIS}$ or OE-NTRC$_{SGPS}$ in comparison to *ntrc*. Instead, in apparent contradiction with the recovery of CO_2 assimilation capacity, the redox states of these enzymes were in fact even more oxidized than in *ntrc* (Fig. 7 in Paper II). This discrepancy may at least partly be explained by the impaired reduction of 2-Cys Prxs in OE-NTRC$_{SAIS}$ and OE-NTRC$_{SGPS}$, since it has been reported that accumulation of oxidized 2-Cys Prxs in the chloroplast may inactivate CBC enzymes (Caporaletti et al., 2007; Eliyahu et al., 2015; Pérez-Ruiz et al., 2017).

5. DISCUSSION

In its natural habitat beyond laboratory growth chambers, a plant leaf experiences constant fluctuation of irradiance. Intricate mechanisms to fine-tune the redox homeostasis in chloroplasts have evolved to avoid energy-expensive futile reactions and even more importantly, photodamage to the photosynthetic apparatus and other biomolecules. The PSII reaction center protein D1 undergoes continuous turnover in light (see recent reviews by Järvi et al., 2015; and Townsend et al., 2017). Recently it has been demonstrated that also PSI and its iron sulfur clusters are especially vulnerable to over-reduction, suggesting that plants have to invest strongly in the protection of the photosynthetic machinery (Tikkanen et al., 2012; Tikkanen and Aro, 2014; Tiwari et al., 2016). Post-translational thiol modifications provide a quick and reversible mechanism to control the activity of a wide variety of processes in the chloroplast, including photosynthesis and regulation of its redox poise. Light-dependent activation of Calvin–Benson cycle (CBC) enzymes and the chloroplast ATP synthase by stromal thioredoxins (TRXs) has been known for decades (for recent reviews, see Michelet et al., 2013; Hisabori et al., 2013; and Buchanan, 2016), but the dynamics of TRX-mediated regulation in changing light environments and the coordination of the activities of two distinct chloroplast TRX systems *in vivo* have only recently been addressed. To this end, I adopted a biophysical and biochemical *in vivo* approach in my PhD work to examine more closely the role of NTRC in chloroplast metabolism. Based on my findings, I propose a specific role for NTRC as a pivotal regulator of the ATP synthase, NDH-dependent cyclic electron flow, non-photochemical quenching as well as the CBC in low light conditions and during sudden increases in light intensity. Moreover, I show that the NTRC and Fd-TRX systems interact with each other to form a plastidial redox regulatory network that allows the constant adjustment of photosynthetic processes necessitated by environmental fluctuations. The two TRX systems have overlapping targets and show partial redundancy, but in certain conditions specificity is required.

5.1. Crosstalk between the chloroplast TRX systems

The experiments with transgenic lines containing mutated NTRC indicated that the two chloroplast TRX systems can interact with each other, and likely exchange reducing equivalents. Firstly, the stunted *ntrc* knockout phenotype was partially recovered in the lines overexpressing NTRC with a mutated reductase domain (OE-$NTRC_{SAIS}$ line) or TRX domain (OE-$NTRC_{SGPS}$) (Paper I). Secondly, NTRC and other TRXs interacted *in vivo* (Paper II), and thirdly, TRX-*f* redox state was increased in low light in both OE-NTRC and OE-$NTRC_{SGPS}$ (Paper II). As these results were obtained from plants with increased *NTRC* expression levels, the importance of the interaction in WT is debatable. However, while *trx-f1* knockout plants have a very WT-like phenotype, the phenotype of *trx-f1 ntrc* double mutants is more severe than that of *ntrc* single mutants (Thormählen et al., 2015). This observation suggests that crosstalk between NTRC and Fd-TRXs has physiological significance also at a wild-type level of NTRC content. Partly, however, the phenotype of *trx-f1 ntrc* plants is likely explained by the fact that NTRC and the Fd-TRX system have overlapping targets in CBC (Paper II, (Buchanan, 2016), ATP synthesis (Paper II, (Schwarz et al., 1997), starch biosynthesis (Michalska et al., 2009; Lepistö et al., 2013; Thormählen et al., 2013), chlorophyll biosynthesis (Luo et al., 2012; Richter et al., 2013), and scavenging of H_2O_2 (Perez-Ruiz et al., 2006; Bernal-Bayard et al., 2014). In line with the proposed functional connection between NTRC and TRX-*f*1, the two TRXs co-localize in the chloroplast (Thormählen et al., 2015). Inter-system crosstalk between the two TRX systems with different reductants may therefore allow flexibility and efficient fine-tuning of the activation states of TRX-regulated plastidial process in response to changes in environmental conditions.

