TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. A I OSA - TOM. 422

ASTRONOMICA - CHEMICA - PHYSICA - MATHEMATICA

NADPH-DEPENDENT THIOREDOXIN SYSTEM IN REGULATION OF CHLOROPLAST FUNCTIONS

by

Anna Lepistö

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2011

From the Laboratory of Molecular Plant Biology
Department of Biochemistry and Food Chemistry
University of Turku
FI-20014 Turku, Finland

Supervised by

Professor Eevi Rintamäki
Laboratory of Molecular Plant Biology
Department of Biochemistry and Food Chemistry
University of Turku
FI-20014 Turku, Finland

Reviewed by

Professor Elina Oksanen
Department of Biology
University of Eastern Finland
FI-80101 Joensuu, Finland

and

Professor Anja Hohtola
Department of Biology
University of Oulu
FI-90014 Oulu, Finland

Opponent

Professor Francisco Javier Cejudo
Department of Biology
University of Seville
ES-41092 Seville, Spain

ISBN 978-951-29-4694-5 (PRINT)
ISBN 978-951-29-4695-2 (PDF)
ISSN 0082-7002
Painosalama Oy – Turku, Finland 2011

LIST OF ORIGINAL PUBLICATIONS

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

I Lepistö A*, Kangasjärvi S*, Luomala EM, Brader G, Sipari N, Keränen M, Keinänen M, Rintamäki E. 2009: Chloroplast NADPH thioredoxin reductase interacts with photoperiodic development in *Arabidopsis thaliana*. Plant Physiology 149:1261-1276

II Lepistö A, Toivola J, Vignols F, Rintamäki E. 2011: Regulation of chloroplast biogenesis by chloroplast NADPH-dependent thioredoxin system. Manuscript.

III Lepistö A, Pakula E, Toivola J, Krieger-Liszkay A, Vignols F, Rintamäki E. 2011: Redox-regulation of starch and antioxidant metabolism by chloroplast NADPH thioredoxin reductase in Arabidopsis grown under various photoperiods. Manuscript.

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

*Equal contributions

Publication I has been reprinted by kind permission of American Society of Plant Biologists. Copyright American Society of Plant Biologists.

Publication IV has been reprinted by kind permission of the Biochemical Society.

ABBREVIATIONS

AGPase	ADP-glucose pyrophosphorylase
ALA	aminolevulinic acid
APX	ascorbate peroxidase
ATP	adenosine triphosphate
CAO	chlorophyllide *a* oxygenase
CAT	catalase
CHLH, CHLI, CHLD	subunits of Mg-chelatase
CRY	cryptochrome
Cyt	cytochrome
DAHP	3-deoxy-d-arabino-heptulosonate-7-phosphate
DHA	dehydroascorbate
DSP4	dual specificity protein phosphatase
FAD	flavin adenine dinucleotide
FNR	ferredoxin-$NADP^+$ oxidoreductase
FTR	ferredoxin-thioredoxin reductase
G3P	glyceraldehyde-3-phosphate
GPX	glutathione peroxidase
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
GUN4	regulator of Mg-chelatase
HEMA1	glutamyl-tRNA reductase
LHC	light-harvesting complex
MDA	monodehydroascorbate
NADPH	nicotinamine adenine dinucleotide phosphate
NTR	NADPH-dependent thioredoxin reductase
NTRC	plastid-localized NADPH-dependent thioredoxin reductase C
P680, P700	reaction center chlorophyll of PSII and PSI, respectively
P_i	inorganic phosphate
PC	plastocyanin
PCR	polymerase chain reaction
PGA	3-phosphoglycerate
PHY	phytocrome
PLB	prolamellar body
POR	NADPH:protochlorophyllide oxidoreductase
PQ	plastoquinone
Prx	peroxiredoxin
PS	photosystem
ROS	reactive oxygen species
SOD	superoxide dismutase
T-DNA	transfer DNA
tRNA	transfer RNA
Trx	thioredoxin

ABSTRACT

Photosynthesis, the process in which carbon dioxide is converted into sugars using the energy of sunlight, is vital for heterotrophic life on Earth. In plants, photosynthesis takes place in specific organelles called chloroplasts. During chloroplast biogenesis, light is a prerequisite for the development of functional photosynthetic structures. In addition to photosynthesis, a number of other metabolic processes such as nitrogen assimilation, the biosynthesis of fatty acids, amino acids, vitamins, and hormones are localized to plant chloroplasts. The biosynthetic pathways in chloroplasts are tightly regulated, and especially the reduction/oxidation (redox) signals play important roles in controlling many developmental and metabolic processes in chloroplasts. Thioredoxins are universal regulatory proteins that mediate redox signals in chloroplasts. They are able to modify the structure and function of their target proteins by reduction of disulfide bonds. Oxidized thioredoxins are restored via the action of thioredoxin reductases. Two thioredoxin reductase systems exist in plant chloroplasts, the NADPH-dependent thioredoxin reductase C (NTRC) and ferredoxin-thioredoxin reductase (FTR). The ferredoxin-thioredoxin system that is linked to photosynthetic light reactions is involved in light-activation of chloroplast proteins. NADPH can be produced via both the photosynthetic electron transfer reactions in light, and in darkness via the pentose phosphate pathway. These different pathways of NADPH production enable the regulation of diverse metabolic pathways in chloroplasts by the NADPH-dependent thioredoxin system.

In this thesis, the role of NADPH-dependent thioredoxin system in the redox-control of chloroplast development and metabolism was studied by characterization of *Arabidopsis thaliana* T-DNA insertion lines of *NTRC* gene (*ntrc*) and by identification of chloroplast proteins regulated by NTRC. The *ntrc* plants showed the strongest visible phenotypes when grown under short 8-h photoperiod. This indicates that i) chloroplast NADPH-dependent thioredoxin system is non-redundant to ferredoxin-thioredoxin system and that ii) NTRC particularly controls the chloroplast processes that are easily imbalanced in daily light/dark rhythms with short day and long night. I identified four processes and the redox-regulated proteins therein that are potentially regulated by NTRC; i) chloroplast development, ii) starch biosynthesis, iii) aromatic amino acid biosynthesis and iv) detoxification of H_2O_2. Such regulation can be achieved directly by modulating the redox state of intramolecular or intermolecular disulfide bridges of enzymes, or by protecting enzymes from oxidation in conjunction with 2-cysteine peroxiredoxins. This thesis work also demonstrated that the enzymatic antioxidant systems in chloroplasts, ascorbate peroxidases, superoxide dismutase and NTRC-dependent 2-cysteine peroxiredoxins are tightly linked up to prevent the detrimental accumulation of reactive oxygen species in plants.

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

ABBREVIATIONS

ABSTRACT

1. INTRODUCTION 9

1.1. CHLOROPLASTS IN PLANTS 9
1.2. PHOTOSYNTHESIS AND OTHER METABOLIC REACTIONS IN CHLOROPLASTS 10
1.2.1. Photosynthetic light reactions 10
1.2.2. Calvin-Benson cycle and other metabolic pathways in chloroplasts 11
1.3. REGULATORY AND ANTIOXIDANT REDOX-SYSTEMS IN CHLOROPLAST 13
1.3.1. Thioredoxin systems 14
1.3.2. Reactive oxygen species in chloroplast 17
1.3.3. Detoxification of reactive oxygen species 18
1.4. CHLOROPLAST BIOGENESIS 20
1.4.1. Chlorophyll biosynthesis 22
1.5. REGULATION OF CHLOROPLAST BIOGENESIS AND ACCLIMATION TO ENVIRONMENTAL CUES 24
1.5.1. Anterograde signaling 25
1.5.2. Chloroplast-to-nucleus retrograde signaling 25
1.5.3. ROS in plant signaling 27

2. AIMS OF THE STUDY 28

3. METHODOLOGICAL ASPECTS 29

3.1. PLANT MATERIAL AND GROWTH CONDITIONS 29
3.2. STRESS TREATMENTS 29
3.3. ANALYSIS OF PIGMENTS AND PROTEINS 30
3.4. MICROARRAY ANALYSIS 30
3.5. BIOPHYSICAL METHODS 30
3.6. MICROSCOPY 31
3.7. IN VIVO-DETECTION OF H_2O_2 AND SUPEROXIDE 31
3.8. ANALYSES OF AMINO ACIDS, HORMONES AND SUGARS 31
3.9. YEAST TWO-HYBRID ANALYSIS 32

4. OVERVIEW OF THE RESULTS 33
4.1. DAILY LIGHT/DARK RHYTHM MODULATES THE PHOTOSYNTHESIS AND GROWTH OF ARABIDOPSIS 33
4.1.1. Impact of photoperiod on growth and photosynthesis in wild type Arabidopsis 33
4.1.2. Impact of photoperiod on the redox metabolism of mesophyll cells 34
4.1.3. Knockout of NTRC impairs the acclimation of Arabidopsis to short photoperiods . 35
4.1.4. Photosynthesis of knockout ntrc lines 35
4.2. IDENTIFICATION OF CHLOROPLAST PROCESSES CONTROLLED BY NADPH-DEPENDENT THIOREDOXIN SYSTEM 36
4.2.1. Impact of NTRC on chloroplast biogenesis 36
4.2.2. Starch and sucrose metabolism in knockout ntrc leaves 38
4.2.3. NTRC and chloroplast ROS metabolism 39
4.2.4. Knockout of NTRC interferes with the homeostasis of metabolites produced in shikimate pathway 39
4.3. CONTRIBUTION OF CHLOROPLAST ASCORBATE PEROXIDASES TO THE ROS METABOLISM IN ARABIDOPSIS 41
5. DISCUSSION 42
5.1. NTRC IN REDOX-REGULATION OF CHLOROPLAST BIOGENESIS 42
5.1.1. Regulation of chloroplast division by NTRC 43
5.1.2. Regulation of chlorophyll biosynthesis by NTRC 44
5.1.3. Thylakoid formation in ntrc mutant lines 45
5.2. REGULATION OF CHLOROPLAST METABOLISM BY NTRC 45
5.2.1. Redox control of starch metabolism in chloroplasts 45
5.2.2. Altered homeostasis of metabolites synthesized from the end products of shikimate acid pathway 46
5.3. THIOREDOXIN- AND ASCORBATE-DEPENDENT SCAVENGING OF ROS IN CHLOROPLASTS .. 47
5.3.1. Detoxification of H_2O_2 by NTRC 47
5.3.2. Impacts of chloroplast APXs on chloroplast redox homeostasis 48
5.4. PHOTOPERIODIC DEVELOPMENT OF PHOTOSYNTHETIC TISSUES 49
5.4.1. Impact of NTRC knockout on the growth of Arabidopsis 50
5.5. CHLOROPLAST-TO-NUCLEUS RETROGRADE SIGNALING 50
6. CONCLUDING REMARKS 52
ACKNOWLEDGEMENTS 53
REFERENCES 54
ORIGINAL PUBLICATIONS 63

“There is a theory which states that if ever anyone discovers
exactly what the Universe is for and why it is here,
it will instantly disappear and be replaced by something
even more bizarre and inexplicable.

There is another theory which states that this has already happened.”

Douglas Adams: The Restaurant at the End of the Universe (1980)

1. Introduction

Chloroplasts arose approximately 1.5 billion years ago when a free-living autotrophic bacterium, an ancestor of present-day cyanobacterium was engulfed by a mitochondrion-containing eukayotic host cell and became an endosymbiont. This event provided mutual benefit for both partners; bacterium occupied an untapped niche and the host received nutrients such as reduced carbon (Glynn, et al., 2007). Since its origin, the chloroplast has become fully integrated to the life cycle of photosynthetic eukaryotes, being essential to the plants themselves but also enabling the existence of heterotrophic life on Earth.

1.1. Chloroplasts in plants

Chloroplasts (Figure 1) are flat disc-shaped organelles, surrounded by an envelope that consists of an outer and inner lipid membrane. Between these two layers is the intermembrane space. Inside the chloroplast, the third membrane system, thylakoids, appear individually as stroma thylakoids or form stacked grana structures. Thylakoids of a single chloroplast form a three-dimensional interconnected network, enclosing a space called lumen (Mustardy and Garab, 2003). The chloroplast content outside the thylakoids, the stroma, corresponds to the cytosol of the original endosymbiotic bacterium, containing one or more molecules of circular DNA as well as ribosomes, proteins, metabolites and temporary starch granules. A typical *Arabidopsis thaliana* (Arabidopsis) leaf cell contains over 100 chloroplasts.

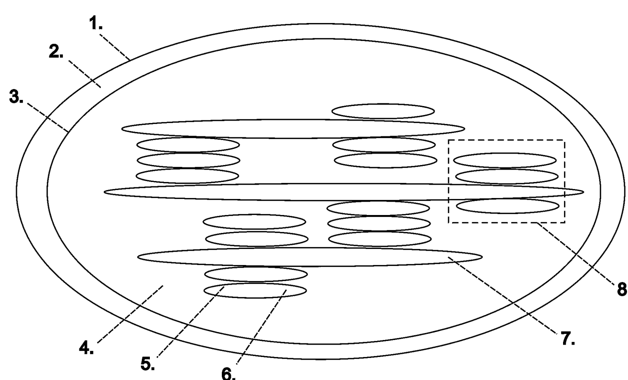


Figure 1. Outline of the chloroplast ultrastructure. 1. outer membrane, 2. intermembrane space, 3. inner membrane (1+2+3 : envelope), 4. stroma, 5. thylakoid membrane 6. thylakoid lumen (inside of thylakoid), 7. stroma thylakoid, 8. granum (stack of thylakoids)

The size of plastidial DNA (plastome) is significantly smaller than the cyanobacterial genome; chloroplast genome encodes 50-200 proteins (Martin, 2003), whereas the genome of a cyanobacterium *Synechocystis* theoretically encodes more than 3600 proteins (Battchikova, et al., 2010). During the course of evolution, genes have been transferred from chloroplast to nucleus where they can be regulated in an integrated

manner (Martin and Herrmann, 1998). Some genes are still retained in chloroplasts, most probably because the expression of those genes is required to be directly and efficiently regulated by the factors present in chloroplast (Allen, et al., 2005).

1.2. Photosynthesis and other metabolic reactions in chloroplasts

The chloroplasts' ability to perform photosynthesis, the conversion of carbon dioxide into organic compounds using the energy of sunlight, is vital for life on Earth. Sugars produced by photosynthesis provide nourishment, either directly or indirectly, to nearly all life forms and the oxygen released in the process as a side product maintains the oxygen level in the atmosphere and enables the living of aerobic life forms. Photosynthesis consists of two distinct phases that both occur inside the chloroplast, yet in different compartments. The light-dependent reactions take place in thylakoid membranes, in which light energy drives electron flow between a series of multi-subunit protein complexes, eventually generating ATP and reducing equivalents. This chemical energy is then spent in the stroma by Calvin-Benson cycle to fix CO_2 into energy-rich sugar phosphates. Sugar phosphates are then exported to the cytosol or stored in the chloroplast as starch.

1.2.1. Photosynthetic light reactions

In plants, the photosynthetic light-induced electron transfer reactions are carried out by two physically separated, thylakoid-membrane-bound multi-subunit protein complexes, photosystem I and II (PSI and PSII) in addition to cytochrome *b6f* (Cyt *b6f*) complex, ATP synthase and mobile electron carriers. The electron transfer reactions begin when a photon of visible light is absorbed by the reaction center chlorophyll molecule in PSII and in PSI (Figure 2). The reaction center pigments of photosystems are able to absorb photons directly, but light is also absorbed by the light-harvesting complex, a set of photosynthetic pigments outside the reaction centers of each photosystem. Light-harvesting complex functions as a solar collector that feeds light energy through resonance energy transfer to the reaction center chlorophyll molecules at photosystems. The absorbed energy boosts the PSII reaction center pigment P680 to an excited state P680* which rapidly transfers an electron to a nearby pheophytin, a PSII-bound pigment. The electron flows from pheophytin via PSII-bound plastoquinones Q_A and Q_B to plastoquinone (PQ) pool, mobile electron carriers that pass electrons to Cyt *b6f* complex. When P680* delivers an electron, $P680^+$ is formed. It is a very strong oxidant capable of extracting electrons from water molecules bound at the manganese cluster in oxygen evolving complex (OEC), which results in splitting water to protons and oxygen.

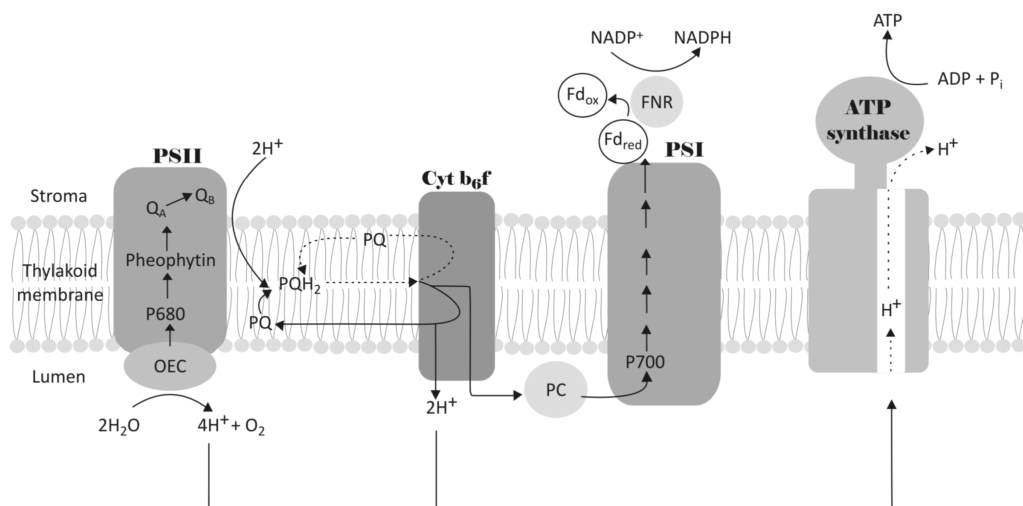


Figure 2. Schematic diagram of linear electron transfer reactions in oxygenic photosynthesis. See text for details.

Electrons from the Cyt b_6f complex are transferred to PSI by plastocyanin (PC), another mobile electron carrier. Before an electron can be accepted by PSI reaction center pigment P700, another photon of light is needed to excite P700 to P700*. When P700* releases its electron to the primary electron acceptor of PSI, $P700^+$ accepts the electron delivered by PC and returns to the ground state. The electrons are finally accepted by ferredoxin (Fd) which then reduces $NADP^+$ to NADPH with the help of ferredoxin-$NADP^+$ oxidoreductase (FNR). Both the proton translocation coupled to electron transfer and the release of protons by the water oxidation reaction contribute to the electrochemical gradient across the thylakoid membrane that drives the synthesis of ATP by ATP synthase. ATP and NADPH are used in the Calvin-Benson cycle in the stroma to fix CO_2 to carbohydrates.

1.2.2. Calvin-Benson cycle and other metabolic pathways in chloroplasts

The reactions in Calvin-Benson cycle catalyze the fixation of carbon dioxide and the reduction of sugars in three stages: (i) the incorporation of a CO_2 molecule into ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate (PGA) in the reaction catalyzed by Rubisco; (ii) the reduction of PGA to glyceraldehyde 3-phosphate (G3P) that is used to form hexose sugars; and (iii) the transformation of five of the six G3P into three ribulose 1,5-bisphosphates to replace the ones that initiated the cycle (Figure 3).