5.2. NTRC and Fd-TRX systems are differentially activated by light conditions

The Fd-TRX system has been regarded as a primary activator of carbon metabolism in light because of its tight coupling to photosynthetic electron transfer reactions. However, the activation of the Fd-TRX system requires sustained illumination at moderate light intensities (Paper II) (Geigenberger et al., 2017). Therefore, initial activation of photosynthetic

processes during dark-to-light transitions as well as adjustment of plastidial redox homeostasis in low light conditions and during sudden changes in irradiance require additional regulatory components. As NADPH is produced also in the oxidative pentose phosphate pathway (OPPP) in darkness, it has been assumed that the NADPH-dependent NTRC system, unlike the Fd-TRX system, can maintain activity in darkness and function as a regulator of chloroplast dark-metabolism (Pérex-Ruiz et al., 2006). In Paper III I showed that not only is the NTRC pool partially reduced in darkness, the amount of reduced NTRC also stays fairly constant irrespective of light intensity, as well as during dark-to-light transitions. This is also true in the NTRC overexpression line (OE-NTRC), in which NTRC content is raised 20-fold in comparison to WT (Paper III). This unique property of NTRC allows it to function as an effective redox regulator in conditions where light or developmental stage of the chloroplast limits the reduction of ferredoxin by the PETC.

One obvious question that arises from these results and conclusions is the following: how does NTRC act as a dynamic regulator of photosynthetic processes in response to changes in environmental conditions if its own redox state is constant? At least two possible explanations can be presented. Firstly, affinities between TRXs and TRX targets may change considerably in different physiological conditions. Changes in stromal pH and ion concentrations, for instance, may affect the activation states and target specificities of TRXs (Setterdahl et al., 2003; Hochmal et al., 2016). For example, NTRC, whose midpoint redox potential has been measured *in vitro* as –275 mV (Yoshida and Hisabori, 2016), is the primary reductant of 2-Cys Prxs (Paper II, Perez-Ruiz et al., 2006; Pulido et al., 2010), whose redox potentials *in vitro* are as low as –315 mV (König et al., 2002). Hence, even though the accumulation of the reduced NTRC form does not change considerably in different light conditions (Paper III), it may only be able to reduce some of its targets in specific circumstances.

Alternatively, the access of NTRC to its substrates may be partly controlled by the redox state of the Fd-TRX system. The *f* and *m*-type TRXs often have more negative redox potentials and a higher affinity to common TRX targets *in vitro* than NTRC (Hirasawa et al., 1999; Collin et al., 2003; Yoshida and Hisabori, 2016). Moreover, the combined amount *f* and *m*-type TRXs in the chloroplast is roughly five times higher than that of NTRC

(Belin et al., 2015). When active, the Fd-TRX system likely outcompetes NTRC for interaction with common targets. NTRC-mediated reduction would therefore be required when the activation state of the Fd-TRX system is low, such as in darkness and low light conditions (Papers II–IV). In OE-NTRC, this dynamic is overturned by increased NTRC content (Papers II–IV). This hypothesis would also contribute to explaining the discrepancies between *in vitro* and *in vivo* experiments of TRX target specificity. NTRC has been found to be an inefficient reductant of TRX targets in the CBC in comparison to TRX-*f* and TRX-*m in vitro*, but *in vivo*, deficiency of NTRC impairs the reduction of CBC enzymes, photosynthetic electron transfer and leaf growth to much greater extent than deficiency of TRX-*f* or TRX-*m* (Paper II) (Yoshida et al., 2015; Thormählen et al., 2015; Yoshida and Hisabori, 2016; Thormählen et al., 2017). These studies demonstrate that NTRC has a specific function in the regulatory network of the chloroplast that cannot be compensated for by other TRXs.

Interestingly, the NTRC and Fd-TRX-*m* systems may have antagonistic roles in regulation of electron transfer in thylakoid membranes. Plants deficient in *m*-type TRXs show *enhanced* PSI yield in low light intensities (Thormählen et al., 2017), similarly NTRC-*overexpressing* plants (Papers II and III). Moreover, opposite to NTRC (Paper III), TRX-*m*4 has been proposed to have an inhibitory effect on NDH-dependent CET (Courteille et al., 2013). Antagonistic effects of the NTRC and Fd-TRX*m* systems on the activity of the NDH complex could allow activation of NDH-mediated CET during dark-to-light transitions and in low light by NTRC, and downregulation under higher light intensities by TRX-*m*4.

5.3. Regulation of photosynthetic processes by NTRC allows maintenance of redox homeostasis during changes in light conditions

Through my research, the NTRC system has emerged a vital regulatory hub that couples the metabolic state of the stroma to the redox poise of the photosynthetic electron transfer chain in low light conditions and during fluctuations in light intensity. This is achieved by simultaneous redox control of the activity of the ATP synthase (Papers II and III), CBC

enzymes (Paper II), cyclic electron transfer around PSI through the NDH complex (Paper III), induction of NPQ (Paper IV), and distribution of excitation energy between PSII and PSI (Paper IV).

5.3.1. NTRC is required both to activate the ATP synthase in low light and to control its activity under fluctuating light conditions

In papers II and III I demonstrated that NTRC has a role in initiating ATP production by activating the chloroplast ATP synthase through reduction of a disulfide in the $CF_1\gamma$ subunit under low light intensities. Reduction of $CF_1\gamma$ by the Fd-TRX system is well established (McKinney et al., 1978; Hisabori et al., 2013), and *f*-type TRXs in particular have been shown to be effective reductants of $CF_1\gamma$ (Schwarz et al., 1997). However, the Fd-TRX system can only compensate for an absence of NTRC in moderate to high light conditions, while in low light NTRC deficiency results in impaired reduction of $CF_1\gamma$, with several consequences (Paper II, Carrillo et al., 2016). Firstly, proton conductivity of the ATP synthase remains low resulting in acidification of the lumen (Papers III). This in turn induces high non-photochemical quenching and inhibition of electron transfer at Cyt *b6f* (Papers II, III and IV). Moreover, low activity of the ATP synthase results in low ATP production, which together with impaired reduction of CBC enzymes (Paper II) contributes to a decreased rate of carbon fixation in low light in NTRC-deficient plants (Paper II) (Perez-Ruiz et al., 2006; Lepistö et al., 2009; Pulido et al., 2010). Despite a low electron transfer rate, PSI is therefore limited on the acceptor side as the electron sink capacity of stromal processes is impaired (Papers II and III). By contrast, overexpression of NTRC causes reduction of $CF_1\gamma$ and ATP synthase activation also in darkness (Paper II). Depending on physiological conditions, this may lead to reverse activity of the ATP synthase; pumping of protons to the lumen driven by hydrolysis of ATP to ADP, or to high conductivity of the thylakoid membrane to protons and dissipation of the proton gradient (Kohzuma et al., 2012; Kohzuma et al., 2017). The thiol regulation of $CF_1\gamma$, which does not occur in cyanobacteria or mitochondria (Hisabori et al., 2013), likely evolved in chloroplasts to prevent these futile dark-reactions.

After the publication of Paper II, the role of NTRC as an ATP synthase activator in low light was also verified by another report with similar results (Carrillo et al., 2016). I therefore propose that NTRC is a non-redundant activator of the ATP synthase in low light conditions. NTRC-mediated activation is required during transitions from dark to low irradiance, and in fluctuating light conditions during transitions to low light intensity.