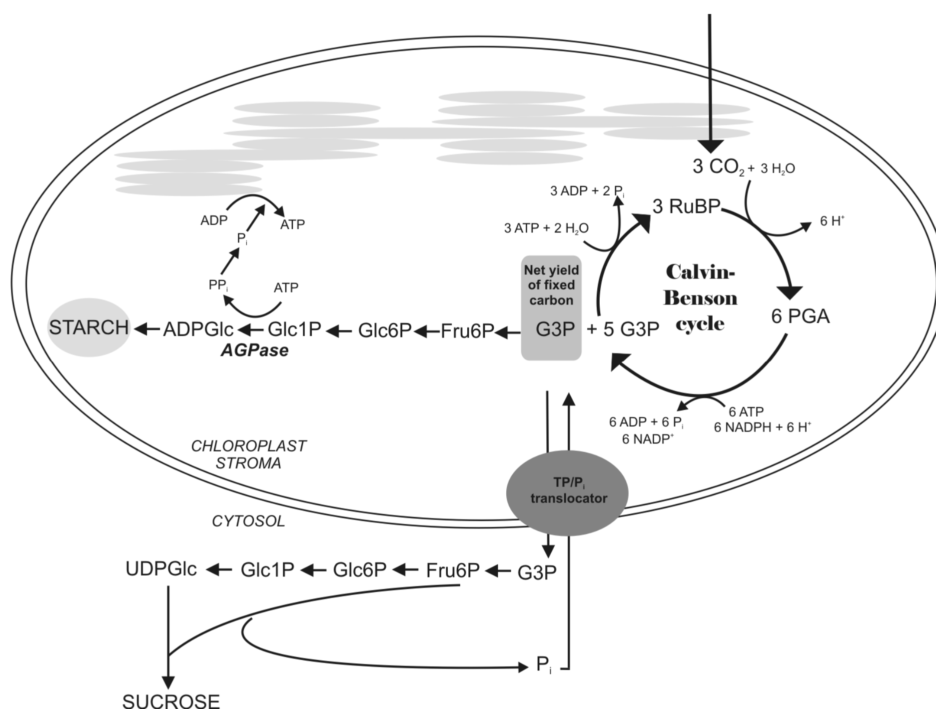


Figure 3. Carbon assimilated via Calvin-Benson cycle is partitioned into fraction retained in chloroplast for starch synthesis and a fraction exported to the cytosol for sucrose synthesis. RuBP, ribulose 1,5-bisphosphate; PGA, 3-phosphoglycerate; G3P, glyceraldehyde 3-phosphate; Fru6P, fructose 6-phosphate; Glc6P, glucose 6-phosphate; Glc1P, glucose 1-phosphate; AGPase, ADP-glucose pyrophosphorylase; ADPGlc, ADP-glucose; PP_i, pyrophosphate; P_i inorganic phosphate; UDPGlc, UDP-glucose; TP/P_i translocator, triose phosphate/P_i translocator.

Photoassimilated carbon, in the form of G3P, is converted into hexose phosphates that serve as precursors for the synthesis of starch and sucrose. Starch is synthesized within the stroma of chloroplasts (recently reviewed by Zeeman, et al., 2007 and 2010). In the key step of starch synthesis, glucose 1-phosphate is activated with ATP in a reaction catalyzed by ADP-glucose pyrophosphorylase (AGPase) to yield ADP-glucose and pyrophosphate that is hydrolyzed to two inorganic phosphate groups (P_i) by alkaline pyrophosphatase. ADP-glucose then transfers the glucosyl group to the nonreducing end of a starch molecule (Figure 3). The P_i released in the synthesis of starch operates as a regulatory link between light reactions and carbon reactions since it is consumed in the synthesis of ATP in the light reactions. Starch is the major storage form of chemical energy in most plants. It is transiently stored in chloroplasts and degraded during dark periods to provide sugars for metabolism and growth throughout the night. Starch degradation begins with the reversible phosphorylation of starch granules by glucan, water dikinase (GWD) and phosphoglucan phosphatase (DSP4). Soluble

glucans are then released from the starch granule by amylases and converted to glucose and maltose that are exported from the chloroplast to the cytosol (Zeeman, et al., 2010).

Sucrose is the transport form of carbohydrates in plants and in contrast to starch, sucrose is synthesized in the cytosol. G3P is transported to cytosol via triose phosphate/P_i translocator that regulates the competing processes, the synthesis of starch and sucrose in mesophyll cells. When the cytosolic P_i concentration is high, G3P is exported to cytosol and converted to sucrose. When the cytosolic P_i concentration is low, starch synthesis is activated inside the chloroplasts (Figure 3).

In the sucrose synthesis glucose 1-phosphate is activated with UTP to form UDP-glucose, which then transfers a glucosyl group to fructose 6-phosphate to form sucrose 6-phosphate. Hydrolysis of sucrose 6-phosphate by sucrose phosphatase yields sucrose (Figure 3).

Besides photosynthesis a number of other metabolic processes are localized to chloroplasts in plants. Nitrogen assimilation, the biosynthesis of fatty acids, amino acids, vitamins, purines, hormones and heme take place in chloroplasts (Galili, 2002; Weber and Flugge, 2002; Boldt and Zrenner, 2003; Lunn, 2007; Tanaka and Tanaka, 2006). These pathways are not necessarily confined exclusively to chloroplasts as part of them is also, partially or alternatively conducted in the cytosol and in the mitochondria. Thus the pathways in different cellular compartments need to be linked together, which is usually attained by integral membrane translocator proteins that transfer intermediate metabolites. The transported metabolites also act as signals that provide information of metabolic conditions in the originating compartment (Lunn, 2007).

1.3. Regulatory and antioxidant redox-systems in chloroplast

Chloroplasts contain numerous redox-active components that function in electron transfer reactions, as oxidants or as specific regulators of other molecules. The redox compounds in chloroplasts may also participate in signaling cascades that control metabolic reactions, gene transcription and translation not only in the chloroplast but via retrograde signaling in the nucleus as well. The redox-active compounds in chloroplasts include photosynthetic electron transport chain components in thylakoid membranes and electron-flow-dependent soluble components such as ROS, thioredoxins and glutaredoxins as well as antioxidants. In this chapter the redox compounds related to this thesis work, thioredoxins, ROS and antioxidative enzymes, are introduced.

1.3.1. Thioredoxin systems

Thioredoxins (Trxs) are small proteins with the conserved amino acid sequence of WCG/PPC that has thiol:disulfide oxidoreductase activity. These regulatory proteins have a low redox potential (between -285 and -350 mV) that gives them strong reductive properties (Gelhaye, et al., 2005). In the reduced state, Trxs are able to reduce the disulfide bridges formed between redox-active cysteines in the Trx target proteins. Subsequently, the oxidized Trxs are reduced by thioredoxin reductases, forming together the so-called thioredoxin system. In contrast to non-photosynthetic organisms, plants contain a particularly large number of Trx isoforms in several cellular compartments, including the cytosol, nucleus, mitochondria and chloroplasts. In Arabidopsis, 42 genes coding for Trx and Trx-like sequences have been identified (Table 1; Meyer, et al., 2008; Chibani, et al., 2009; Arsova, et al., 2010), and many of these proteins are located in the chloroplasts or at least predicted to be imported into the chloroplasts. Chloroplasts contain *f*-, *m*-, *x*-, *y*-, *z*- type Trxs and a drought-induced stress protein (CDSP)32; *h*-type Trxs are present both in cytosol and mitochondria, and *o*-type Trxs are present only in mitochondria. The crystal structures of *f*-, *m*- and *h*-type Trxs and their target proteins have been resolved (Chiadmi, et al., 1999; Johansson, et al., 1999; Capitani, et al., 2000; Fermani, et al., 2001; Falini, et al., 2003; Coudevylle, et al., 2005; Peterson, et al., 2005; Maeda, et al., 2010) whereas even the function of other Trxs, especially that of the Trx-like proteins is yet fairly unknown. The reasons for the high number of Trxs in plants compared to other living organisms are still unclear. It has been suggested to be related to the increased generation of ROS during photosynthesis and also to genome duplications in terrestrial plants (Meyer, et al., 2008).

Oxidized Trxs become reduced via the action of thioredoxin reductases. Cytosolic and mitochondrial Trxs in plants are reduced by NADPH-dependent thioredoxin reductases (NTRs) whereas the reduction of Trxs in chloroplasts is accomplished via the action of two systems, NADPH-dependent thioredoxin reductase C (NTRC) and ferredoxin-thioredoxin reductase (FTR). Arabidopsis has three genes encoding the isoforms of NTRs. Two isoforms, NTRA and NTRB are localized both in cytosol and mitochondria and constitute the Trx-reduction system in those cellular compartments (Serrato, et al., 2004). NTRC is a 55-kDa fusion protein localized to chloroplasts (Serrato, et al., 2004). It consists of two domains, a thioredoxin reductase domain in the N-terminus and a Trx domain in the C-terminus (Figure 4, Serrato, et al., 2004) that is missing from other NTR isoforms (Serrato, et al., 2004). Both NTRC domains possess a redox-active cysteine pair.

Table 1. Thioredoxins in Arabidopsis. From Meyer et al. (2008).

Thioredoxin type	AGI ID	Common name	Subcellular localization in Arabidopsis
F	AT3G02730	f1	Chloroplast
	AT5G16400	f2	Chloroplast
M	AT1G03680	m1	Chloroplast
	AT4G03520	m2	Chloroplast
	AT2G15570	m3	Chloroplast
	AT3G15360	m4	Chloroplast
X	AT1G50320	x	Chloroplast
Y	AT1G76760	y1	Chloroplast
	AT1G43560	y2	Chloroplast
Z	AT3G06730	z	Chloroplast
CDSP32	AT1G76080	CDSP32	Chloroplast
HCF164	AT4G37200	HCF164	Chloroplast
WCRKC	AT5G06690	WCRKC1	Chloroplast (homology)
	AT5G04260	WCRKC2	Chloroplast (homology)
Lilium	AT1G08570	Lilium1	Chloroplast (homology)
	AT4G29670	Lilium2	Chloroplast (homology)
	AT5G61440	Lilium3	Chloroplast (homology)
	AT2G33270	Lilium4	Chloroplast (homology)
	AT1G07700	Lilium5	Chloroplast (homology)
O	AT2G35010	o1	Mitochondria
	AT1G31020	o2	?
h subtype I	AT3G51030	h1	Cytosol
	AT5G42980	h3	Cytosol
	AT1G19730	h4	Cytosol
	AT1G45145	h5	Cytosol
h subtype II	AT5G39950	h2	Cytosol
	AT1G59730	h7	Cytosol (homology)
	AT1G69880	h8	Cytosol (homology)
h subtype III	AT2G40790	CxxS1	Cytosol (homology)
	AT3G08710	h9	Cytosol (homology)
	AT1G11530	CxxS2	Cytosol (homology)
	AT3G56420	CxxC2	Cytosol (homology)
WCGVC	AT3G53220	WCGVC	Cytosol (homology)
Cf-6 Interacting	AT3G06730	CI	?
Clot TRP14	AT5G42850	Clot	Cytosol (homology)
TDX	AT3G17880	TDX	Cytosol/nucleus
Nucleoredoxin	AT1G60420	Nucleor1	Nucleus (homology)
	AT4G31240	Nucleor2	Nucleus (homology)
Picot	AT4G04950	Picot1	Cytosol (homology)
	AT4G32580	Picot2	Cytosol (homology)
TARWCGPC	AT1G52990	TARWCGPC	Secretion (homology)
NTRC	AT2G41680	NTRC	Chloroplast

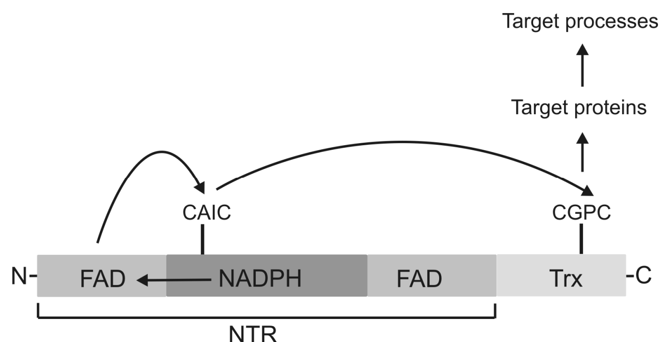


Figure 4. Schematic model of NTRC domains and the reaction mechanism. NTR, NADPH-dependent thioredoxin reductase domain containing binding sites for NADPH and FAD; Trx, thioredoxin domain; CAIC and CGPS, redox-active sites of NTR domain and Trx domain, respectively. Arrows represent electron transfer routes; see text for details.

Several NTR crystal structures have been resolved; most of them are bacterial enzymes (Kuriyan, et al., 1991; Lennon, et al., 1999 and 2000; Waksman, et al., 1994; Akif, et al., 2005; Hernandez, et al., 2008; Ruggiero, et al., 2009), but also two structures for plant enzymes, Arabidopsis NTRB (Dai, et al., 1996) and *Hv*NTR2 from barley (Kirkensgaard, et al., 2009) have been published. Based on those structures, a model for NTRC reaction mechanism has been proposed (Perez-Ruiz and Cejudo, 2009). According to this model, NTRC functions as a homodimer. Both subunits are able to transfer electrons from NADPH to FAD bound to NTR domain, and from FAD to the adjacent redox-active disulphide in the NTR domain (Figure 4). From the NTR domain of one subunit, electrons are then transferred via inter-subunit reaction to the redox-active site of Trx domain of the other subunit. The Trx domain is then capable of reducing its target proteins (Figure 4). As NADPH can also be produced during the dark period via the action of pentose phosphate pathway (Neuhaus and Emes, 2000), it is suggested that NADPH-dependent reduction of thioredoxins play an important role in plant chloroplasts during the night or under low light, when the level of reduced ferredoxin is low (Perez-Ruiz, et al., 2006).

Another plastidial thioredoxin reductase, FTR has been intensively studied since its discovery in the 1970's (Wolosiuk and Buchanan, 1978). FTR uses ferredoxin, reduced by electrons transported through the photosystems during photosynthesis, as an electron donor, thereby converting a light-activated electron signal to thiol signal. FTR in higher plants is an αβ-heterodimer encoded by the nuclear genes. FTR is comprised of a 13-kDa large catalytic subunit containing both a [4Fe-4S] cluster and a proximal redox-active disulfide, and a 8-12 kDa variable subunit (Schürmann and Jacquot, 2000; Schürmann and Buchanan, 2008). FTR is a versatile enzyme regarding its reactivity as it efficiently reduces *f*- and *m*-type Trxs (Schürmann and Buchanan, 2008). Specificity arises from the interaction between Trxs and target enzyme. Trx *f* functions primarily

in the activation of enzymes in photosynthetic metabolism like Calvin-Benson cycle enzymes (reviewed by Schürmann and Buchanan, 2008), Rubisco activase (Zhang and Portis, 1999) and ATP synthase (Schwarz, et al., 1997; Stumpp, et al., 1999). These enzymes interact less efficiently with Trx *m*. NADP-malate dehydrogenase was originally considered to be specifically activated by Trx *m*, but was later shown to be even more efficiently activated by Trx *f* (Hodges, et al., 1994; Geck, et al., 1996). Both Trx *f* and Trx *m* are also active in enzyme deactivation (Schürmann and Buchanan, 2008). For example, the STN7 protein kinase that phosphorylates thylakoid light-harvesting proteins loses its activity after treatment with Trx *f* and Trx *m* (Rintamäki, et al., 2000).

The development of proteomic approaches during recent years has allowed the identification of numerous proteins linked to thioredoxins. In addition to photosynthesis, thioredoxins appear to play important roles in a number of physiological processes in plants, including housekeeping metabolism, development and abiotic and biotic stress defense mechanisms (Montrichard, et al., 2009). However, although thioredoxins are well characterized in the control of photosynthesis (reviewed by Schürmann and Buchanan, 2008), the functions of many newly identified thioredoxin target proteins still remain to be explored.

1.3.2. Reactive oxygen species in chloroplast

Photosynthesis produces intermediates with extreme redox potentials: PSI generates a strong reductant capable of reducing $NADP^+$ and PSII reduces the electron donors to PSI and generates a very strong oxidant capable of oxidizing water to molecular oxygen. These basic photosynthetic reactions pose a significant risk for electron transfer to oxygen in chloroplast. Such reactions are harmful to the organism since oxygen can be considered as a double-edged molecular sward as it plays two different roles in biological systems; it is vital for aerobic metabolism but at the same time the reduction of molecular oxygen may result in formation of reactive oxygen species (ROS) that can harm the constituents of the cells and ultimately even cause a cell death.

The PSI and PSII complexes in thylakoid membranes are indeed the major generation sites of ROS in chloroplasts. Photoreduction of oxygen to hydrogen peroxide (H_2O_2) in PSI was discovered over 50 years ago by (Mehler, 1951). The primary step in this reaction generates superoxide radical ($O_2^{\cdot-}$), and its spontaneous or enzymatic disproportionation produces hydrogen peroxide (H_2O_2) and O_2 (Figure 5; Asada, et al., 1974). On the other hand, in PSII, oxygen of the ground (triplet) state ($^3O_2$) may be excited to singlet state ($^1O_2$) by the triplet state of excited chlorophyll (Telfer, et al., 1994; Hideg, et al., 1998; Krieger-Liszkay, 2005). The production of ROS in light is largely affected by physiological and environmental factors; the rate is enhanced under conditions where electron transport chain is overreduced, that is when light absorbed exceeds the dissipation and utilization capacity of light energy in photosynthesis (Li, et

al., 2009). Due to the high reactivity of ROS they pose a continuous threat to cellular constituents for uncontrolled oxidation of lipids, nucleic acids and amino acids.

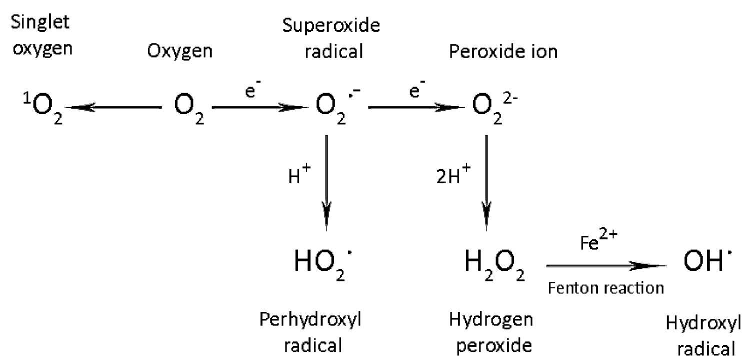


Figure 5. Generation of different forms of ROS from oxygen. See text for details. Figure drawn according to Gill and Tuteja (2010).

H_2O_2 is a moderately reactive form of oxygen. It is not a free radical and therefore less reactive and longer-lived compound than superoxide anion. On the other hand, due to its lower reactivity compared to other ROS, H_2O_2 can diffuse out of the chloroplasts and within and between cells and act beyond the generation site (Mubarakshina, et al., 2010). In principle, H_2O_2 is relatively stable in the absence of transition metals. However, with molecules containing Fe^{2+} or other transition metals, hydroxyl radicals can be formed from H_2O_2 via Fenton reaction (Figure 5; reviewed by Blokhina et al., 2003). Hydroxyl radical is highly toxic ROS that can potentially react with all important biological molecules including DNA, proteins and lipids (Gill and Tuteja, 2010).