In addition to the on/off thiol switch of $CF_1\gamma$, the activity of the ATP synthase is modulated by other, less characterized mechanisms that allow maintenance of redox homeostasis between the electron transfer chain and stromal metabolism during changes in environmental conditions (Kanazawa and Kramer, 2002; Rott et al., 2011; Kohzuma et al., 2013; Kanazawa et al., 2017). Decrease of proton efflux through the ATP synthase results in acidification of lumen and thus plays an important regulatory role in induction of NPQ and photosynthetic control (Kanazawa and Kramer, 2002; Kanazawa et al., 2017). It has been suggested that the function of the PGR5 protein may be related to such regulation of ATP synthase activity. (Avenson et al., 2005; Tikkanen et al., 2015; Kanazawa et al., 2017; Armbruster et al., 2017). This hypothesis is based on the inability of the *pgr5* mutant to downregulate thylakoid conductivity to protons in high light, which results in loss of *pmf* and an inability to induce NPQ or photosynthetic control at Cyt *b6f* (Avenson et al., 2005; Suorsa et al., 2012) (Paper III). Moreover, it is possible that the putative PGR5-dependent regulation is redox-dependent, because NTRC overexpression, but only in the presence of PGR5, intensified the repression of proton conductivity of the thylakoid membrane upon transitions from low to high irradiance (Paper III). PGR5 contains one conserved cysteine residue (Munekage et al., 2002), which could form an intermolecular disulfide with PGRL1 (Hertle et al., 2013) or be subject to other thiol modifications that control the suggested association of PGR5 with the ATP synthase. I propose that reduction of the conserved Cys residue by TRX would allow PGR5 to disassociate from PGRL1 and to subsequently interact with the ATP synthase.

Overexpression of NTRC in *pgr5* background restored *pmf* in high light to a level comparable to WT, most likely via increased activation of NDH-dependent CET (Paper III). This was not, however, sufficient to restore the ability to induce photosynthetic control or NPQ upon increases in light

intensity. This indicates that the impaired control of electron transfer in *pgr5* does not only derive from diminished *pmf*, but the PGR5 protein itself is essential for the normal induction of photosynthetic control and NPQ and thus for protection of PSI from excessive reduction and photodamage, as suggested previously (Suorsa et al., 2013; Tikkanen et al., 2015).

As in *pgr5*, in *ntrc* the PQ pool is reduced during dark-to-growth light transitions, but in contrast to *pgr5*, the donor side of PSI limits electron transfer (Paper III). This suggests over-activation of photosynthetic control at Cyt *b6f*. A decreased content of PGR5, as observed in *ntrc* (Paper III), may constitute an attempt to decrease PGR5-dependent photosynthetic control and alleviate the excitation pressure in the PQ pool. These results further imply that the putative direct function of PGR5 in photosynthetic control would be dependent on stromal thiol redox state.

In summary, the activation state of the chloroplast ATP synthase is regulated by TRXs by two distinct mechanisms: i) upon onset of illumination a disulfide bridge in the $CF_1\gamma$ subunit is reduced by TRX, which decreases the *pmf* threshold required for activation of the ATP synthase. There is redundancy between the chloroplast TRX systems as both NTRC and TRX-*f* can reduce $CF_1\gamma$, but NTRC-mediated reduction is particularly important in low light conditions. ii) I propose that the chloroplast TRX systems also regulate the activation state of the ATP synthase indirectly during transitions to high irradiance by inducing an inhibitory response that may involve a redox-dependent function of the PGR5 protein.