1.3.3. Detoxification of reactive oxygen species

To minimize the potentially hazardous reactions initiated by ROS, plants have evolved an extensive antioxidant defence system with low-molecular mass non-enzymatic antioxidants such as ascorbate, glutathione, tocopherols, carotenoids and phenolic compounds, and antioxidant enzymes that respond to redox imbalances (Mittler, et al., 2004). Under physiological steady state conditions plants typically maintain ROS at low levels, but in the induction of photo-oxidative stress both the production of ROS and the pool sizes of low-molecular mass antioxidants and antioxidant enzymes increase (Foyer, et al., 1997). In contrast to most organisms, plants have multiple genes encoding for antioxidant enzymes. Different isoforms are specifically targeted to

chloroplasts, mitochondria, peroxisomes, as well as to the cytosol and apoplast (Apel and Hirt, 2004; Mittler, et al., 2004).

Detoxification of superoxide anions and H_2O_2 in chloroplasts is catalyzed by four antioxidant enzyme systems presented in Figure 6. Superoxide anions produced by PSI are rapidly dismutated to H_2O_2 either spontaneously or by superoxide dismutases (SODs) (Foyer et al 1997; Asada, 1999) (Figure 6A). Subsequently, H_2O_2 is converted to water by ascorbate peroxidases (APXs) and ascorbate, by peroxiredoxins (Prxs) and thioredoxins, or by glutathione peroxidase (GPX) cycle (Foyer, et al., 1997; Asada, 1999; Mittler, 2002; Rouhier and Jacquot, 2002).

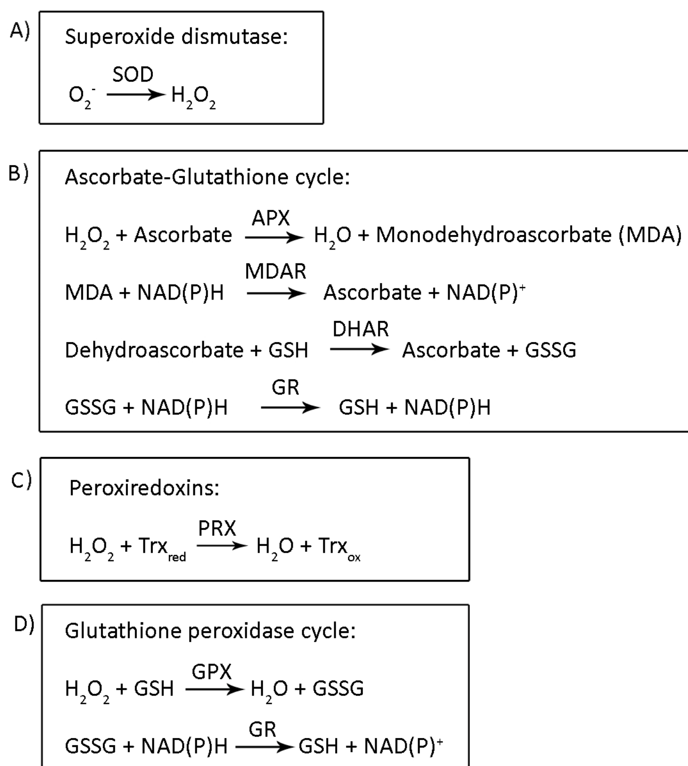


Figure 6. Overview of the enzymatic ROS scavenging reactions by SODs (A), APXs (B), Prxs (C) and GPXs (D) in chloroplasts. See text for details.

APXs are haem-binding enzymes that reduce H_2O_2 with ascorbate as an electron donor. Plants contain five APX isoenzymes in different cellular compartments. Two chloroplastic isoforms, a 38 kDa thylakoid-bound APX (tAPX) and a 33 kDa stromal APX (sAPX), which is dually targeted also to mitochondria, are both encoded by a

single nuclear gene in Arabidopsis. 25 kDa APX1 and APX2 are located in the cytoplasm and a 31 kDa APX3 has been found in peroxisomes and oilseed glyoxysomes. In the first APX-catalyzed step of ascorbate-glutahione cycle (Figure 6B), H_2O_2 is reduced to water with electrons from ascorbate which is oxidized into monodehydroascorbate (MDA). In the second step, MDA reductase (MDAR) reduces MDA into ascorbate with the help of NAD(P)H. MDA can also become oxidized spontaneously to dehydroascorbate (DHA) that is reduced to ascorbate by DHA reductase (DHAR) or by glutaredoxins with the help of GSH that is oxidized to GSSG. The cycle closes with glutathione reductase (GR) that rereduces GSSG back into GSH with NADPH (Asada, 1999).

Prxs form a large group of 17-22 kDa enzymes which contain a conserved cysteine in the N-terminus of a protein that is responsible for peroxidase activity (Rouhier and Jacquot, 2002). In Arabidopsis, four Prxs are found in chloroplasts, 2-cysteine (2-Cys) Prxs A and B, PrxQ and PrxIIE. Like APXs, Prxs rely on external electron donor in order to reduce H_2O_2 to water (Figure 6C). A regular electron donor for chloroplastic Prx isoforms is a reduced thioredoxin (Rouhier and Jacquot, 2002), which is oxidized in the reaction and then needs to be re-reduced by thioredoxin reductase.

The GPX cycle converts H_2O_2 into water using reducing equivalents from glutathione (GSH) (Figure 6D) (Ursini, et al., 1995) or thioredoxins (Herbette, et al., 2002; Jung, et al., 2002). Oxidized GSSG is again converted into GSH by glutathione reductase with NADPH as a reducing agent.

1.4. Chloroplast biogenesis

Chloroplast biogenesis in higher plants is defined here as a process in which chloroplasts develop from proplastids, small precursor organelles that lack organized internal membranes and are primarily present in meristematic cells. As meristematic cells differentiate to leaf mesophyll cells, proplastids differentiate into chloroplasts (Vothknecht and Westhoff, 2001). In young, developing leaves the conversion of proplastids to chloroplasts can occur directly or indirectly via etioplast and etio-chloroplast stages (Figure 7). Direct transformation of proplastids to chloroplasts requires high light intensity, while etioplasts and etio-chloroplasts are developed under darkness and also under low light and natural light-dark cycles (Solymosi and Schoefs, 2010). In contrast to proplastids, etioplasts contain an inner membrane system which is organized in highly regular paracrystalline structures called prolamellar bodies (PLBs), several proteins involved in photosynthesis (Blomqvist, et al., 2008; Kanervo, et al., 2008) and protochlorophyllide, a precursor for chlorophyll (Schoefs and Franck, 2003). Under natural light-dark cycles PLBs appear during dark periods and disappear in the beginning of the light period (Solymosi and Schoefs, 2010). PLB membranes are precursors for thylakoid membranes that are formed during greening when etioplasts are converted to chloroplasts.

During the conversion of proplastids into chloroplasts, the plastid volume increases up to 100-fold (Gutierrez-Nava, et al., 2004). Plastids also need to increase their number by binary fission as their 'host' cells expand. The total volume of chloroplasts in cell and the size of mesophyll cell are strictly controlled in plant species (Pyke, 1999) but the regulation mechanisms are still fairly unknown. Plastid division requires the operation of several plastid division proteins (recently reviewed by Miyagishima and Kabeya, 2010). FtsZ proteins self-assemble beneath the inner envelope membrane to form the core ring structure (Z-ring) on which other division components assemble. The correct position of the Z-ring in the middle of the chloroplast is determined by a complex of Min proteins and ARC3 which is localized in the ends of chloroplast thus preventing the assembly of the Z-ring at incorrect position (Maple and Møller, 2007).

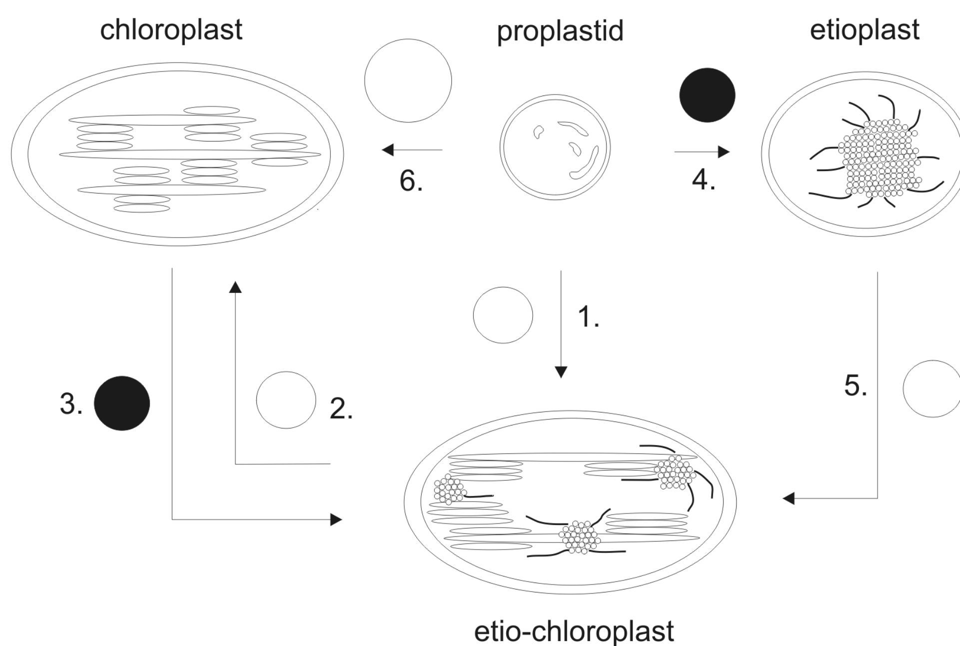


Figure 7. Different pathways for chloroplast development. Under low light intensities and under natural light-dark cycles, proplastids differentiate to etio-chloroplasts (1.) before conversion to chloroplasts (2.). This route is reversible during the dark periods until chloroplasts are fully maturated (3.). The proplastids of seedlings germinating in the absence of light differentiate into etioplasts (4.). When provided with light, etioplasts are transformed into chloroplasts via the etio-chloroplast stage (5. and 2.). Under high light intensities, proplastids differentiate directly to chloroplasts (6.). Figure drawn according to Solymosi and Schoefs (2010).

Division and expansion of chloroplasts also require active synthesis of membranes in chloroplasts. New thylakoid membranes are formed from lipids that are either derived from precursors assembled de novo in the plastid or imported from the endoplasmic reticulum (Benning, 2009). Thylakoid membranes are constructed via an orchestrated process in which membrane lipids, proteins and pigments are assembled into functional, three-dimensional structures (Vothknecht and Westhoff, 2001). Since relatively small number of genes resides in chloroplasts, the majority of plastid proteins are encoded in the nucleus, translated in the cytosol and imported into chloroplasts. Chloroplast multiprotein complexes such as ribosomes and photosystems are patchworks of subunits encoded by both chloroplast and nuclear genomes. In order to attain functional thylakoid membranes, biosynthesis and import of these components must be well-balanced. Mutations that interfere with the synthesis of major protein components of thylakoid membranes, e.g. major subunits of photosystems (Meurer, et al., 1998; Baena-Gonzalez, et al., 2003; Ido, et al., 2009), protein import apparatus (Bauer, et al., 2000) or pigment biosynthesis (Falbel and Staehelin, 1994; Masuda, et al., 2003) may cause defects in thylakoid formation. Furthermore, specific chloroplast proteins involved in thylakoid formation have been recently identified. THYLAKOID FORMATION 1 (THF1) was shown to control an important step required for the normal organization of membrane vesicles into mature thylakoid stacks (Gao, et al., 2006), and loss of FZO-LIKE (FZL) protein function resulted in altered chloroplast and thylakoid morphology in Arabidopsis (Wang, et al., 2004). Also VESICLE INDUCING PLASTID PROTEIN 1 (VIPP1) has been suggested to be important for thylakoid membrane formation, since mutant plants with greatly reduced expression of *vipp1* gene show inhibited chloroplast vesicle transport and probably therefore have lost the ability to build up a proper thylakoid membrane system (Kroll, et al., 2001; Aseeva, et al., 2007).

1.4.1. Chlorophyll biosynthesis

Chlorophyll, the green pigment, is vital for photosynthesis as it absorbs light energy and directs the light energy towards photosystems. In plants, chlorophyll biosynthesis takes place in chloroplasts, and all participating enzymes are encoded by the nucleus. Chlorophyll biosynthesis (reviewed by Mochizuki et al., 2010) starts with the formation of 5-aminolevulinic acid (ALA) from glutamyl-tRNA^{Glu} by glutamyl-tRNA reductase (HEMA1) and glutamate 1-semialdehyde aminotransferase (Figure 8). Eight molecules of ALA are then assembled via three enzymatic steps into uroporphyrinogen III, which can continue in the chlorophyll biosynthesis pathway or be converted to siroheme, the prosthetic group for both nitrite and sulfite reductases in plants. In the subsequent step of chlorophyll biosynthesis, uroporphyrinogen III is decarboxylated to form protoporphyrinogen IX, which is further oxidized to protoporphyrin IX. Protoporphyrin IX can be converted to heme via insertion of Fe^{2+} or to chlorophyll via insertion of Mg^{2+}, thus being the second branchpoint of the pathway. In the chlorophyll branch, insertion of Mg^{2+} is catalyzed by Mg-chelatase, a multi-subunit protein complex. Mg-protoporphyrin IX is then modified to Mg-protoporphyrin monomethyl

ester by Mg-protoporphyrin IX methyltransferase (CHLM), and in subsequent step catalyzed by Mg-protoporphyrin monomethylester cyclase (CRD1) to form protochlorophyllide which is then reduced to chlorophyllide *a*. The reaction of protochlorophyllide reduction to chlorophyllide, mediated by NADPH:protochlorophyllide oxidoreductase (POR), is catalyzed by the energy of light. Chlorophyllide *a* is esterified with a phytol chain to give chlorophyll *a*, some of which is reversibly converted to chlorophyll *b*.

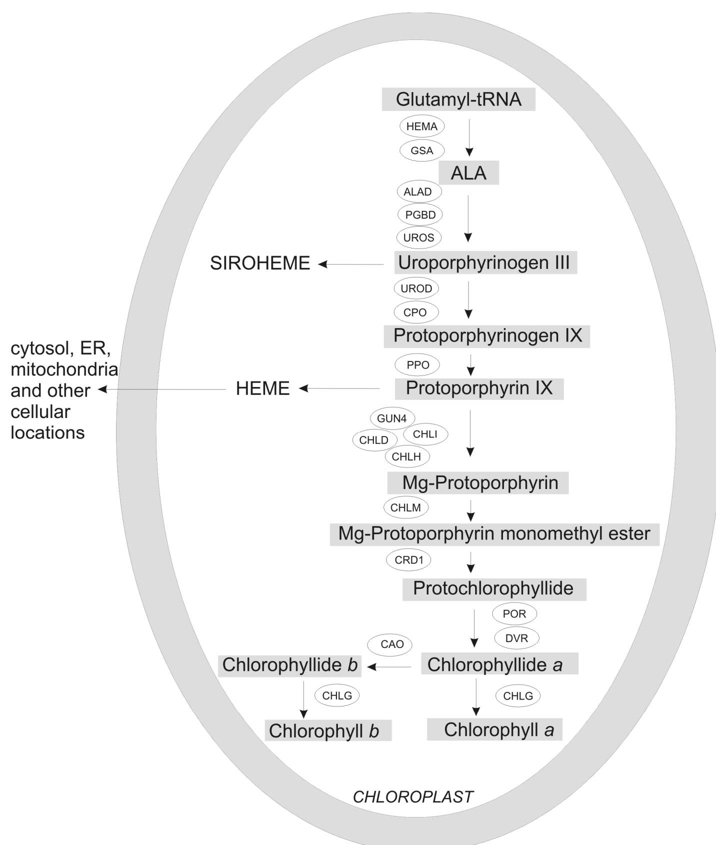


Figure 8. Schematic illustration of the chlorophyll biosynthesis pathway. HEMA, glutamyl-tRNA reductase; GSA, glutamate-1-semialdehyde 2,1-aminomutase; ALAD5-aminolevulinate dehydratase; PBGD, porphobilinogen deaminase; UROS, uroporphyrinogen III synthase; UROD, uroporphyrinogen III decarboxylase; CPO, coproporphyrinogen III oxidase; PPO, protoporphyrinogen IX oxidase; GUN4, regulator of Mg-chelatase; CHLD, Mg-chelatase D-subunit; CHLI, Mg-chelatase I-subunit; CHLH, Mg-chelatase H-subunit; CHLM, Mg-protoporphyrin IX methyltransferase; CRD1, Mg-protoporphyrin IX monomethylester cyclase; POR, NADPH:protochlorophyllide oxidoreductase; DVR, divinyl-protochlorophyllide reductase; CHLG, chlorophyll synthase; CAO, chlorophyllide *a* oxygenase. Figure drawn according to Mochizuki et al. (2010).

The enzymes of the chlorophyll biosynthesis pathway from glutamyl-tRNA to protoporphyrinogen are localized in chloroplast stroma (Joyard, et al., 2009). Subsequent enzymes from protoporphyrinogen oxidase through to POR are associated with both chloroplast envelope and thylakoid membranes. However, the localization of Mg-chelatase is puzzling, since GUN4 and different subunits of Mg-chelatase have been found in both soluble and membrane-containing fractions of purified chloroplast (Guo, et al., 1998; Nakayama, et al., 1998; Larkin, et al., 2003; Joyard, et al., 2009) CHLI is localized in the stroma and CHLD has been suggested to be localized predominantly in stroma but to some extent in thylakoid membranes as well (Guo, et al., 1998; Joyard, et al., 2009). CHLH is localized in both stroma and envelope membranes (Nakayama, et al., 1998). Moreover, Mg-chelatase has been reported to respond to Mg^{2+} concentration and to associate with envelope membranes when Mg^{2+} concentration is high but to dissociate from membranes when Mg^{2+} concentration decreases (Guo, et al., 1998; Nakayama, et al., 1998). Of the enzymes catalyzing the final steps of chlorophyll biosynthesis, chlorophyll synthase is localized in thylakoid membranes (Joyard, et al., 2009) but chlorophyllide *a* oxygenase (CAO) has been suggested to be present in envelope membranes (Eggink, et al., 2004).

Chlorophyll molecules that are not properly assembled to protein complexes are able to direct the light energy to unappropriate targets, such as molecular oxygen, which results in the formation of ROS that can harm the cell constituents. Since chlorophyll is synthesized in particularly large amounts during chloroplast biogenesis it is clear that chlorophyll biosynthesis is tightly regulated. The regulation is achieved by strong transcriptional control of specific genes together with post-translational regulation in chloroplasts which allow rapid changes in the flow rate of the pathway. *HEMA1, CHLH, CRD1* and *CAO* have been demonstrated to comprise a group of genes that strongly respond to light and circadian signals (Matsumoto, et al., 2004). The rate-limiting step in the pathway is the activity of glutamyl-tRNA reductase and the synthesis of ALA, which is feedback-inhibited by the accumulation of the end products of both heme branch and chlorophyll branch. Feedback-inhibition of HEMA1 in response to accumulation of protochlorophyllide in the chlorophyll branch is achieved via activation of FLU that suppresses the activity of HEMA1 (Meskauskiene, et al., 2001; Goslings, et al., 2004). CHLI, one of the three subunits of Mg-chelatase (Ikegami, et al., 2007), has been identified as a target for thioredoxin-dependent regulation. Also the activity of CAO is modified post-translationally as it is destabilized in response to accumulation of chlorophyll *b* (Yamasato, et al., 2005).

1.5. Regulation of chloroplast biogenesis and acclimation to environmental cues

The chloroplast differentiation process is modulated by environmental cues, of which the most important is light, and it depends on coordinated action of nuclear and organellar gene expression. To sense light, plants use a range of different

photoreceptors that cover a broad spectrum of light signals. Sensitivity to red and far-red light is mediated by phytochromes, the family of which contains five proteins (phyA-phyE) in Arabidopsis. Two of these, phyA and phyB, are particularly important during de-etiolation, the conversion of an etioplast to chloroplast (Reed, et al., 1994; Mazzella, et al., 2001). Blue light is sensed by cryptochromes (cry1 and cry2 in Arabidopsis; Cashmore, et al., 1999), of which cry 1 has a dominant role during de-etiolation under blue light (Mazzella, et al., 2001).