5.3.2. NTRC activates NDH-dependent CET

It has been suggested recently that NDH-mediated CET contributes to photosynthetic redox poise and generation of the *pmf* specifically in low light conditions and during increases in light intensity (Martin et al., 2015; Yamori et al., 2015; Yamori et al., 2016). The NDH complex is reduced by ferredoxin (Yamamoto et al., 2011), which functions as a light-dependent redox hub, controlling the distribution of electrons to FNR, FTR, and several other stromal acceptors in addition to both CET pathways (Hanke and Mulo, 2013). Therefore, strict regulation of NDH activity is likely necessary for concerted function of all these processes under fluctuating

light conditions, in order to maintain redox balance in the chloroplast. In Paper III I showed that this modulation of NDH activity is likely mediated by stromal TRX systems. In particular, my results indicate that NTRC has a role in activating the NDH complex during dark-to-light transitions and under low irradiance. NTRC overexpression enhanced non-photochemical PQ reduction in darkness, and increased the magnitude of *pmf* and PSI yield in comparison to WT in low light and upon increases in light intensity. This, however, only occurred if the NDH complex was present (Paper III). As these effects were not due to changes in NDH content or amount of substrate, NTRC may be directly involved in regulation of NDH activity.

Based on interaction assays, mobility shift assays, and analysis of amino acid sequences (Paper III), I propose that a target of TRX-regulation exists close to the ferredoxin binding and oxidation site on the stromal side of the chloroplast NDH complex (Peltier et al., 2016; Shikanai, 2016). The most likely candidate to form an internal or intermolecular regulatory disulfide is NdhS (Paper III). TRX-mediated regulation of such a disulfide would provide a quick and reversible way to dynamically regulate the ferredoxin:plastoquinone oxidoreductase activity of the NDH complex according to physiological conditions in the chloroplast by connecting the stromal thiol redox state with photosynthetic electron transfer.

Increased activation of NDH-CET is not, however, sufficient to fully explain the elevation of *pmf* in NTRC-overexpressing plants. In some conditions elevation of *pmf* without an increase in linear electron transfer or decrease in thylakoid proton conductivity occurred also in the OE-NTRC *ndho* line. This could be caused by enhanced activity of PGR5/PGRL1-dependent CET. However, results in Paper III suggested that NTRC does not directly activate PGR5/PGRL1-dependent CET, and that TRX-*m* is the primary reductant of PGRL1, as proposed by (Hertle et al., 2013). Nevertheless, as NTRC was shown to interact with TRX-*m* in Paper II, overexpression of NTRC may enhance the activity of TRX-*m* and thus indirectly cause activation of PGR5/PGRL1-dependent CET.

5.3.3. NTRC mediates ΔpH-independent downregulation of NPQ

The major, energy dependent qE component of NPQ depends on the magnitude of the transthylakoid proton gradient, because effective

induction of qE requires protonation of both PsbS and association of the xanthophyll cycle enzyme VDE with the thylakoid membrane (Holt et al., 2004; Takizawa et al., 2007; Arnoux et al., 2009). Acidification of the lumen therefore correlates with induction of NPQ under different light intensities in wild type plants (Paper IV). However, the level of NPQ shows decreased correlation with the *pmf* in NTRC-transgenic lines (Paper IV). In *ntrc*, high *pmf* caused by impaired activation of the ATP synthase partly explains the elevation of NPQ in low light. In growth and higher light intensities ATP synthase is activated normally by the Fd-TRX system and *pmf* does not differ from WT (Papers II and III) and (Carrillo et al., 2016). Nevertheless, elevated NPQ is also observed in these conditions in *ntrc*. To some extent this may be explained by increased PsbS content in *ntrc* (Paper IV), which likely increases NPQ (Li et al., 2002; Zia et al., 2011). Indeed, NPQ is decreased in *ntrc psbs* double mutants when compared to *ntrc,* and growth of the plants is partly recovered (Naranjo et al., 2016). PsbS-dependent quenching (qE) characteristically relaxes quickly upon cessation of illumination (Li et al., 2000; Zia et al., 2011), but NPQ in *ntrc* showed very slow relaxation kinetics in darkness (Paper IV). Also increased accumulation of zeaxanthin (Zx) and antheraxanthin (Ax) was detected in *ntrc* in growth and high light (Paper IV). This correlates with the high NPQ but not with the wild-type level of *pmf* in those conditions, and therefore implies altered regulation of the Xanthophyll cycle enzymes VDE or ZE. Redox-regulation of VDE and ZE by TRXs has been proposed (Hall et al., 2010; Yu et al., 2014; Da et al., 2017), but no difference in their *in vivo* redox states, however, have been observed between WT and *ntrc* (Naranjo et al., 2016). In OE-NTRC, the relative proportion of Zx from total xanthophyll content is increased in high light (Paper IV), which most likely results in enhanced activation of VDE (Jahns et al., 2009) due to increased ΔpH (Papers III and IV). Despite the increased content of de-epoxidated xanthophylls, however, NPQ is decreased in comparison to WT in OE-NTRC in high light (Paper IV).