1.5.1. Anterograde signaling

Since chloroplast biogenesis is tightly bound to the development of its 'host' cell and largely dependent on the import of nuclear-encoded proteins, communication between nucleus and chloroplast is required in order to adjust gene expression in both compartments to ensure co-regulation of genes whose products function together. Anterograde (nucleus-to-organelle) mechanisms coordinate gene expression in organelles in response to endogenous and environmental signals perceived by the nucleus. Nuclear-encoded proteins primarily control the gene expression in the plastids, mostly by post-transcriptional mechanisms (Woodson and Chory, 2008), but also chloroplast division and differentiation are dependent on nuclear-encoded proteins. Chloroplasts of higher plants contain two RNA polymerases, nuclear-encoded one (NEP) and plastid-encoded one (PEP) whose activity is, however, fine-tuned by nuclear-encoded sigma factors (Maliga, 1998). The two RNA polymerases and different nuclear-encoded sigma factors are essential during different stages of Arabidopsis development (Kanamaru and Tanaka, 2004). It is assumed that NEP and PEP act sequentially during the chloroplast development: NEP is active in proplastids transcribing housekeeping genes, and PEP initiates the transcription of photosynthesis-related genes and takes over the transcription of housekeeping genes in developing chloroplasts (Maliga, 1998). Of the 6 Arabidopsis nuclear-encoded sigma factors, SIG2 is essential for chloroplast development as it has a role in transcription of glutamate-specific tRNA gene. Thus SIG2 has the potential for controlling the flux of glutamyl-tRNA for both protein synthesis and chlorophyll synthesis in chloroplasts. SIG5 instead responses to various stress conditions and contributes to the repair of the damaged PSII reaction center proteins (Kanamaru and Tanaka, 2004).

1.5.2. Chloroplast-to-nucleus retrograde signaling

In order to maintain mature chloroplasts optimally functional, chloroplast operations need to be adjusted according to the environmental cues. Changes in the light conditions result in changes in the photosynthetic flux, and damage of chloroplasts by high light or pathogens induces repairing mechanisms. Coordinated regulation of gene expression in the nucleus and plastids is crucial for the acclimation of plants as the

intracellular communication between organelles establishes the proper balance of gene expression products in a changing environment.

Retrograde (organelle-to-nucleus) signalling plays a key role in optimizing plastid functions. Retrograde mechanisms transmit signals that have originated in the organelle to regulate gene expression in nucleus that can then modify anterograde signals. Thus retrograde signals provide information of the metabolic and developmental stage of organelles for the nucleus, and it is now obvious that these signals are produced by several different processes in plastids, including photosynthesis, pigment biosynthesis and metabolism (Beck, 2005; Nott, et al., 2006; Piippo, et al., 2006). Plastidial signals can be classified into several groups depending on where they originate from: (i) redox processes in photosynthesis, (ii) pigment biosynthesis, (iii) metabolite pool changes, and (iv) generation of ROS.

The redox state of photosynthetic electron transport chain and the levels of ROS that are continuously formed as byproducts of photosynthesis depict the chloroplast's photosynthetic performance which needs to be adjusted according to environmental changes. Relevant redox-active components that can be a source of signal are the PQ pool and the PSI acceptor site molecules (NADPH, thioredoxin and glutaredoxin) (Baier and Dietz, 2005). However, it seems unlikely that either ROS or redox compounds could themselves act as signalling molecules that traverse to cytosol.

The chlorophyll biosynthesis intermediate, Mg-protoporphyrin IX, has been considered to act directly as a signalling molecule (Strand, et al., 2003) and to travel to cytosol (Ankele, et al., 2007), but recent precise and reproducible experiments have questioned the hypothesis (Mochizuki, et al., 2008; Moulin, et al., 2008). Nevertheless, studies with a unicellular red alga *Cyanidioschyzon merolae* have shown that nuclear DNA replication, which precedes cell division, is regulated by tetrapyrrole signals, namely protoporphyrin IX and and Mg-protoporphyrin IX (Kobayashi, et al., 2009). Furthermore, another composer of chlorophyll biosynthesis, H-subunit of Mg-chelatase, might still mediate plastid signalling as was suggested before by (Mochizuki, et al., 2001). Nonetheless, protein export from chloroplasts has not been demonstrated and the protein import apparatus is known to act unidirectionally (Kleine, et al., 2009).

Metabolites, especially carbohydrates, are strong candidates for signalling molecules as photosynthesis is tightly integrated with cellular metabolism. It is well known that increased levels of photosynthetic end products, glucose and sucrose, repress the expression of photosynthetic genes (reviewed by Rolland, et al., 2006). Moreover, small amounts of hexokinase, a cytosolic enzyme important for sensing and responding to intracellular glucose signals, are found in the nucleus (Cho, et al., 2006) thus implying a direct metabolic connection between photosynthesis and nuclear gene expression.

1.5.3. ROS in plant signaling

ROS are toxic compounds but they are also involved in signaling networks in plants. Singlet oxygen, superoxide and hydrogen peroxide produced in chloroplasts are implicated to participate in chloroplast-to-nucleus retrograde signaling. The connection between H_2O_2 and signaling networks has been well-documented for many biotic and abiotic stress responses (Larkindale and Knight, 2002; Apel and Hirt, 2004; Mateo, et al., 2006). In fact, H_2O_2 is the ROS that has been recognized to induce the largest changes in the levels of gene expression in plants, and this is probably due to its relative stability (Dat, et al., 2000; Bechtold, et al., 2008; Fahnenstich, et al., 2008; Foyer and Noctor, 2009). Yet it is not clear whether H_2O_2 is actually the signal itself, or whether H_2O_2 oxidizes other molecules to generate an intracellular signal (Desikan, et al., 2004). In microbes, proteins with H_2O_2 sensing capabilities have been identified (Lee and Helmann, 2006; Toledano, et al., 2004; Manchado, et al., 2000; Zheng, et al., 1998). These sensors share a mechanism in which H_2O_2 reacts with a unique cysteine residue, coupling H_2O_2 metabolism and thiol redox signaling. To date, no H_2O_2 receptor has been definitively identified in plants (Foyer and Noctor, 2009). However, signal molecules are usually present in cells in very low amounts, and plant cells seem to tolerate higher concentrations of H_2O_2 than animal cells. The endogenous concentration of H_2O_2 in plant cells is reported to range from nanomoles to several hundred micromoles of H_2O_2 per gram fresh mass (Willekens, et al., 1997; Karpinski, et al., 1999; Veljovic-Jovanovic, et al., 2002), while H_2O_2 is toxic for most animal cells at levels of about 10–100 µM (Slesak, et al., 2007). It has been suggested that plants tolerate high H_2O_2 levels due to the fact that plant antioxidant response systems are designed more for the control of the cellular redox state than for complete elimination of H_2O_2 (Slesak, et al., 2007). Superoxide and singlet oxygen are highly reactive compounds and therefore possibly able to bind and modify the activity of some kinases and phosphatases (Reinbothe, et al., 2010). Superoxide and singlet oxygen accumulating in chloroplasts during photosynthesis have been proposed to trigger refined signaling cascades involving plant hormones that modify plant growth (Reinbothe, et al., 2010; Pilon, et al., 2011). However, both superoxide and singlet oxygen produced in chloroplasts have only limited ability to cross membranes thus being unable to affect signaling pathways outside of the chloroplast (Apel and Hirt, 2004).

The limitation of H_2O_2 and other ROS as signaling compounds is the fact that they lack specificity; they are simple molecules, unable to store or transmit complex information, and most of them are very reactive and thus short-lived. It has been proposed that rather than ROS themselves, peptides derived from degradation of ROS-damaged proteins could be more specific and selective secondary messengers to the nucleus (Møller and Sweetlove, 2010). Nonetheless, due to its dual role as a damage-inducing and a signal-inducing compound, the levels of H_2O_2 in the cells must be carefully balanced.

2. Aims of the study

Plants contain a particularly large number of thioredoxin isoforms in different cellular compartments, including the cytosol, nucleus, mitochondria and chloroplasts. Chloroplasts are different from other cell organelles since in addition to numerous thioredoxin isoforms located to chloroplasts, they also contain two different thioredoxin reductase systems. Chloroplasts are also unique because of the high production of ROS in light-dependent reactions, and thioredoxins are closely linked with the antioxidant systems scavenging hydrogen peroxide. In my thesis work, I aimed i) to identify chloroplast processes regulated by the NADPH-dependent thioredoxin system involving NTRC, the thioredoxin reductase that was recently discovered when the experimental work for my doctoral thesis was about to start; and ii) to reveal the impact of NTRC and other chloroplast antioxidant enzymes on the acclimation of plants to various light regimes.

3. Methodological aspects

3.1. Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia plants were used in all experiments. T-DNA insertion mutants (Alonso et al. 2003) *ntrc* (At2g41680; SALK_096776 and SALK_114293), *sapx* (At4g08390; SALK_083737) and *tapx* (At1g77490; SALK_027804) were obtained from the Salk Institute (http://signal.salk.edu/). The homozygosity of the mutants was assessed by PCR with two gene-specific primers and one primer specific to T-DNA insertion. A *tapx sapx* double mutant was created by crossing *tapx* and *sapx* single mutants, and was identified from the F2 generation by PCR analysis by using the same set of primers which were used to identify the single mutants.

Plants were grown at 23 °C under moderate white light (growth light; 100-150 µmol photons m^{-2} s^{-1}) in 8-h light/16-h dark periods (short-day-plants) (Papers I-IV), 16-h light/8-h dark periods (long-day-plants) (Papers I-III), continuous light (Papers I-II) or several distinct light-dark periods (Papers II-III). In Paper IV, plants were treated with high light intensities (300-1300 µmol photons m^{-2} s^{-1}) or low temperature (10 °C) for 2 h to 2 weeks. In Paper I, plants were also germinated under different spectral qualities of light: red (30 µmol photons m^{-2} s^{-1}), blue (3 and 30 µmol photons m^{-2} s^{-1}) and far-red light. Mature leaves or rosettes of 3-4 weeks old Arabidopsis plants were used as material in most of the experiments in Papers I-IV. 5-day-old and 10-day-old seedlings were also used in Paper I, and 7-day old seedlings in Paper IV.

3.2. Stress treatments

For short-term stress treatments, 4-week-old plants grown under growth light and an 8-h photoperiod were shifted to higher light intensities, described in more detail in Paper IV, for 2 to 6 hours. For long term stress treatments, plants were first grown under growth light for 2 weeks, and thereafter shifted to higher light intensities or low temperature, described in more detail in Paper IV, for 2 weeks.

The sensitivity of mature rosettes to methyl viologen -induced photo-oxidative stress was explored by spraying the plants with 50 µM methyl viologen at the end of the dark period. In the subsequent morning, plants were illuminated under growth light or high light as described in the Paper IV. The extent of cell death and membrane disruption was measured as ion leakage of excised rosettes to ion-exchanged water with a conductivity meter (Radiometer).

The sensitivity of germination to photo-oxidative stress was explored by germinating seeds on filter paper on Petri dishes containing half-strength Murashige and Skoog medium supplemented with 1,5 µM methyl viologen and following greening under growth light.

3.3. Analysis of pigments and proteins

Chlorophyll content of leaf disks was determined in DMF (dimethylformamide) according to Inskeep and Bloom (1985). Anthocyanin content of leaf disks was measured according to Neff and Chory (1998) with small modifications described in Paper I. Isolation of thylakoid membranes and total root and leaf extracts was performed as described in Paper I. Chlorophyll content of isolated thylakoids was measured in HEPES-buffered acetone according to Porra et al. (1989), and the protein contents of the total and soluble extracts were determined with the Bio-Rad Protein Assay Kit. Proteins were separated with 12% (w/v) SDS-PAGE and detected using specific antibodies described in the respective papers.

3.4. Microarray analysis

For microarray analysis, total RNA was isolated with Trizol reagent and labeled by the aminoallyl method with Cy3 or Cy5 fluorescent dyes. RNA isolation, cDNA synthesis, labeling, hybridization and the data analysis were performed as described in detail in Papers I and IV.

3.5. Biophysical methods

The photoinhibition state of PSII was monitored as a ratio of variable to maximal chlorophyll fluorescence, $F\text{v}/F\text{max}$ ($F\text{v}$ is a difference between maximal, $F\text{max}$, and initial, $F\text{o}$, fluorescence), measured from intact leaves with a Hansatech PEA fluorometer after a 30 min dark incubation. Gas exchange of intact Arabidopsis plants was measured with CIRAS-1 combined IR gas analysis system (PP Systems) equipped with an Arabidopsis pot chamber (PP Systems). The response of net photosynthesis (A_n) to the reference CO_2 was measured under PPDF (photosynthetically active photon flux density) that was saturating for net photosynthesis (500 µmol m^{-2} s^{-1} at 20 °C). The parameters for maximal carboxylation rate of Rubisco ($V\text{cmax}$, µmol of CO_2 m^{-2} s^{-1}), maximal electron transport rate ($J\text{max}$, µmol m^{-2} s^{-1}) and rate of mitochondrial respiration in light ($R\text{d}$, µmol m^{-2} s^{-1}) were obtained by modelling the response of net CO_2 assimilation to increasing extracellular CO_2 concentration according to Farquhar et al. (1980).

3.6. Microscopy

Confocal microscope images in Papers I and IV were obtained with an inverted confocal laser-scanning microscope (Zeiss LSM510 META) using a 20x/0.50 water objective. Chlorophyll fluorescence was excited at 488 nm with an argon diode laser, and detected with a 650-710 nm passing emission filter. Maximal projections of sequential confocal images were created with the Zeiss LSM Image Browser software. The samples for light microscopy and electron microscopy were prepared according to Pätsikkä et al. (2002). Electron microscope images were obtained with a transmission electron microscope (JEOL JEM-1200EX).

3.7. In vivo-detection of H_2O_2 and superoxide

Accumulation of H_2O_2 and superoxide in the leaves was detected using DAB (diaminobenzidine; Sigma–Aldrich) and NBT (nitroblue tetrazolium, Sigma-Aldrich), respectively, as substrates. Rosettes were excised at the end of the light period, and incubated on Petri dishes containing a solution of DAB or NBT overnight in darkness. In the subsequent morning, the dishes were transferred to growth light for 1 h and thereafter the rosettes were incubated in ethanol until chlorophyll was bleached. Finally the rosettes were photographed.

3.8. Analyses of amino acids, hormones and sugars

Plant material for amino acid analysis was treated and extracted as described in Paper I. Amino acid content of the extracts were further extracted and derivatized with the Ez:faast liquid chromatography-mass spectrometry kit (Phenomenex) using the procedure described by Husek (1998) and analyzed as propyl chloroformates with HPLC-ESI/MS. Plant hormones were analyzed in Paper I by gas chromatography-mass spectrometry analysis using a modified vapour-phase extraction method described by Schmelz et al. (2003). Soluble sugars were extracted from the plant material as described in Paper III. Sucrose, D-fructose and D-glucose were measured spectrophotometrically with Sucrose/Fructose/D-Glucose assay kit (Megazyme). For measurement of starch content, the remaining leaf material was dried and ground in liquid nitrogen. Starch was solubilised and the total starch content was measured spectrophotometrically with Total Starch assay kit (Megazyme).

3.9. Yeast two-hybrid analysis

The yeast strain CY306 (Vignols, et al., 2005) that carries deletions of endogenous genes encoding cytosolic TRXs was used throughout the yeast two-hybrid experiments. *Escherichia coli* DH5α –strain was used in molecular cloning. For the yeast two-hybrid prey constructs, Riken RAFL (Riken, BRC, Japan) cDNA clones were used as templates to generate PCR products from open reading frames of Arabidopsis genes and to clone them cloned in frame with the activator or binding Gal4 domains in pGAD.T7 and pGBK.T7 vectors (Clontech). For the yeast two-hybrid bait constructs, full length open reading frame of NTRC gene or NTRC gene construct including mutated cysteines (C217S, C220S, C457S) was used as a template in PCR to generate truncated NTR and TRX domains. Double transformants in CY306 strain were selected as the cells were grown on YNB -agar Petri dishes in the presence of histidine, uracile, lysine, adenine and methionine but in the absence of leucine and tryptophan. Cells bearing interacting proteins were further selected on the medium lacking tryptophan, leucine and histidine.

4. Overview of the results

4.1. Daily light/dark rhythm modulates the photosynthesis and growth of Arabidopsis

Characterization of the *ntrc* lines lacking chloroplast NADPH-dependent thioredoxin reductase demonstrated that the mutant phenotype strongly depended on the photoperiod, to which plants were acclimated during the growth (Paper I). Therfore I studied in detail how the daily light/dark rhythm modulates the growth and metabolism of Arabidopsis in general (Papers I-III). The results in this thesis demonstrate that like acclimation to various light intensities and CO_2 concentrations, daily photoperiods modulate the photosynthetic structures of wild type Arabidopsis leaves and induce changes in the vegetative growth of plants.

4.1.1. Impact of photoperiod on growth and photosynthesis in wild type Arabidopsis

The growth rate of Arabidopsis is determined by the efficiency to convert the assimilated carbon to biomass (Zeeman et al. 1998, Gibon et al. 2004). Like low light intensity and low CO_2 concentration, short daily photoperiods decreased the photosynthetic carbon assimilation and resulted in deceleration in the vegetative growth of plants (Paper I). In Papers II and III, the effect of the photoperiod on the growth of Arabidopsis was further studied by growing plants in continuous light and under various light-dark regimes, some of which contained an interrupted dark period or was deviated from the natural 24-h rhythm. Short photoperiods in combination with long nights reduced the growth of Arabidopsis rosettes, measured as a reduction in biomass production (Paper III). Light-dark cycles deviating from the natural 24-h-period did not diminish the growth when the dark period was short. Furthermore, long photoperiods were not able to compensate for the disadvantages caused by long dark periods (10 h light/10 h dark, 12 h light/12 h dark vs. 16 h light/16 h dark). Thereby the reduction in growth is not determined by the total duration of the light available for photosynthesis during the growth, but instead by the changes in metabolism induced by the light-dark rhythm itself. Accordingly, slow growth rates observed under short photoperiods correlate well with the efficiency of starch metabolism; especially with the lower starch degradation rates under short photoperiods (see 4.2.2).

In this thesis work it is further demonstrated that similar to light intensity (reviewed by Kim et al., 2005) also the length of the daily light/dark periods modulates the structure of the leaf and the development of mesophyll cells and chloroplasts in Arabidopsis

(Papers I and II). Also adjustments to the photosynthetic apparatus were observed that are comparable to changes induced by light intensity (Papers I-II). Arabidopsis wild type plants grown under short photoperiods with long nights resemble low-light-grown plants with poorly developed, roundish palisade mesophyll cells and thin leaves. On the contrary, plants grown under long photoperiods or continuous light resemble high-light-acclimated plants with thick leaves and well-developed, tightly packed and vertically elongated palisade mesophyll cells (Paper II; Yano and Terashima, 2001). In addition to adjustments in the anatomy of the palisade mesophyll, an increase of 40% in the stomatal index, which refers to the number of stomata per leaf area, was observed in plants grown under long photoperiods. As the gas exchange and transpiration through stomata are crucial determinants of the photosynthetic performance of plants, also the net CO_2 assimilation per rosette was 20 to 40 % higher in long-day-grown leaves than in short-day-grown leaves at both ambient CO_2 concentration and in saturating light intensity (Paper I). Plants grown under long days have also higher chlorophyll content per leaf area due to thicker leaves and a higher chlorophyll *a*/*b* ratio when compared to plants grown under short days (Paper I). High chlorophyll *a*/*b* ratio of long-day-grown leaves in comparison to short-day-grown leaves implies that the photoperiod has an influence on the composition of the light-harvesting complexes in the thylakoid membranes. Indeed, the grana stacks were smaller in chloroplasts of plants grown under long days (Paper II) and accordingly, the relative amount of the trimeric chlorophyll a/b-binding proteins of the PSII antenna in thylakoid membranes was smaller in long-day-grown plants when compared to short-day-grown plants. However, no significant difference was observed in the relative proportion of the representative subunits of PSII, PSI and Cyt *b6f* complexes in plants grown under short or long photoperiods (Paper I).

4.1.2. Impact of photoperiod on the redox metabolism of mesophyll cells

As shown in Papers I-II, different light-dark rhythms induce adjustments in the photosynthetic structures in Arabidopsis leaves. The underlying mechanism for such modifications remains to be resolved, but the length of the photoperiod also induced notable changes in ROS metabolism (Paper III). Short photoperiods increased the production of ROS in Arabidopsis leaves (Paper III), as especially H_2O_2 accumulated in the leaves during the light periods. Also thylakoids isolated from short-day-grown plants produced more ROS than thylakoids isolated from long-day-grown plants (Paper III). However, the analysis of the steady-state levels of ROS scavenging enzymes revealed that no significant compensatory changes occur in the accumulation of chloroplast antioxidative enzymes. Only the amount of chloroplast PrxIIE was slightly higher in plants grown under short photoperiods compared to those under long photoperiods. Apparently the antioxidative systems in chloroplasts are not able to intensify to counteract the increased production of ROS under short photoperiods. On the other hand, the production of ROS may also be of physiological expedient, standing for the regulation of metabolism during acclimation of plants to different light-dark rhythms. If the changes in the redox homeostasis are crucial to induce

acclimation of plant to various light regimes, thioredoxins that are abundant in chloroplasts can be considered as significant regulatory components in the acclimation processes.

4.1.3. Knockout of NTRC impairs the acclimation of Arabidopsis to short photoperiods

Plants lacking chloroplast NADPH-dependent thioredoxin reductase showed more distinct photoperiod-dependent phenotypes than wild type Arabidopsis. Indeed, the growth rate, biomass production and chlorophyll accumulation varied strongly in *ntrc* plants under different daily light-dark-cycles (Papers I-III). Cotyledons of *ntrc* plants were visually indistinguishable from those of wild type plants, but upon the emergence of the first true leaves the mutant phenotype became evident especially under short photoperiods. During the first month of growth under 8-hour photoperiod, *ntrc* plants formed small rosettes with pale green leaves and had a low chlorophyll content. Upon ageing, the *ntrc* leaves started to green and eventually the rosettes gained the size similar to mature wild type plants. Moreover, the flowering time of *ntrc* plants was significantly delayed under short photoperiods (Paper I). In general, the chlorophyll accumulation in wild type plants was significantly lower upon the short 8-hour photoperiod, suggesting that the length of the photoperiod is a critical factor regulating chlorophyll accumulation. In plants deficient in NTRC, such reduction of chlorophyll accumulation under short photoperiods, less than nine hours, was even more pronounced than in wild type plants (Papers I-II). Low chlorophyll content was associated with reduced number of chloroplast per *ntrc* mesophyll cell (Papers I-II). Also the biomass production was more prominently reduced in *ntrc* plants grown under short photoperiod with long nights than in wild type plants (Paper III). The growth under longer photoperiods, 16 h light/8h dark or continuous light, enhanced the accumulation of both chlorophyll and biomass as well as the growth rate in *ntrc* plants. Also, the transition to flowering took place approximately at the same time in *ntrc* and in wild type plants (Paper I). Thus it appears that NTRC is essential for the proper acclimation of Arabidopsis to short photoperiods combined with long nights.

4.1.4. Photosynthesis of knockout ntrc lines

Low chloroplast number per cell and the occurrence of abnormal chloroplasts in *ntrc* plants, especially under short photoperiod, was accompanied with low CO_2 fixation capacity and high CO_2 compensation point of photosynthesis (Paper I). The *ntrc* plants grown under short photoperiod suffered also from enhanced photoinhibition of PSII, measured as a decrease in F_v/F_m in growth light intensity. Consistent with the high CO_2 compensation point, *ntrc* lines had doubled respiration rate when compared to Col-0 leaves (Paper I). The transcript profiling of short-day-grown *ntrc* plants also showed induction of several photorespiratory genes (Paper I) that suggests an elevated

utilization of light energy in photorespiration. Under long photoperiod, the differences in the net CO_2 assimilation, CO_2 compensation point and photoinhibition of PSII between *ntrc* and wild type plants were less distinct. However, the rate parameters of photosynthesis and the pattern of photosynthetic thylakoid membrane protein complexes in *ntrc* grown under both short and long photoperiods was comparable to that of wild type plants (Paper I) implicating that the basic structure and function of light and carbon fixation reactions were not affected by the knockout of NTRC enzyme in Arabidopsis.

4.2. Identification of chloroplast processes controlled by NADPH-dependent thioredoxin system

The results in this thesis demonstrate that the thioredoxin system has higher impact on the development, metabolism and acclimation of chloroplast than was previously assumed in the light of studies with ferredoxin-thioredoxin system. The two thioredoxin systems in plant chloroplasts, the ferredoxin-dependent system and the NADPH-dependent system, have distinct roles in redox-regulation of chloroplast functions. By comparing the growth, responses to environment, biochemical properties and gene expression in wild type plants and plants lacking chloroplast NADPH-dependent thioredoxin reductase, I conclude that NTRC regulates chloroplast biogenesis and carbon metabolism subsequent to Calvin-Benson cycle such as starch metabolism and shikimate pathway, as well as is involved in defence reactions against oxidative stress. On the contrary, ferredoxin-thioredoxin system seems to be the key regulator of primary photosynthetic reactions such as Calvin-Benson cycle and redox-regulated enzymes therein.

4.2.1. Impact of NTRC on chloroplast biogenesis

The characterization of the *ntrc* knockout lines demonstrated that NTRC is involved in regulation of chloroplast biogenesis. The chloroplast number in palisade mesophyll cells was reduced in mature *ntrc* leaves, especially in plants acclimated to short photoperiods (Papers I and II). The chloroplasts were also heterogeneous in size. Transmission electron micrographs demonstrated developmental disorders in the ultrastructure of chloroplasts in *ntrc* cells (Paper II). Some of the *ntrc* chloroplasts were malformed and irregularly elongated in shape, exhibiting protrusions devoid of thylakoid membranes. Also the distribution of the thylakoid membranes inside chloroplasts was varying as several types of chloroplasts were observed in *ntrc* cells: regular chloroplasts containing grana and stroma thylakoids identical to wild type plants, chloroplasts with central areas well occupied with grana and stroma thylakoids but one or both ends deficient in internal membranes and chloroplasts with only few thylakoid membranes. Importantly, a population of chloroplasts with dissimilar structure/function exists in a single *ntrc* mesophyll cell (Paper II). The different types

of chloroplasts appeared often in a series in a single cell in which unimpaired plastids resided next to chloroplasts with unbalanced distribution of thylakoids, and the plastids with significant reduction in thylakoid membranes being situated at the end of the chain. The largest number of anomalous chloroplasts was detected in mature *ntrc* leaves grown under short photoperiods, but they were also observed already in the 5-day-old cotyledons, in the young developing leaves, and in the mature leaves of plants grown under long photoperiods or under continuous light (Paper II). The increasing appearance of abnormal chloroplasts in maturating *ntrc* leaves implies that NTRC is essential for the proper biogenesis of chloroplasts, possibly via regulation of chloroplast division, chlorophyll biosynthesis and thylakoid formation.

FtsZ proteins form a core component of the inner division machinery in chloroplast (Yang, et al., 2008). They are tubulin-like GTPases that form the ring structure on the stromal surface of the envelope (Miyagishima, et al., 2006; Schmitz, et al., 2009). FtsZ ring functions as a scaffold structure, on which the other division components assemble (Maple, et al., 2005; Miyagishima, et al., 2006; Glynn, et al., 2008). Unlike cyanobacteria that have only one FtsZ protein, plants have two FtsZ proteins, called FtsZ1 and FtsZ2 (Osteryoung, et al., 1998). Both proteins localize at the division site, and loss of either protein impairs chloroplast division (Schmitz, et al., 2009). Interestingly, FtsZ has been identified by thioredoxin proteomics (Balmer, et al., 2003), suggesting that thioredoxins may control the formation of chloroplast division machinery. To get further insights into this assumption the interaction of NTR and TRX domains of NTRC with FtsZ1 and FtsZ2 was tested by yeast two-hybrid approach. In the test, an interaction between FtsZ1 and the Trx domain of NTRC was observed (Paper II).

Besides the reduced number of chloroplasts in *ntrc* cells and the developmental disorders in the ultrastructure of chloroplasts, also chlorophyll biosynthesis was impaired in *ntrc* plants. Short light periods decreased significantly the accumulation of chlorophyll in *ntrc* leaves in comparison to wild type plants (Papers I-II). In Paper I, comparative transcript profiling of *ntrc* and wild type plants revealed alterations in the expression of specific genes relating to chlorophyll biosynthesis. Among the most up-regulated genes in short-day-grown *ntrc* leaves were two genes encoding the key enzymes of the chlorophyll biosynthesis pathway, glutamyl-tRNA reducatse (HEMA1) and the H-subunit of Mg-chelatase (GUN5), suggesting an unbalanced biosynthesis of chlorophyll in *ntrc* leaves. A yeast two-hybrid approach was used to test if NTR or TRX domains of NTRC interacts with chlorophyll biosynthesis enzymes HEMA1, Mg-protoporphyrin IX methyltransferase and subunits of Mg-chelatase, GUN4 and I-subunit. The analysis revealed no interaction between the I-subunit of Mg-chelatase and the thioredoxin domain of NTRC (Paper II). HEMA1, Mg-protoporphyrin IX methyltransferase and GUN4 showed only weak interaction with the thioredoxin domain of NTRC (Paper II; Jouni Toivola, unpublished results).

The comparative transcript profiling revealed also changes in the expression of genes involved in the thylakoid formation. The *ntrc* plants showed lowered accumulation of transcripts for *THYLAKOID MEMBRANE ORGANIZATION-LIKE* (*FZL*) and

THYLAKOID FORMATION 1 (*THF1*) as well as down-regulation of the expression of *LHCB1*, *LHCB2* and *LHCB3* that encode for protein components of the light-harvesting complex of PSII. The reduced accumulation of *LHCB*, *FZL* and *THF1* transcripts may be a consequence of the impaired chlorophyll biosynthesis, since the proper formation of thylakoid membranes requires the simultaneous assembly of membrane lipids, protein components and pigments to attain functional, three-dimensional structures.

4.2.2. Starch and sucrose metabolism in knockout ntrc leaves

The acclimation of Arabidopsis to different photoperiods was accompanied by altered sugar metabolism in vegetative leaves (Paper III). Wild type Arabidopsis plants grown under short days had a higher rate of starch synthesis during the first hour of light period and a lower rate of starch degradation in night than plants grown under long days. Sucrose accumulated with the same rate under both short and long photoperiods, the amounts left after dark period being lower in short-day-grown plants. The content of hexoses, glucose and fructose increased in wild type plants during the first hours of light period, but stayed rather stable or decreased towards the end of the day. The content of glucose was similar in both short-day- and long-day-grown wild type plants whereas the amount of fructose was lower in plants grown under short days.

The *ntrc* plants grown under both short and long photoperiods produced less sucrose, glucose and fructose when compared to the wild type plants (Paper III). Under both photoperiods, *ntrc* plants also accumulated less starch when compared to wild type plants (Paper III). Partly it is due to decreased photosynthetic productivity caused by the low chloroplast number per cell and the occurrence of abnormal chloroplasts, especially in *ntrc* plants acclimated to short photoperiods. However, the reduced apparent rates of starch accumulation was also detected in *ntrc* plants acclimated to long photoperiods (Paper III), in which no reduction in chloroplast number was observed (Paper II). Furthermore, the amount of starch in wild type plants in short photoperiod began to rise directly after the onset of the light period and the accumulation of starch was accelerated towards the end of the light period both under short-day- and long-day-grown wild type Arabidopsis leaves. Under both conditions, *ntrc* plants were not capable of accelerating starch biosynthesis in the end of the light period, suggesting a requirement of NTRC in a redox control of enzymes in starch synthesis. Also the degradation of starch during the first hour of subsequent dark period was slightly slower in *ntrc* than in wild type plants under both photoperiods. Nevertheless, no significant differences between *ntrc* and Col-0 were recorded in the amount of starch left in the leaves after the dark period.

An ADP-glucose pyrophosphorylase (AGPase) and a dual specificity protein phosphatase (DSP4) play important roles in the synthesis and degradation pathways of starch, respectively (Neuhaus and Stitt, 1990; Sokolov, et al., 2006). Both enzymes are also shown to be activated by thioredoxins (Ballicora, et al., 2000; Sokolov, et al.,

2006). Furthermore, AGPase has been suggested to be regulated by NTRC (Michalska, et al., 2009). Thereby the yeast two-hybrid approach was used to test if NTR or TRX domains of NTRC interact with AGPase and DSP4. The analysis revealed an interaction between the thioredoxin domain of NTRC and the small subunit of AGPase (Paper III), but no specific interaction with DSP4 polypeptide and TRX of NTRC was detected.

4.2.3. NTRC and chloroplast ROS metabolism

Earlier reports have shown that NTRC is able to reduce chloroplast 2-Cys Prxs oxidized in the elimination reactions of H_2O_2 (Perez-Ruiz, et al., 2006; Kirchsteiger, et al., 2009; Perez-Ruiz and Cejudo, 2009). In yeast two-hybrid screen (Paper III) a strong interaction between NTRC and 2-Cys Prx was observed. The analysis of the steady-state levels of ROS scavenging enzymes (Paper III) revealed that under normal growth conditions, *ntrc* plants show distinctive modulations only in the levels of APX-dependent and 2-Cys-Prx-dependent detoxification systems of H_2O_2. The protein level of 2-Cys Prxs was significantly reduced and the levels of SOD and sAPX increased in *ntrc* leaves grown both under short or long photoperiod when compared to wild type plants (Paper III). 2-Cys Prxs are labile in *ntrc* plants, probably because of the proteolytic degradation of the oxidized forms of 2-Cys Prx that cannot be reduced in the absence of NTRC. Despite the shutdown of NTRC-dependent 2-Cys Prx system, H_2O_2 or superoxide did not accumulate in significantly higher levels in leaves of NTRC-deficient plants under normal growth conditions in comparison to wild type Arabidopsis (Paper III). This can be explained by the compensation of 2-Cys Prx system with ascorbate-dependent SOD and sAPX enzymes (Paper III) in the detoxification of H_2O_2 in NTRC-deficient plants. Accordingly, knockout of both chloroplast APXs induced an enhanced accumulation of 2-Cys Prxs in high-light acclimated Arabidopsis leaves (Paper IV) indicating the tight interlinkage between the ascorbate-dependent APX and NTRC-dependent 2-Cys Prx systems in the control of H_2O_2 in chloroplast.

4.2.4. Knockout of NTRC interferes with the homeostasis of metabolites produced in shikimate pathway

Aromatic amino acids are synthesized in chloroplasts via shikimic acid pathway and they serve as precursors for the biosynthesis of auxin and flavonoids. Under short photoperiods where the growth of *ntrc* plants is slowest, adjustments in the amount of aromatic amino acids and metabolites derived from them were observed. i) The *ntrc* seedlings grown under short photoperiod contained significantly less auxin than wild type plants (Paper I). ii) *ntrc* plants were not capable of enhancing the accumulation of anthocyanins in response to low temperature and senescence (Paper I). iii) The accumulation of amino acids in the wild type and *ntrc* plants showed photoperiod-

dependent modulations (Paper I). The amounts of glycine, alanine and threonine were significantly lower in both wild type and *ntrc* plants grown under long photoperiods than under short photoperiods. Aromatic amino acids tryptophan, phenylalanine and tyrosine accumulated more in *ntrc* plants than in wild type plants, and the differences were more pronounced under short photoperiods. Furthermore, under short photoperiods, *ntrc* accumulated more arginine, asparagine and histidine while the growth under long photoperiods diminished the differences.

In preliminary yeast two-hybrid analysis we tested if NTRC interacts with two enzymes involved in shikimate pathway (Jouni Toivola, unpublished results). 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) is the first enzyme in shikimate pathway that is responsible for the formation of 3-dehydroquianate from phosphoenolpyruvate and erythrose 4-phosphate, and tryptophan synthase β subunit (TSB) catalyzes the conversion of indole to tryptophan which in turn can serve as a precursor for auxin. Both enzymes have been identified as subjects for thiol-redox-regulation (Entus, et al., 2002; Balmer, et al., 2006; Kolbe, et al., 2006) and both enzymes gave specific interaction with TRX-domain of NTRC (Figure 9, Jouni Toivola, unpublished results).

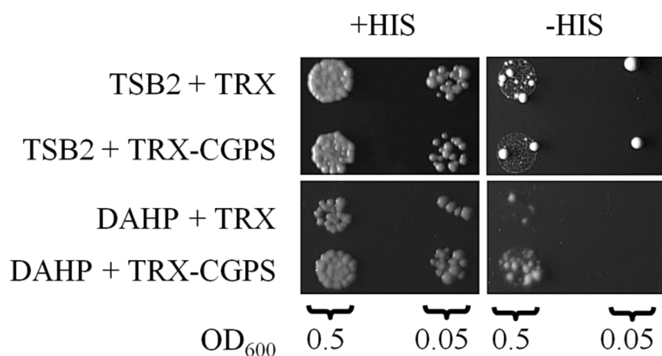


Figure 9. Interaction of the TRX domain of NTRC with TSB2 and DAHP synthase in yeast two-hybrid assay. Yeast CY306 cells co-expressing TRX domain of NTRC (native domain, or domain carrying a mutation replacing the second cysteine of redox center CGPC by a serine) as bait and target candidate (either TSB2, or DAHP synthase) proteins were grown into stationary phase and adjusted to an OD_{600} of 0.5 and 0.05 before spotting onto plates containing histidine (+HIS control panel) or without histidine (-HIS panel), respectively. Result indicates that both TSB2 and DAHP synthase are able to interact with TRX domain of NTRC.

4.3. Contribution of chloroplast ascorbate peroxidases to the ROS metabolism in Arabidopsis

Arabidopsis plants contain five APX isoenzymes, two of which are located in chloroplasts, thylakoid-bound APX (tAPX) and stromal APX (sAPX). In addition to chloroplasts, sAPX is dual targeted to mitochondria as well. APXs have long been considered as key enzymes in detoxification of H_2O_2, but their functional specificities have been poorly understood. Characterization of single *tapx* and *sapx* mutant lines and double *tapx sapx* mutant line of Arabidopsis (Paper IV) showed that sAPX is particularly important for photoprotection during the early greening process in germinating seedlings. In mature leaves, tAPX and sAPX are functionally redundant, and crucial upon sudden onset of oxidative stress. The analysis of the steady-state levels of other, possibly compensatory ROS scavenging enzymes in *tapx* and *sapx* single mutant plants and *tapx sapx* double mutant plants revealed that under normal growth conditions no distinct modulations in the levels of H_2O_2-detoxifying enzymes were observable for *tapx* and *sapx* single mutant plants or *tapx sapx* double mutant plants (Paper IV). Only high-light-stress-acclimated *tapx sapx* double mutant plants showed increased level of 2-Cys Prxs, and low-temperature-acclimated wild type and *tapx* plants showed upregulation of sAPX (Paper IV). As mentioned in the section 4.2.3., plants deficient in chloroplast APXs compensate for the lack of APXs by upregulating 2-Cys Prxs, whereas plants deficient in NTRC upregulate SOD and sAPX (Paper III). Thus the two H_2O_2-detoxifying systems in chloroplast, ascorbate-dependent APX and NTRC-dependent 2-Cys Prxs are tightly linked up to prevent the detrimental accumulation of ROS in chloroplast.