These observations indicate that in addition to altered activity of the ATP synthase, PsbS content, and activity of the xanthophyll cycle, an unknown factor independent of transthylakoid ΔpH is upregulating NPQ in *ntrc* and downregulating it in OE-NTRC. Inhibition of a slow-relaxing constituent of NPQ has been shown to depend on SUPPRESSOR OF QUENCHING 1 (SOQ1), an integral thylakoid membrane protein that contains a lumenal

thioredoxin-like domain (Brooks et al., 2013; Malnoë et al., 2017). Importantly, this mechanism is ΔpH-independent, as in addition to *soq1* knockout plants, a slow-relaxing NPQ component remains elevated in plants lacking both SOQ1 and PsbS (Brooks et al., 2013). A similar slowly reversible NPQ component was detected also in *ntrc* (Paper IV), and SOQ1 was identified as a putative NTRC interactor by Co-IP/MS (Paper III). It may be that absence of NTRC incapacitates SOQ1-dependent downregulation of NPQ, while *NTRC* overexpression would result in over-activation of this inhibitory mechanism. Reducing equivalents are likely mediated from stromal NTRC to the luminal TRX-like motif of SOQ1 via the CcdA and HCF164 proteins in the thylakoid membrane. This effect can result from direct reduction of CcdA by NTRC, or from increased activation of TRX-*m* in OE-NTRC, since TRX-*m* has been shown to efficiently reduce CcdA *in vitro* (Motohashi and Hisabori, 2010). Therefore, I suggest that reduction of the TRX-like motif of SOQ1 via the CcdA-HCF164 pathway induces suppression of NPQ in low light, while oxidation of SOQ1 under higher light intensities inactivates the suppression mechanism and allows induction of lipocalin-dependent quenching (qH) (Malnoë et al., 2017). It has indeed been reported that *lncp* mutants, which cannot induce qH, show increased sensitivity to high-light stress (Levesque-Tremblay et al., 2009; Malnoë et al., 2017).

Another putative target of thiol regulation of NPQ is the CP29 protein (Lhcb4), which partakes in hetero-oligomers with CP24 and an LHCII-M trimer whose disassociation is prerequisite to induction of the qE component of NPQ (Betterle et al., 2009). CP29 contains a conserved S-nitrosylated cysteine residue (Puyaubert et al., 2014) and was identified as a putative NTRC interactor by Co-IP/MS (Suppl. Table 2 in Paper III). Similarly to OE-NTRC, knockout mutants of all three CP29 isoforms show decreased NPQ, but also increased P_{700} oxidation and enhanced state transition capacity (de Bianchi et al., 2011). Control of CP29 function by a reversible thiol modification could therefore constitute a regulatory switch between NPQ induction and state II transitions.