5. Discussion

Plant thioredoxins were initially defined as regulators of the enzymes in Calvin-Benson cycle and malate valve in chloroplasts (Schurmann and Jacquot, 2000), but the release of Arabidopsis genome (The Arabidopsis Genome Initiative, 2000) and the development of proteomic approaches (Verdoucq, et al., 1999; Motohashi, et al., 2001; Balmer, et al., 2003; Maeda, et al., 2004; Marchand, et al., 2004; Hägglund, et al., 2008) have allowed the identification of numerous novel thioredoxin targets in chloroplasts as well as in cytosol, mitochondria and nucleus. Up to date, more than 400 thioredoxin target proteins have been identified in plants (Montrichard, et al., 2009) and they are implicated in almost all aspects of plants' life from housekeeping metabolism to stress defense mechanisms. However, the implication of thioredoxin in the functions of many thioredoxin target proteins identified by proteomic approaches still remains to be resolved.

Plants contain two main types of thioredoxin reductases, ferredoxin-dependent thioredoxin reductase (FTR) located in plastids and NADPH-dependent thioredoxin reductases (NTRs) located in cytosol, mitochondria and chloroplasts (Laloi, et al., 2001; Serrato, et al., 2004; Reichheld, et al., 2005). Originally, ferredoxin-thioredoxin system was thought to be solely responsible for thiol-redox regulation in chloroplasts but the discovery of chloroplastic NADPH-dependent thioredoxin reductase (NTRC; Serrato, et al., 2004) has broadened the view of redox regulation of chloroplastic proteins. Ferredoxin-thioredoxin system is dependent on ferredoxin reduced in light during photosynthesis, while besides the light reactions, NADPH can be produced in darkness via oxidative carbon metabolism. In this thesis, the role of NTRC in plant development, acclimation and detoxification of ROS was studied. As shown in Papers I, II and III, knockout of NTRC led to severe photoperiod- and age-dependent developmental disorders that were especially pronounced under short photoperiods. This is supported by an earlier report of Perez-Ruiz et al. (2006) that plants lacking NTRC are hypersensitive to prolonged darkness, thus indicating that the two plastidial thioredoxin reductases, FTR and NTRC, are functionally nonredundant.

5.1. NTRC in redox-regulation of chloroplast biogenesis

The reduced amount and altered ultrastructure of chloroplasts (Papers I-II) in plants deficient in NTRC indicate that chloroplast differentiation is deteriorated in the absence of NTRC. Since the formation of functional chloroplasts is vital for plant survival, chloroplast differentiation requires tightly coordinated regulation of multiple processes including chloroplast division, formation of thylakoid membranes, chlorophyll biosynthesis and assembly of photosynthetic protein complexes. As regards to thiol-redox regulation of these processes, three proteins in chlorophyll biosynthesis and one protein in chloroplast division have been implicated as targets for

thioredoxin-dependent regulation (Balmer, et al., 2003; Montrichard, et al., 2009), supporting the hypothesis that NTRC has a role in controlling chloroplast biogenesis. NADPH-thioredoxin system, rather than ferredoxin-thioredoxin system, is a preferential candidate to regulate chloroplast biogenesis since metabolically produced NADPH is available in differentiating chloroplasts prior the assembly of the light reactions.

5.1.1. Regulation of chloroplast division by NTRC

Plants have two FtsZ proteins, FtsZ1 and FtsZ2 form heterodimers that polymerize to a ring-like structure (Z-ring) at the chloroplast division site and both proteins are essential for correct chloroplast division (reviewed by Miyagishima and Kabeya, 2010). FtsZ protein has been identified by thioredoxin proteomics (Balmer, et al., 2003), suggesting that thioredoxins may be involved in regulating the chloroplast division machinery. From the FtsZ proteins, an interaction between the thioredoxin domain of NTRC and FtsZ1, but not with FtsZ2 protein, was observed in yeast two-hybrid screen (Paper II). In a predicted amino acid sequence FtsZ1 protein contains a concerved Cys that is missing in FtsZ2 protein (Paper II). If NTRC is involved in regulating the formation of the FtsZ ring, the assembly of division machinery is impaired in plants lacking NTRC resulting in lower accumulation of chloroplasts in cells. Alternatively, the structural disorders in *ntrc* chloroplasts and the existence of small plastid-like organelles in *ntrc* cells (Paper II) may refer to asymmetrical division of chloroplasts. The correct position of the Z-ring in the middle of chloroplast is mediated by a complex consisting of Min proteins and ARC3 protein (Maple and Møller, 2007). FtsZ1 – but not FtsZ2 – has been shown to interact with Min/ARC3 complex (Maple and Møller, 2007). Min/ARC3 protein complex is localized at the ends of a chloroplast, where the formation of the Z-ring is prevented by the binding of the FtsZ1 to Min/ARC3 (Maple and Møller, 2007). The Min/ARC3 complex is absent from the middle part of the chloroplast, thus allowing the Z-ring to assemble. Accordingly, NTRC protein has been shown to be localized in clusters in the chloroplast (Perez-Ruiz, et al., 2009). The role of NTRC in the regulation of chloroplast division remains to be elucidated in the future but I hypothesize that a disulphide bridge is formed between FtsZ1 and Min/ARC3 complex when they interact with each other. The reduction of the disulphide bridge by a thioredoxin, presumably NTRC, is needed to release the FtsZ1 protein from the complex and only a free FtsZ1 protein is capable to polymerize with FtsZ2 protein in the middle of a chloroplast. The lack of a sufficient thiol reductant may disturb the division resulting in the miscellaneous population of plastids in *ntrc* cell.

5.1.2. Regulation of chlorophyll biosynthesis by NTRC

The characterization of *ntrc* mutant lines addressed to altered chlorophyll synthesis in the absence of NTRC. The *ntrc* plants showed significant reduction in chlorophyll content (Papers I-II; Perez-Ruiz, et al., 2006) and the transcript profiling of *ntrc* leaves revealed an upregulation of specific genes coding for enzymes related to chlorophyll biosynthesis (Paper I). Furthermore, NTRC gene expression has been shown to be co-regulated with genes involved in chlorophyll biosynthesis (Stenbaek, et al., 2008). It has been previously reported that the key enzyme in the chlorophyll branch of tetrapyrrole biosynthesis, Mg-chelatase (reviewed by Stenbaek and Jensen, 2010) is regulated by thioredoxin system (Balmer, et al., 2003; Ikegami, et al., 2007). The Mg-chelatase consists of three subunits, CHLD, CHLH and CHLI, and is regulated by GUN4 protein by an unknown mechanism (Larkin, et al., 2003). CHLI subunit catalyses the hydrolysis of ATP that is stimulated by chloroplast thioredoxins (Ikegami, et al., 2007). However, the thioredoxin-dependent regulation of Mg-chelatase is still obscure, because thioredoxin treatment in vitro did not affect the overall magnesium chelation activity of the enzyme (Ikegami, et al., 2007). Accordingly, the yeast two-hybrid assay that failed to show any interaction between NTRC and I-subunit of Mg-chelatase (Paper II) suggesting that Mg-chelatase is not a target enzyme of NTRC. The weak interaction between GUN4 and NTRC in yeast two-hybrid assay needs further elucidation before any conclusion can be drawn.

Stenbaek et al. (2008) showed that when plants were fed with ALA in darkness, the intermediates of chlorophyll biosynthesis, Mg-protoporphyrin and Mg-protoporphyrin monomethyl ester accumulated in higher amounts in *ntrc* than in wild type leaves, suggesting that the reaction catalyzed by Mg-protoporphyrin IX methyltransferase (CHLM) and Mg-protoporphyrin IX monomethylester cyclase (CRD1) (Figure 8) are imbalanced in the absence of NTRC. A weak interaction between NTRC and CHLM was detected in yeast two-hybrid assay, suggesting that NTRC may regulate the activity of CHLM. Furthermore, slight increase in the activity of CHLM was observed in vitro in the presence of NTRC (Anne Stenbaek, PhD thesis) that further supports the thiol-redox regulation of CHLM enzyme. NTRC may also interfere indirectly with chlorophyll biosynthesis enzymes by protecting enzymes from oxidation that potentially occurs during chlorophyll synthesis (Stenbaek and Jensen, 2010). The latter hypothesis is supported by in vitro experiments of (Stenbaek, et al., 2008) which showed that in combination with 2-Cys Prx, NTRC was able to stimulate the activity of CRD1, enzyme acting downstream of CHLM in the chlorophyll biosynthesis pathway (Figure 8).

5.1.3. Thylakoid formation in ntrc mutant lines

Transmission electron micrographs of chloroplasts demonstrated defective formation of thylakoid membranes in *ntrc* mesophyll cells (Paper II). Microarray analysis on *ntrc* (Paper I) also revealed a down-regulation of genes whose products act on formation of thylakoid membranes, *THYLAKOID MEMBRANE ORGANIZATION-LIKE* (*FZL*) and *THYLAKOID FORMATION 1* (*THF1*) (Wang, et al., 2004; Gao, et al., 2006). During the conversion of etioplasts to chloroplasts, thylakoid membranes initiate from prolamellar bodies, tubular membrane aggregates arranged in a clustered manner inside chloroplasts (recently reviewed by Solymosi and Schoefs, 2010). Localization of NTRC protein in clusters in chloroplasts (Perez-Ruiz, et al., 2009) may alternatively be linked with the redox-control of thylakoid formation by NTRC in chloroplasts. Otherwise, the malformation of thylakoids observed in *ntrc* chloroplast may be a secondary effect caused by the irregular plastid division and impaired chlorophyll biosynthesis discussed in chapters 5.1.1. and 5.1.2.

5.2. Regulation of chloroplast metabolism by NTRC

5.2.1. Redox control of starch metabolism in chloroplasts

Transient formation of starch in light and degradation in darkness coordinates the carbon assimilation and allocation to growth in Arabidopsis leaves (Zeeman, et al., 2007; Paper III). The coordinated synthesis and remobilization of starch presumes the regulatory signals from photosynthesis and respiration to the enzymes metabolizing starch. The enzyme regulation is achieved via feed-back control by metabolites, reversible protein phosphorylation and redox-regulation (Zeeman, et al., 2007; Kötting, et al., 2010). ADP-glucose pyrophosphorylase (AGPase) is the key enzyme in starch synthesis that controls the flux from carbon to starch. AGPase is a heterotetrameric enzyme consisting of large and small subunits, which is redox-activated in light by the reduction of a disulphide bridge between small subunits (Hendriks, et al., 2003). AGPase is also allosterically controlled by the level of P_i and PGA, which act as an inhibitor and as an activator, respectively (Zeeman, et al., 2007). Also the enzymes involved in starch degradation, glucan, water dikinase (GWD), dual specificity protein phosphatase (DSP4) and β-amylase 1 (BAM1) have been shown to be under redox control (Mikkelsen, et al., 2005; Sokolov, et al., 2006; Sparla, et al., 2006). Prior to the degradation by amylases, starch granules are reversibly phosphorylated by GWD and DSP4 (Zeeman, et al., 2007). This reversible phosphorylation has been proposed to disrupt the crystalline structure of amylopectin and mutant analyses have shown that both enzymes are necessary to efficient remobilization of starch in Arabidopsis (Ritte, et al., 2002; Yu, et al., 2001; Zeeman, et al., 2010). All these enzymes have been

reported to be regulated by thioredoxins (Hendriks, et al., 2003; Mikkelsen, et al., 2005; Sokolov, et al., 2006).

In *ntrc* plants, starch metabolism is impaired since particularly the accumulation of starch was significantly lower when compared to wild type plants (Paper III). However, starch remobilization was less affected in *ntrc* plants, suggesting that NTRC preferably regulates starch synthesis than degradation in chloroplasts. NTRC has been shown to monomerize the small subunits of AGPase in vitro in the presence of NADPH (Michalska, et al., 2009). The yeast two-hybrid analysis (Paper III) confirmed the interaction between AGPase and the thioredoxin domain of NTRC. On the contrary, no interaction between NTRC domains and DSP4 was observed in yeast two-hybrid assay. These results, together with the observation of the more severe effect of NTRC knockout on starch synthesis rate than starch degradation rate in *ntrc* leaves, attest the hypothesis that NTRC controls the starch synthesis but not the initial steps of starch mobilization in Arabidopsis. However, NTRC may still be involved in regulating other redox-controlled enzymes in starch degradation, since the maximal apparent starch degradation rate was lower in *ntrc* plants when compared to wild type plants (Paper III).

5.2.2. Altered homeostasis of metabolites synthesized from the end products of shikimate acid pathway

The shikimate pathway, present only in plants and micro-organisms, links metabolism of carbohydrates to biosynthesis of aromatic compounds. In a sequence of seven metabolic steps, phosphoenolpyruvate and erythrose 4-phosphate are converted to chorismate, the precursor of the aromatic amino acids tyrosin, tryptophan and phenylalanine. These aromatic amino acids can then be used for protein synthesis or they can be converted via several enzymatic steps to aromatic secondary metabolites such as glucosinolates, phenylpropanoids including anthocyanins, and indole alkaloids including growth hormone auxin (reviewed by Tzin and Galili, 2010).

The first enzyme in the pathway, 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) which catalyzes the formation of 3-dehydroquianate from phosphoenolpyruvate and erythrose 4-phosphate, has been reported to require reduced thioredoxin for its activity (Entus, et al., 2002). Other enzymes in the pathway up to chorismate are less well characterized and mechanisms that regulate their activity remain to be elucidated. In *ntrc* plants the amounts of aromatic amino acids as well as the amounts of auxin and anthocyanins were pronouncedly different from wild type (Paper I). Shikimate-derived pathways are regulated by complex networks, including feedback control by aromatic amino acids and environmental factors (Ishihara, et al., 2007). Thus the lack of NTRC alters the homeostasis among the metabolic pathways that derive from the shikimate pathway leading up to differential accumulation of aromatic amino acids and to decreased accumulation of secondary metabolites auxin and anthocyanins. Furthermore, in yeast two-hybrid analysis both DAHP synthase and

tryptophan synthase β subunit gave specific interactions with thioredoxin domain of NTRC (Figure 9), thus implicating that NTRC may be involved in regulating shikimate pathway.

5.3. Thioredoxin- and ascorbate-dependent scavenging of ROS in chloroplasts

5.3.1. Detoxification of H_2O_2 by NTRC

Thioredoxins participate in the avoidance of oxidative stress as they supply reducing power to reductase enzymes involved in antioxidative metabolism, such as peroxiredoxins (Rey, et al., 2005; Collin, et al., 2003; Finkemeier, et al., 2005) and glutathione peroxidases (Herbette, et al., 2002; Jung, et al., 2002) (see 1.3.2.). The reaction mechanism for peroxide reduction by peroxiredoxins involves a cysteine residue that attacks the peroxide and becomes transiently oxidized to sulphenic acid. This intermediate is subsequently attacked by the second cysteine residue yielding water or the corresponding alcohol, and the two cysteine residues become oxidized and form a disulphide bridge that needs to be reduced for a new catalytic cycle (Dietz, 2003; Konig, et al., 2003; Hall, et al., 2009). In chloroplasts, two pathways have been proposed to reduce 2-Cys Prxs; one consists of FTR and free plastidial thioredoxins of which Trx *x* is the most efficient (Collin, et al., 2003), another is based on NTRC (Perez-Ruiz, et al., 2006; Kirchsteiger, et al., 2009; Perez-Ruiz and Cejudo, 2009). Recently Pulido et al. (2010) demonstrated that NTRC is in fact the most relevant pathway for chloroplast 2-Cys Prx reduction *in vivo*. The interaction between NTRC and 2-Cys PrxB was confirmed also by the yeast two-hybrid analysis in Paper III.

Knockout of one antioxidative system in chloroplasts has demonstrated that the detoxification of ROS never relies only on a single mechanism (Papers III and IV). The decrease in the activity of NTRC/2-Cys Prxs was compensated by the elevation of ascorbate-dependent antioxidative system in *ntrc* plants (Paper III; Pulido, et al., 2010). Similarly, high-light-acclimated double mutant plants lacking both tAPX and sAPX upregulated the accumulation of 2-Cys Prxs (Paper IV), indicating that chloroplast APX-dependent and NTRC/2-Cys Prx-dependent systems are compensatory in the detoxification of H_2O_2. Accordingly, plants deficient in NTRC do not accumulate excess amount of ROS in leaf cells when compared to wild type (Paper III). Nevertheless, the photoinhibition measurements, the activation state of malate dehydrogenase and the carbonylation of proteins speak for the moderate oxidative stress in *ntrc* leaves (Papers I and III; Pulido et al. 2010), indicating that ascorbate-dependent detoxification system could not entirely balance the ROS metabolism in chloroplasts. The total elimination of one antioxidant system may generally modify the homeostasis of antioxidants in chloroplast and thereby impair the redox regulation of metabolic enzymes.

5.3.2. Impacts of chloroplast APXs on chloroplast redox homeostasis

Chloroplastic ascorbate peroxidases localized in the thylakoid membrane (tAPX) and stroma (sAPX) have been unequivocally considered to play an essential role in scavenging H_2O_2 in chloroplasts (Asada, 1999). The oxidation of the chloroplast APXs by high concentration of H_2O_2 (Miyake, et al., 2006) has attenuated their physiological significance regarding the defence response to photo-oxidative stress. In Paper IV, the physiological function of tAPX and sAPX is clarified by characterization of single *tapx* and *sapx*, and double *tapx sapx* lines. It is demonstrated that chloroplast APXs are crucial in the protection against oxidative stress during greening of seedlings and upon the short-term and long-term oxidative stress in mature leaves.

During seed germination, the mobilization of food storage by oxidative phosphorylation in mitochondria generates ROS. In addition, light absorption of chlorophylls (Hideg, et al., 2001) and deficient coupling between the two photosystems support ROS formation in chloroplasts (Hutin, et al., 2003). To protect the developing seedlings against ROS, activation of the antioxidant systems takes place early during seedling development. Photo-oxidative stress during germination led to bleaching of chloroplasts in seedlings lacking sAPX and especially in plants lacking both sAPX and tAPX, whereas plants lacking only tAPX were equally resistant to photo-oxidative stress during germination as wild type plants (Paper IV). Thus chloroplast APXs contribute to antioxidant defence during the greening process, sAPX being primarily important. Previously, both sAPX and tAPX have been shown to accumulate in pea etioplasts in darkness and to decrease during de-etiolation and greening process (Kanervo, et al., 2008), depicting their importance for photoprotection in developing chloroplasts. In addition, the expression of genes encoding chloroplast APXs and Prxs is upregulated during the first days after germination in Arabidopsis (Pena-Ahumada, et al., 2006). Also glutaredoxin (Kanervo, et al., 2008), SOD and glutathione transferase (Yang, et al., 2007) have been shown to be highly expressed in etiolated plants and down-regulated upon greening of seedlings in light. Thereby the proper induction of antioxidant systems in germinated seeds is crucial for the survival of a seedling.