5.4. The impact of 2-Cys Prxs on chloroplast redox state

It was recently proposed that the severity of the *ntrc* phenotype and the impairment of reduction of CBC enzymes in *ntrc* (Paper II) results indirectly from oxidation of TRX-*f* (and possibly other TRXs) due to an increase in the amount of oxidized 2-Cys Prxs (Pérez-Ruiz et al., 2017). This conclusion was based on partial recovery of the *ntrc* phenotype in the *ntrc Δ2cp* triple mutant, which has a considerably lowered content of 2-Cys Prxs in addition to the lack of NTRC. However, I detected no impairment of TRX-*f* reduction in *ntrc*, OE-NTRC$_{SAIS}$ or OE-NTRC$_{SGPS}$, which all have more oxidized 2-Cys Prx pools than WT (Paper II). On the contrary, in OE-NTRC$_{SGPS}$, in which the most severe impairment of 2-Cys Prx reduction was observed, showed slightly enhanced reduction of TRX-*f* in low light conditions. Moreover, considering that 2-Cys Prx content is decreased in *ntrc*, the absolute amount of oxidized 2-Cys Prxs is not considerably higher than in WT (Paper II). Oxidized 2-Cys Prx has been shown to have an oxidative effect on chloroplast enzymes (Dangoor et al., 2012; Eliyahu et al., 2015; Pérez-Ruiz et al., 2017), and 2-Cys Prx deficiency also increases the amount of reduced CBC enzymes in the presence of NTRC in *Δ2cp* (Pérez-Ruiz et al., 2017). Moreover, the amounts of reduced CBC enzymes and reduced 2-Cys Prx do correlate in NTRC-overexpressing lines (Paper II). Therefore, the hypothesis of Pérez-Ruiz et al. cannot be excluded, but the partial recovery of the *ntrc* phenotype in *ntrc Δ2cp* does not necessitate that the *ntrc* phenotype is exclusively caused by impairment of 2-Cys Prx reduction. Nevertheless, it is clear that the phenotype of *ntrc* is highly pleiotropic, and any single physiological symptom of the mutant is likely a sum of multiple factors. This complexity always needs to be considered when interpreting results from *ntrc*.

5.5. The effect of NTRC overexpression on photosynthetic redox balance and plant fitness

NTRC overexpression allows faster balancing of photosynthetic redox poise and efficient electron transfer in low light and upon onset of illumination or fluctuations in light intensity by maintaining elevated activation states of the CBC enzymes FBPase and PRK (Paper II), and by

enhancing the capacity to re-distribute excitation energy among PSII and PSI (Paper IV). Increased activation of CBC enzymes results in higher capacity of the stroma to accept electrons from PSI immediately upon onset of illumination and in low light. Moreover, increased dark-activation of the NDH complex in OE-NTRC (Paper III) results in increased reduction of the PQ pool in dark, which in turn causes phosphorylation of LHCII proteins through activation of the STN7 kinase (Vener et al., 1997; Shapiguzov et al., 2016) and an increase in the relative size of PSI antenna cross section (Paper IV). Hence, the quantum yield of PSI is high immediately upon illumination in dark-adapted OE-NTRC leaves. Importantly, however, efficient PSI oxidation does not occur when either the NDH complex or PGR5 is missing (Paper III), emphasizing the importance of those systems for controlling the redox poise.

Improvement of photosynthetic efficiency in low light likely increases the vegetative growth of shaded leaves, which at least partly explains the enhanced leaf growth and biomass yield in OE-NTRC in comparison to WT (Paper I). In many ways, overexpression of NTRC overrides the natural thiol-dependent regulatory mechanisms that in wild-type plants limit the activity of the CBC and photosynthetic electron transfer in order to provide more efficient photoprotection and plasticity in unpredictable natural environments. In artificially stable growth conditions, such as in laboratories or cultivated agricultural crops however, these mechanisms are largely redundant and likely limit biomass yield (Kromdijk et al., 2016).

5.6. NTRC is a master regulator of photosynthetic processes on thylakoids and in stroma during changes in light conditions

Based on the results in Papers I–IV, I present a schematic summary of NTRC-mediated regulation of photosynthetic redox poise in Figure 7. Upon the onset of illumination of leaves, the NTRC pool is already partially active due to NADPH produced in the OPPP in darkness. In low light, NTRC rapidly activates the ATP synthase by reducing the $CF_1\gamma$ subunit. NTRC also contributes to activation of the Calvin–Benson cycle, which together with activation of NDH-dependent CET enhances the electron sink capacity of the stroma and alleviates acceptor side limitation of PSI. Nonetheless, binding of PQH_2 to Cyt b_6f results in phosphorylation of