In mature leaves, tAPX and sAPX appear to be functionally redundant, and exhibit a key role in the maintenance of chloroplast functionality upon sudden onset of oxidative stress (Paper IV). In contrast, in the course of long-term acclimation to various stress conditions, the chloroplast APXs can be compensated by other components of the chloroplast antioxidative system (Paper IV). Therefore the stress-acclimation involves the operations of multiple H_2O_2 detoxification systems to efficiently control the H_2O_2 levels in chloroplasts (see Figure 6). It was demonstrated in this thesis that in the absence of both tAPX and sAPX, plants are able to scavenge H_2O_2 up to a certain level, but if the production of H_2O_2 suddenly exceeds the threshold level, the lack of tAPX and sAPX is not fully compensated by other components of the antioxidant

network, suggesting that chloroplast APXs are indispensable under short-term changes in the oxidative state of chloroplast, e.g. by strong sun flecks in nature. Accordingly, high-light-acclimation of the *tapx sapx* double mutant plants enhanced the level of 2-Cys Prxs (Paper IV), that can compensate the APXs in regard to H_2O_2 detoxification (Dietz, et al., 2006). All high-light-acclimated plants, wild type, *tapx*, *sapx* and *tapx sapx* double mutant plants accumulated also cytoplasmic APX (Paper IV). These results suggest that both chloroplastic Prxs and cytoplasmic APX are more potent in terms of H_2O_2 removal than chloroplast APXs under condition of elevated H_2O_2 production.

5.4. Photoperiodic development of photosynthetic tissues

Plant development is controlled by a number of external factors, of which light quantity and quality and the length of the daily light period are the most essential ones. As described in Papers I-II, the length of the daily light period induces developmental alterations in Arabidopsis that are comparable to changes induced by light intensity. Like plants grown under high light intensity (reviewed by Kim et al., 2005), long-day-grown plants possess thick leaves, vertically elongated palisade mesophyll cells, and small grana stacks in chloroplasts when compared to plants grown under short days. The length of the photoperiod also induces alterations in the physiological state of the leaves. As described in Paper III, plants grown under short photoperiods show higher accumulation of ROS after switching the light on subsequent to the dark period. Also thylakoids isolated from short-day-grown plants produce more ROS than thylakoids isolated from long-day-grown plants (Paper III). In respect of components of antioxidative systems, the amounts of PrxIIE, 2-Cys Prxs and chloroplast GPX only slightly increase in short-day-grown plants, being not sufficient to eliminate the higher accumulation of ROS (Paper III). Accordingly, no significant differences could be detected in the total content of ascorbate and glutathione between the short-day-grown and long-day-grown plants (Queval, et al., 2007). The elevated production of ROS and the low production of compensatory antioxidants in plants grown under short days (Paper III; Becker, et al., 2006) may suggest that instead of oxidative stress, ROS have a regulatory role in short-day-grown plants balancing metabolic reactions and the regulation of development. Interestingly, Becker et al. (2006) showed that in plants grown under long days, the expression of genes encoding several antioxidative enzymes, including tAPX and sAPX, is strongly increased. This also indicates that ROS-mediated signalling is specific for short photoperiods since under long photoperiods ROS is aimed to be removed. All these studies collectively indicate that an interaction between the day length and redox-mediated acclimation signals exists in plants.

Diurnal metabolism of starch is one example of redox-regulated metabolic pathways that balances the carbon fixation, carbon storage and the mobilization of stored carbon according to the plant growth rate. As shown in Paper III, short photoperiods reduce the growth of Arabidopsis. Both enzymes involved in starch synthesis, AGPase, and

several enzymes involved in starch degradation, GWD, DSP4 and β-amylase1 (BAM1) have been shown to be under redox control (Mikkelsen, et al., 2005; Sokolov, et al., 2006; Sparla, et al., 2006). The higher accumulation of ROS in the leaves of short-day-grown plants soon after the onset of light (Paper III) suggests that chloroplasts encounter higher oxidative pressure under short photoperiods than under long photoperiods. Higher oxidation state in chloroplasts challenges the thioredoxin systems and interferes with the redox-regulation of enzymes thus decreasing their optimal activities.

5.4.1. Impact of NTRC knockout on the growth of Arabidopsis

The pleiotropic phenotype of *ntrc* plants suggests that there is no single reason for growth reduction in plants deficient in NTRC. I have shown in my thesis that NTRC contributes to important developmental, metabolic and defence processes in chloroplasts, such as plastid division, biosynthesis of chlorophyll, starch and amino acids and detoxification of H_2O_2. The significant reduction in chloroplast number and the extent of irregularly developed chloroplasts observed in *ntrc* cells is probably the most concrete factor that depresses the growth of *ntrc* plants. In addition, imbalances in the important metabolic pathways in chloroplasts, starch metabolism and shikimate pathway that leads to production of aromatic amino acids and secondary metabolites, result in disorders in the functions of chloroplasts thus diminishing their optimal performance. As confirmed by several reports, NTRC is a primary reductant for 2-Cys Prxs (Paper III , (Perez-Ruiz, et al., 2006; Kirchsteiger, et al., 2009; Perez-Ruiz and Cejudo, 2009; Pulido, et al., 2010). Thus plants lacking NTRC lack also one component of the ROS scavenging mechanism in chloroplast resulting in increased susceptibility to oxidative stress. However, it is noteworthy that the double mutant plants deficient in both 2-Cys Prx A and B did not show any phenotype significantly different from that of the wild type plants (Pulido, et al., 2010), indicating that the reduced growth of *ntrc* line is mainly due to the turn down of the other NTRC target processes. Since the highest growth reduction in *ntrc* plants was observed under short photoperiods, it suggests that the demand for reductive capacity in the developmental and metabolic processes, mediated by NTRC, is increased especially under short photoperiods.

5.5. Chloroplast-to-nucleus retrograde signaling

The prevailing condition in *ntrc* cells - the existence of heterogeneous types of chloroplasts in a single mesophyll cell - poses extraordinary challenges for the retrograde signalling from chloroplast to nucleus that controls the nuclear gene expression (reviewed by Nott et al., 2006 and Kleine et al., 2009). In plant cells, all chloroplasts are autonomous in regard to biogenesis and function and they communicate with the nucleus separately from each other (Yu, et al., 2007).

Heterogenous chloroplast population in *ntrc* cells (Paper II) may send contradictory signals to nucleus depending on their functional status, thereby confusing the nuclear-controlled developmental processes. Similar irregularities in chloroplasts have been reported in other mutant lines of chloroplast proteins (Knappe, et al., 2003; Hricova, et al., 2006; Yu, et al., 2007). The deterioration of morphological development may be caused by heterogeneous signals from chloroplasts that interfere with the development of young leaves.

Light is the most eminent external factor that controls the plant development in nature. Light is perceived by a set of wavelength-specific photoreceptors that initiate signalling cascades which ultimately lead to adjustments in gene expression. Both photoperiodic and photomorphogenetic development are regulated by the phytochromes, which perceive red and far-red light, and the cryptochromes, which respond to blue and UVA light (Jiao, et al., 2007). Transcript levels of genes coding for cryptochrome 2 and the far-red light-impaired response regulator FRS3 are strongly down-regulated in *ntrc* (Paper I). We also demonstrated that that the blue and far-red light-mediated signaling is affected in *ntrc* plants (Paper I). Whether the down-regulation of genes encoding blue light receptor and far-red-signaling component is caused by the signals derived from the irregularly differentiated plastid in *ntrc* cell, remains to be elucidated.

The transcript levels of four chloroplast-located proteases are elevated in *ntrc* (Paper I). In the course of plant development, the quality and quantity of plastid proteins need to be carefully controlled by chaperones and proteases. Proteases in plastids have two types of function; firstly, they are involved in protein maturation and secondly, they degrade damaged and unnecessary proteins to free amino acids (reviewed by Sakamoto, 2006). Oxidation of proteins which takes place in a close vicinity to the major cellular sites of ROS production induces damages to proteins and is a major contributor to protein degradation. It was recently suggested that rather than ROS themselves, peptides deriving from proteolytic degradation of oxidized proteins would act as secondary ROS messengers thus contributing to retrograde signalling during oxidative stress (Møller and Sweetlove, 2010).

6. Concluding remarks

I have demonstrated in this thesis that the acclimation of Arabidopsis to short photoperiods/long nights increases the accumulation of ROS. This, in turn, increases the demand for reductive systems to maintain the activities of redox-controlled proteins involved in metabolic and developmental processes. Plants deficient in NTRC show photoperiod-dependent phenotypes with more severe reduction in growth rate and chlorophyll accumulation under short photoperiods than under long photoperiods. These findings provide evidence that NTRC is particularly important regulator of enzyme activities in plants grown under short photoperiods. In this thesis work, NTRC was identified to be involved in controlling metabolic processes including starch metabolism and shikimate pathway. Regulation by NTRC can be achieved directly by mediating redox regulation of intramolecular or intermolecular disulfide bridges of enzymes, or alternatively by protecting enzymes from oxidation via removal of H_2O_2 in conjunction with 2-Cys Prxs. This thesis work also demonstrated that chloroplast APXs, SOD and 2-Cys Prxs are tightly linked up to prevent the detrimental accumulation of ROS in plants. Furthermore, it was shown that NTRC is needed for regular biogenesis of chloroplasts in plant cells. However, the precise impact of NTRC on the assembly and function of chloroplast division machinery and/or on the enzymes of chlorophyll biosynthesis needs to be further clarified experimentally. Also the search for novel NTRC target proteins by screening of the cDNA library with NTRC baits is currently under process.

Acknowledgements

This work was carried out in the Laboratory of Molecular Plant Biology at the University of Turku. Financial support from the Academy of Finland, the Finnish Graduate School in Plant Biology and the Turku University Foundation is gratefully acknowledged. My supervisor, Professor Eevi Rintamäki is warmly thanked for giving me the opportunity to work in this project, and for excellent supervision and support in scientific, as well as in non-scientific matters. Professor Eva-Mari Aro is thanked for providing excellent working facilities in Biocity, and for her valuable scientific advice and encouragement throughout these years.

Professors Elina Oksanen and Anja Hohtola are acknowledged for critically reviewing this thesis.

I wish to acknowledge all my co-authors for excellent collaboration. Above all of the previous and present co-workers I want to thank Saijaliisa Kangasjärvi for sharing her knowledge and instructing me in the practical laboratory work with the peculiar organism called Arabidopsis. Thank you Saija for letting me "watch you working"!

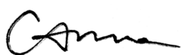
Ulla-Maija Suoranta and Mirja Jaala are acknowledged for keeping the laboratory working in Biocity. I also want to thank Mika Keränen and Kurt Ståhle for their invaluable help with all kinds of technical issues, and especially warm thanks are given to Kurt who has patiently programmed the growth chambers to various light-dark rhythms more than gazillion times.

"Transient group members" Ninni and Eve are warmly thanked for their support and friendship. I am also grateful to everybody who has worked with me in Biocity during these years; without you the working environment wouldn't have been as pleasant and fun and creative and supportive!

Of people outside the laboratory, enormous thanks are given to my sister Ullariikka and her family for their love and encouragement. I also wish to thank my friends for their support. And last, but definitely not least - Mika, thank you for being there for me as we once promised to each other, "for better or for worse, and in sickness and in health".

Kiitos!

Kaarina July 2011



References

Akif M, Suhre K, Verma C, Mande SC (2005) Conformational flexibility of Mycobacterium tuberculosis thioredoxin reductase: crystal structure and normal-mode analysis. Acta Crystallogr D Biol Crystallogr **61:** 1603-1611

Allen JF, Puthiyaveetil S, Strom J, Allen CA (2005) Energy transduction anchors genes in organelles. Bioessays **27:** 426-435

Ankele E, Kindgren P, Pesquet E, Strand A (2007) In vivo visualization of Mg-protoporphyrin IX, a coordinator of photosynthetic gene expression in the nucleus and the chloroplast. Plant Cell **19:** 1964-1979

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55:** 373-399

Arsova B, Hoja U, Wimmelbacher M, Greiner E, Ustun S, Melzer M, Petersen K, Lein W, Bornke F (2010) Plastidial thioredoxin z interacts with two fructokinase-like proteins in a thiol-dependent manner: evidence for an essential role in chloroplast development in Arabidopsis and Nicotiana benthamiana. Plant Cell **22:** 1498-1515

Asada K (1999) THE WATER-WATER CYCLE IN CHLOROPLASTS: Scavenging of Active Oxygens and Dissipation of Excess Photons. Annu Rev Plant Physiol Plant Mol Biol **50:** 601-639

Asada K, Kiso K, Yoshikawa K (1974) Univalent reduction of molecular oxygen by spinach chloroplasts on illumination. J Biol Chem **249:** 2175-2181

Aseeva E, Ossenbuhl F, Sippel C, Cho WK, Stein B, Eichacker LA, Meurer J, Wanner G, Westhoff P, Soll J, Vothknecht UC (2007) Vipp1 is required for basic thylakoid membrane formation but not for the assembly of thylakoid protein complexes. Plant Physiol Biochem **45:** 119-128

Baena-Gonzalez E, Allahverdiyeva Y, Svab Z, Maliga P, Josse EM, Kuntz M, Maenpaa P, Aro EM (2003) Deletion of the tobacco plastid psbA gene triggers an upregulation of the thylakoid-associated NAD(P)H dehydrogenase complex and the plastid terminal oxidase (PTOX). Plant J **35:** 704-716

Baier M, Dietz KJ (2005) Chloroplasts as source and target of cellular redox regulation: a discussion on chloroplast redox signals in the context of plant physiology. J Exp Bot **56:** 1449-1462

Ballicora MA, Frueauf JB, Fu Y, Schurmann P, Preiss J (2000) Activation of the potato tuber ADP-glucose pyrophosphorylase by thioredoxin. J Biol Chem **275:** 1315-1320

Balmer Y, Koller A, del Val G, Manieri W, Schurmann P, Buchanan BB (2003) Proteomics gives insight into the regulatory function of chloroplast thioredoxins. Proc Natl Acad Sci U S A **100:** 370-375

Balmer Y, Vensel WH, Hurkman WJ, Buchanan BB (2006) Thioredoxin target proteins in chloroplast thylakoid membranes. Antioxid Redox Signal **8:** 1829-1834

Battchikova N, Vainonen JP, Vorontsova N, Keranen M, Carmel D, Aro EM (2010) Dynamic changes in the proteome of Synechocystis 6803 in response to CO(2) limitation revealed by quantitative proteomics. J Proteome Res **9:** 5896-5912

Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, Schnell D, Kessler F (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature **403:** 203-207

Bechtold U, Richard O, Zamboni A, Gapper C, Geisler M, Pogson B, Karpinski S, Mullineaux PM (2008) Impact of chloroplastic- and extracellular-sourced ROS on high light-responsive gene expression in Arabidopsis. J Exp Bot **59:** 121-133

Beck CF (2005) Signaling pathways from the chloroplast to the nucleus. Planta **222:** 743-756

Becker B, Holtgrefe S, Jung S, Wunrau C, Kandlbinder A, Baier M, Dietz KJ, Backhausen JE, Scheibe R (2006) Influence of the photoperiod on redox regulation and stress responses in Arabidopsis thaliana L. (Heynh.) plants under long- and short-day conditions. Planta **224:** 380-393

Benning C (2009) Mechanisms of lipid transport involved in organelle biogenesis in plant cells. Annu Rev Cell Dev Biol **25:** 71-91

Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot **91 Spec No:** 179-194

Blomqvist LA, Ryberg M, Sundqvist C (2008) Proteomic analysis of highly purified prolamellar bodies reveals their significance in chloroplast development. Photosynth Res **96:** 37-50

Boldt R, Zrenner R (2003) Purine and pyrimidine biosynthesis in higher plants. Physiol Plant **117:** 297-304

Capitani G, Markovic-Housley Z, DelVal G, Morris M, Jansonius JN, Schurmann P (2000) Crystal structures of two functionally different thioredoxins in spinach chloroplasts. J Mol Biol **302:** 135-154

Cashmore AR, Jarillo JA, Wu YJ, Liu D (1999) Cryptochromes: blue light receptors for plants and animals. Science **284:** 760-765

Chiadmi M, Navaza A, Miginiac-Maslow M, Jacquot JP, Cherfils J (1999) Redox signalling in the chloroplast: structure of oxidized pea fructose-1,6-bisphosphate phosphatase. EMBO J **18:** 6809-6815

Chibani K, Wingsle G, Jacquot JP, Gelhaye E, Rouhier N (2009) Comparative genomic study of the thioredoxin family in photosynthetic organisms with emphasis on Populus trichocarpa. Mol Plant **2:** 308-322

Cho YH, Yoo SD, Sheen J (2006) Regulatory functions of nuclear hexokinase1 complex in glucose signaling. Cell **127:** 579-589

Collin V, Issakidis-Bourguet E, Marchand C, Hirasawa M, Lancelin JM, Knaff DB, Miginiac-Maslow M (2003) The Arabidopsis plastidial thioredoxins: new functions and new insights into specificity. J Biol Chem **278:** 23747-23752

Coudevylle N, Thureau A, Hemmerlin C, Gelhaye E, Jacquot JP, Cung MT (2005) Solution structure of a natural CPPC active site variant, the reduced form of thioredoxin h1 from poplar. Biochemistry **44:** 2001-2008

Dai S, Saarinen M, Ramaswamy S, Meyer Y, Jacquot JP, Eklund H (1996) Crystal structure of Arabidopsis thaliana NADPH dependent thioredoxin reductase at 2.5 A resolution. J Mol Biol **264:** 1044-1057

Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inze D, Van Breusegem F (2000) Dual action of the active oxygen species during plant stress responses. Cell Mol Life Sci **57:** 779-795

Desikan R, Cheung MK, Bright J, Henson D, Hancock JT, Neill SJ (2004) ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. J Exp Bot **55:** 205-212

Dietz KJ (2003) Plant peroxiredoxins. Annu Rev Plant Biol **54:** 93-107

Dietz KJ, Jacob S, Oelze ML, Laxa M, Tognetti V, de Miranda SM, Baier M, Finkemeier I (2006) The function of peroxiredoxins in plant organelle redox metabolism. J Exp Bot **57:** 1697-1709

Eggink LL, LoBrutto R, Brune DC, Brusslan J, Yamasato A, Tanaka A, Hoober JK (2004) Synthesis of chlorophyll b: localization of chlorophyllide a oxygenase and discovery of a stable radical in the catalytic subunit. BMC Plant Biol **4:** 5

Entus R, Poling M, Herrmann KM (2002) Redox regulation of Arabidopsis 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. Plant Physiol **129:** 1866-1871

Fahnenstich H, Scarpeci TE, Valle EM, Flugge UI, Maurino VG (2008) Generation of hydrogen peroxide in chloroplasts of Arabidopsis overexpressing glycolate oxidase as an inducible system to study oxidative stress. Plant Physiol **148:** 719-729

Falbel TG, Staehelin LA (1994) Characterization of a family of chlorophyll-deficient wheat (Triticum) and barley (Hordeum vulgare) mutants with defects in the magnesium-insertion step of chlorophyll biosynthesis. Plant Physiol **104:** 639-648

Falini G, Fermani S, Ripamonti A, Sabatino P, Sparla F, Pupillo P, Trost P (2003) Dual coenzyme specificity of photosynthetic glyceraldehyde-3-phosphate dehydrogenase interpreted by the crystal structure of A4 isoform complexed with NAD. Biochemistry **42:** 4631-4639

Farquhar GD, von Caemmerer S, Berry JA (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta **149, 1:** 78-90