LHCII proteins by the STN7 kinase and an increase in the relative size of PSI antenna cross section (state II) (Vener et al., 1997). Under low irradiance, NTRC mediates downregulation of the lipocalin-dependent qH component of NPQ (Malnoë et al., 2017). This likely occurs because NTRC increases the transmission of redox equivalents from the stroma to the lumen via the CcdA–HCF164 pathway, either directly or indirectly through crosstalk with *m*-type TRXs. This results in reduction of the SOQ1 protein and repression of the qH component of NPQ. Upon a sudden increase in light intensity, NTRC promotes PGR5-dependent inhibition of ATP synthase activity. The mechanism may involve cleavage of an intermolecular disulfide between PGR5 and PGRL1, which would permit suppression of ATP synthase activity by PGR5 (Avenson et al., 2005; Kanazawa et al., 2017). As a result, conductivity of the thylakoid membrane to protons is decreased and the lumen is acidified, which induces qE and photosynthetic control at Cyt *b6f*. All of the events described above contribute to prevention of over-reduction of the electron transfer chain and allow efficient oxidation of PSI, protecting it from photodamage.

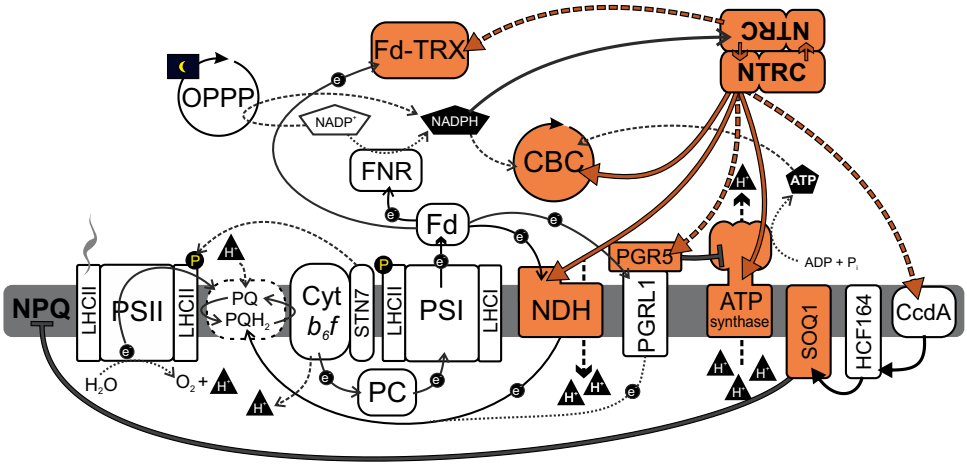

Figure 7. Schematic model of TRX-mediated regulation of photosynthetic redox homeostasis. Orange colour and arrows represent thiol regulation by NTRC. Dotted lines depict putative effects, and the stunted dark grey lines represent inhibitory effects. P=phosphorylation, while e^- denotes electron transfer. See the text for details.

6. CONCLUDING REMARKS

In my PhD research I have demonstrated that the ferredoxin- and NADPH-dependent chloroplast thioredoxin systems form a cooperative regulatory network that allows dynamic response to a variety of environmental changes, particularly fluctuations in light conditions. I have discovered novel target processes for the NTRC system in regulation of the Calvin–Benson cycle, ATP synthesis, cyclic electron flow and NPQ. Changes in the NTRC content of leaves also affect a wide variety of chloroplast functions indirectly through crosstalk with the Fd-TRX system and by generally modifying the stromal thiol redox state. As the redox state of the NTRC pool itself is maintained fairly constant in all light conditions by NADPH produced both by the light reactions and by the oxidative pentose phosphate pathway, NTRC can function as a master regulator of photosynthetic redox poise in low light and upon sudden changes in light conditions. Furthermore, my results suggest that overexpression of NTRC via a simple genetic modification could considerably improve photosynthetic efficiency and biomass yield in shaded leaves and in fluctuating light conditions. Therefore, as global climate change and population growth will increasingly necessitate enhancement of agricultural production, the potential of TRX overexpression as a bioengineering tool to improve the yields of crop plants or biofuel production might be worth considering (Nikkanen et al., 2017).

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