Fermani S, Ripamonti A, Sabatino P, Zanotti G, Scagliarini S, Sparla F, Trost P, Pupillo P (2001) Crystal structure of the non-regulatory A(4)isoform of spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase complexed with NADP. J Mol Biol **314:** 527-542

Finkemeier I, Goodman M, Lamkemeyer P, Kandlbinder A, Sweetlove LJ, Dietz KJ (2005) The mitochondrial type II peroxiredoxin F is essential for redox homeostasis and root growth of Arabidopsis thaliana under stress. J Biol

Chem **280:** 12168-12180

Foyer CH, Noctor G (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxid Redox Signal **11:** 861-905

Foyer CH, Lopez-Delgado H, Dat JF, Scott IM (1997) Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiol Plantarum **100:** 241-254

Galili G (2002) New insights into the regulation and functional significance of lysine metabolism in plants. Annu Rev Plant Biol **53:** 27-43

Gao H, Sage TL, Osteryoung KW (2006) FZL, an FZO-like protein in plants, is a determinant of thylakoid and chloroplast morphology. Proc Natl Acad Sci U S A **103:** 6759-6764

Geck MK, Larimer FW, Hartman FC (1996) Identification of residues of spinach thioredoxin f that

influence interactions with target enzymes. J Biol Chem **271:** 24736-24740

Gelhaye E, Rouhier N, Navrot N, Jacquot JP (2005) The plant thioredoxin system. Cell Mol Life Sci **62:** 24-35

Gibon Y, Blasing OE, Palacios-Rojas N, Pankovic D, Hendriks JH, Fisahn J, Hohne M, Gunther M, Stitt M (2004) Adjustment of diurnal starch turnover to short days: depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post-translational activation of ADP-glucose pyrophosphorylase in the following light period. Plant J **39:** 847-862

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem **48:** 909-930

Glynn JM, Miyagishima SY, Yoder DW, Osteryoung KW, Vitha S (2007) Chloroplast division. Traffic **8:** 451-461

Glynn JM, Froehlich JE, Osteryoung KW (2008) Arabidopsis ARC6 coordinates the division machineries of the inner and outer chloroplast membranes through interaction with PDV2 in the intermembrane space. Plant Cell **20:** 2460-2470

Goslings D, Meskauskiene R, Kim C, Lee KP, Nater M, Apel K (2004) Concurrent interactions of heme and FLU with Glu tRNA reductase (HEMA1), the target of metabolic feedback inhibition of tetrapyrrole biosynthesis, in dark- and light-grown Arabidopsis plants. Plant J **40:** 957-967

Guo R, Luo M, Weinstein JD (1998) Magnesium-chelatase from developing pea leaves. Plant Physiology **116:** 605-615

Gutierrez-Nava MdlL, Gillmor CS, Jimenez LF, Guevara-Garcia A, Leon P (2004) CHLOROPLAST BIOGENESIS genes act cell and noncell autonomously in early chloroplast development. Plant Physiol **135:** 471-482

Hägglund P, Bunkenborg J, Maeda K, Svensson B (2008) Identification of thioredoxin disulfide targets using a quantitative proteomics approach based on isotope-coded affinity tags. J Proteome Res **7:** 5270-5276

Hall A, Karplus PA, Poole LB (2009) Typical 2-Cys peroxiredoxins--structures, mechanisms and functions. FEBS J **276:** 2469-2477

Hendriks JH, Kolbe A, Gibon Y, Stitt M, Geigenberger P (2003) ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. Plant Physiol **133:** 838-849

Herbette S, Lenne C, Leblanc N, Julien JL, Drevet JR, Roeckel-Drevet P (2002) Two GPX-like proteins from Lycopersicon esculentum and Helianthus annuus are antioxidant enzymes with phospholipid hydroperoxide glutathione peroxidase and thioredoxin peroxidase activities. Eur J Biochem **269:** 2414-2420

Hernandez HH, Jaquez OA, Hamill MJ, Elliott SJ, Drennan CL (2008) Thioredoxin reductase from Thermoplasma acidophilum: a new twist on redox regulation. Biochemistry **47:** 9728-9737

Hideg E, Kalai T, Hideg K, Vass I (1998) Photoinhibition of photosynthesis in vivo results in singlet oxygen production detection via nitroxide-induced fluorescence quenching in broad bean leaves. Biochemistry **37:** 11405-11411

Hideg EE, Ogawa K, Kalai T, Hideg K (2001) Singlet oxygen imaging in Arabidopsis thaliana leaves under photoinhibition by excess photosynthetically active radiation. Physiol Plant **112:** 10-14

Hodges M, Miginiac-Maslow M, Decottignies P, Jacquot JP, Stein M, Lepiniec L, Cretin C, Gadal P (1994) Purification and characterization of pea thioredoxin f expressed in Escherichia coli. Plant Mol Biol **26:** 225-234

Hricova A, Quesada V, Micol JL (2006) The SCABRA3 nuclear gene encodes the plastid RpoTp RNA polymerase, which is required for chloroplast biogenesis and mesophyll cell proliferation in Arabidopsis. Plant Physiol **141:** 942-956

Husek P (1998) Chloroformates in gas chromatography as general purpose derivatizing agents. J Chromatogr B Biomed Sci Appl **717:** 57-91

Hutin C, Nussaume L, Moise N, Moya I, Kloppstech K, Havaux M (2003) Early light-induced proteins protect Arabidopsis from photooxidative stress. Proc Natl Acad Sci U S A **100:** 4921-4926

Ido K, Ifuku K, Yamamoto Y, Ishihara S, Murakami A, Takabe K, Miyake C, Sato F (2009) Knockdown of the PsbP protein does not prevent assembly of the dimeric PSII core complex but impairs accumulation of photosystem II

supercomplexes in tobacco. Biochim Biophys Acta **1787:** 873-881

Ikegami A, Yoshimura N, Motohashi K, Takahashi S, Romano PG, Hisabori T, Takamiya K, Masuda T (2007) The CHLI1 subunit of Arabidopsis thaliana magnesium chelatase is a target protein of the chloroplast thioredoxin. J Biol Chem **282:** 19282-19291

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

Ishihara A, Matsuda F, Miyagawa H, Wakasa K (2007) Metabolomics for metabolically manipulated plants: effects of tryptophan overproduction. Metabolomics **3:** 319-334

Jiao Y, Lau OS, Deng XW (2007) Light-regulated transcriptional networks in higher plants. Nat Rev Genet **8:** 217-230

Johansson K, Ramaswamy S, Saarinen M, Lemaire-Chamley M, Issakidis-Bourguet E, Miginiac-Maslow M, Eklund H (1999) Structural basis for light activation of a chloroplast enzyme: the structure of sorghum NADP-malate dehydrogenase in its oxidized form. Biochemistry **38:** 4319-4326

Joyard J, Ferro M, Masselon C, Seigneurin-Berny D, Salvi D, Garin J, Rolland N (2009) Chloroplast proteomics and the compartmentation of plastidial isoprenoid biosynthetic pathways. Mol Plant **2:** 1154-1180

Jung BG, Lee KO, Lee SS, Chi YH, Jang HH, Kang SS, Lee K, Lim D, Yoon SC, Yun DJ, Inoue Y, Cho MJ, Lee SY (2002) A Chinese cabbage cDNA with high sequence identity to phospholipid hydroperoxide glutathione peroxidases encodes a novel isoform of thioredoxin-dependent peroxidase. J Biol Chem **277:** 12572-12578

Kanamaru K, Tanaka K (2004) Roles of chloroplast RNA polymerase sigma factors in chloroplast development and stress response in higher plants. Biosci Biotechnol Biochem **68:** 2215-2223

Kanervo E, Singh M, Suorsa M, Paakkarinen V, Aro E, Battchikova N, Aro EM (2008) Expression of protein complexes and individual proteins upon transition of etioplasts to chloroplasts in pea (Pisum sativum). Plant Cell Physiol **49:** 396-410

Karpinski S, Reynolds H, Karpinska B, Wingsle G, Creissen G, Mullineaux P (1999) Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Science **284:** 654-657

Kim GT, Yano S, Kozuka T, Tsukaya H (2005) Photomorphogenesis of leaves: shade-avoidance and differentiation of sun and shade leaves. Photochem Photobiol Sci **4:** 770-774

Kirchsteiger K, Pulido P, Gonzalez M, Cejudo FJ (2009) NADPH Thioredoxin reductase C controls the redox status of chloroplast 2-Cys peroxiredoxins in Arabidopsis thaliana. Mol Plant **2:** 298-307

Kirkensgaard KG, Hagglund P, Finnie C, Svensson B, Henriksen A (2009) Structure of Hordeum vulgare NADPH-dependent thioredoxin reductase 2. Unwinding the reaction mechanism. Acta Crystallogr D Biol Crystallogr **65:** 932-941

Kleine T, Voigt C, Leister D (2009) Plastid signalling to the nucleus: messengers still lost in the mists? Trends Genet **25:** 185-192

Knappe S, Lottgert T, Schneider A, Voll L, Flugge UI, Fischer K (2003) Characterization of two functional phosphoenolpyruvate/phosphate translocator (PPT) genes in Arabidopsis--AtPPT1 may be involved in the provision of signals for correct mesophyll development. Plant J **36:** 411-420

Kobayashi Y, Kanesaki Y, Tanaka A, Kuroiwa H, Kuroiwa T, Tanaka K (2009) Tetrapyrrole signal as a cell-cycle coordinator from organelle to nuclear DNA replication in plant cells. Proc Natl Acad Sci U S A **106:** 803-807

Kolbe A, Oliver SN, Fernie AR, Stitt M, van Dongen JT, Geigenberger P (2006) Combined transcript and metabolite profiling of Arabidopsis leaves reveals fundamental effects of the thiol-disulfide status on plant metabolism. Plant Physiol **141:** 412-422

Konig J, Lotte K, Plessow R, Brockhinke A, Baier M, Dietz KJ (2003) Reaction mechanism of plant 2-Cys peroxiredoxin. Role of the C terminus and the quaternary structure. J Biol Chem **278:** 24409-24420

Kötting O, Kossmann J, Zeeman SC, Lloyd JR (2010) Regulation of starch metabolism: the age of enlightenment? Curr Opin Plant Biol **13:** 320-328

Krieger-Liszkay A (2005) Singlet oxygen production in photosynthesis. J Exp Bot **56:** 337-346

Kroll D, Meierhoff K, Bechtold N, Kinoshita M, Westphal S, Vothknecht UC, Soll J, Westhoff P (2001) VIPP1, a nuclear gene of Arabidopsis thaliana essential for thylakoid membrane formation. Proc Natl Acad Sci U S A **98:** 4238-4242

Kuriyan J, Krishna TS, Wong L, Guenther B, Pahler A, Williams CH,Jr, Model P (1991) Convergent evolution of similar function in two structurally divergent enzymes. Nature **352:** 172-174

Laloi C, Rayapuram N, Chartier Y, Grienenberger JM, Bonnard G, Meyer Y (2001) Identification and characterization of a mitochondrial thioredoxin system in plants. Proc Natl Acad Sci U S A **98:** 14144-14149

Larkin RM, Alonso JM, Ecker JR, Chory J (2003) GUN4, a regulator of chlorophyll synthesis and intracellular signaling. Science **299:** 902-906

Larkindale J, Knight MR (2002) Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. Plant Physiol **128:** 682-695

Lee JW, Helmann JD (2006) The PerR transcription factor senses H2O2 by metal-catalysed histidine oxidation. Nature **440:** 363-367

Lennon BW, Williams CH,Jr, Ludwig ML (2000) Twists in catalysis: alternating conformations of Escherichia coli thioredoxin reductase. Science **289:** 1190-1194

Lennon BW, Williams CH,Jr, Ludwig ML (1999) Crystal structure of reduced thioredoxin reductase from Escherichia coli: structural flexibility in the isoalloxazine ring of the flavin adenine dinucleotide cofactor. Protein Sci **8:** 2366-2379

Li Z, Wakao S, Fischer BB, Niyogi KK (2009) Sensing and responding to excess light. Annu Rev Plant Biol **60:** 239-260

Lunn JE (2007) Compartmentation in plant metabolism. J Exp Bot **58:** 35-47

Maeda K, Finnie C, Svensson B (2004) Cy5 maleimide labelling for sensitive detection of free thiols in native protein extracts: identification of seed proteins targeted by barley thioredoxin h isoforms. Biochem J **378:** 497-507

Maeda K, Hägglund P, Björnberg O, Winther JR, Svensson B (2010) Kinetic and thermodynamic properties of two barley thioredoxin h isozymes, HvTrxh1 and HvTrxh2. FEBS Lett **584:** 3376-3380

Maliga P (1998) Two plastid RNA polymerases of higher plants: an evolving story. Trends Plant Sci **3:** 4-6

Manchado M, Michan C, Pueyo C (2000) Hydrogen peroxide activates the SoxRS regulon in vivo. J Bacteriol **182:** 6842-6844

Maple J, Aldridge C, Moller SG (2005) Plastid division is mediated by combinatorial assembly of plastid division proteins. Plant J **43:** 811-823

Maple J, Møller SG (2007) Interdependency of formation and localisation of the Min complex controls symmetric plastid division. J Cell Sci **120:** 3446-3456

Marchand C, Le Marechal P, Meyer Y, Miginiac-Maslow M, Issakidis-Bourguet E, Decottignies P (2004) New targets of Arabidopsis thioredoxins revealed by proteomic analysis. Proteomics **4:** 2696-2706

Martin W (2003) Gene transfer from organelles to the nucleus: frequent and in big chunks. Proc Natl Acad Sci U S A **100:** 8612-8614

Martin W, Herrmann RG (1998) Gene transfer from organelles to the nucleus: how much, what happens, and Why? Plant Physiol **118:** 9-17

Masuda T, Fusada N, Oosawa N, Takamatsu K, Yamamoto YY, Ohto M, Nakamura K, Goto K, Shibata D, Shirano Y, Hayashi H, Kato T, Tabata S, Shimada H, Ohta H, Takamiya K (2003) Functional analysis of isoforms of NADPH: protochlorophyllide oxidoreductase (POR), PORB and PORC, in Arabidopsis thaliana. Plant Cell Physiol **44:** 963-974

Mateo A, Funck D, Muhlenbock P, Kular B, Mullineaux PM, Karpinski S (2006) Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. J Exp Bot **57:** 1795-1807

Matsumoto F, Obayashi T, Sasaki-Sekimoto Y, Ohta H, Takamiya K, Masuda T (2004) Gene expression profiling of the tetrapyrrole metabolic pathway in Arabidopsis with a mini-array system. Plant Physiol **135:** 2379-2391

Mazzella MA, Cerdan PD, Staneloni RJ, Casal JJ (2001) Hierarchical coupling of phytochromes and cryptochromes reconciles stability and light modulation of Arabidopsis development. Development **128:** 2291-2299

Mehler AH (1951) Studies on reactions of illuminated chloroplasts. I. Mechanism of the reduction of oxygen and other Hill reagents. Arch Biochem Biophys **33:** 65-77

Meskauskiene R, Nater M, Goslings D, Kessler F, op den Camp R, Apel K (2001) FLU: a negative regulator of chlorophyll biosynthesis in Arabidopsis thaliana. Proc Natl Acad Sci U S A **98:** 12826-12831

Meurer J, Plucken H, Kowallik KV, Westhoff P (1998) A nuclear-encoded protein of prokaryotic origin is essential for the stability of photosystem II in Arabidopsis thaliana. EMBO J **17:** 5286-5297

Meyer Y, Siala W, Bashandy T, Riondet C, Vignols F, Reichheld JP (2008) Glutaredoxins and thioredoxins in plants. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1783:** 589-600

Michalska J, Zauber H, Buchanan BB, Cejudo FJ, Geigenberger P (2009) NTRC links built-in thioredoxin to light and sucrose in regulating starch synthesis in chloroplasts and amyloplasts. Proc Natl Acad Sci U S A **106:** 9908-9913

Mikkelsen R, Mutenda KE, Mant A, Schurmann P, Blennow A (2005) Alpha-glucan, water dikinase (GWD): a plastidic enzyme with redox-regulated and coordinated catalytic activity and binding affinity. Proc Natl Acad Sci U S A **102:** 1785-1790

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci **7:** 405-410

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci **9:** 490-498

Miyagishima SY, Kabeya Y (2010) Chloroplast division: squeezing the photosynthetic captive. Curr Opin Microbiol **13:** 738-746

Miyagishima S, Froehlich JE, Osteryoung KW (2006) PDV1 and PDV2 mediate recruitment of the dynamin-related protein ARC5 to the plastid division site. Plant Cell **18:** 2517-2530

Miyake C, Shinzaki Y, Nishioka M, Horiguchi S, Tomizawa K (2006) Photoinactivation of ascorbate peroxidase in isolated tobacco chloroplasts: Galdieria partita APX maintains the electron flux through the water-water cycle in transplastomic tobacco plants. Plant Cell Physiol **47:** 200-210

Mochizuki N, Tanaka R, Grimm B, Masuda T, Moulin M, Smith AG, Tanaka A, Terry MJ (2010) The cell biology of tetrapyrroles: a life and death struggle. Trends Plant Sci **15:** 488-498

Mochizuki N, Tanaka R, Tanaka A, Masuda T, Nagatani A (2008) The steady-state level of Mg-protoporphyrin IX is not a determinant of plastid-to-nucleus signaling in Arabidopsis. Proc Natl Acad Sci U S A **105:** 15184-15189

Mochizuki N, Brusslan JA, Larkin R, Nagatani A, Chory J (2001) Arabidopsis genomes uncoupled 5 (GUN5) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. Proceedings of the National Academy of Sciences **98:** 2053-2058

Møller IM, Sweetlove LJ (2010) ROS signalling--specificity is required. Trends Plant Sci **15:** 370-374

Montrichard F, Alkhalfioui F, Yano H, Vensel WH, Hurkman WJ, Buchanan BB (2009) Thioredoxin targets in plants: the first 30 years. J Proteomics **72:** 452-474

Motohashi K, Kondoh A, Stumpp MT, Hisabori T (2001) Comprehensive survey of proteins targeted by chloroplast thioredoxin. Proc Natl Acad Sci U S A **98:** 11224-11229

Moulin M, McCormac AC, Terry MJ, Smith AG (2008) Tetrapyrrole profiling in Arabidopsis seedlings reveals that retrograde plastid nuclear signaling is not due to Mg-protoporphyrin IX accumulation. Proc Natl Acad Sci U S A **105:** 15178-15183

Mubarakshina MM, Ivanov BN, Naydov IA, Hillier W, Badger MR, Krieger-Liszkay A (2010) Production and diffusion of chloroplastic H2O2 and its implication to signalling. J Exp Bot **61:** 3577-3587

Mustardy L, Garab G (2003) Granum revisited. A three-dimensional model--where things fall into place. Trends Plant Sci **8:** 117-122

Nakayama M, Masuda T, Bando T, Yamagata H, Ohta H, Takamiya K (1998) Cloning and expression of the soybean chlH gene encoding a subunit of Mg-chelatase and localization of the Mg2+ concentration-dependent ChlH protein within the chloroplast. Plant Cell Physiol **39:** 275-284

Neff MM, Chory J (1998) Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during Arabidopsis development. Plant Physiol **118:** 27-35

Neuhaus HE, Emes MJ (2000) Nonphotosynthetic metabolism in plastids. Annu Rev Plant Physiol Plant Mol Biol **51:** 111-140

Neuhaus HE, Stitt M (1990) Control analysis of photosynthate partitioning: Impact of reduced activity of ADP-glucose pyrophosphorylase or plastid phosphoglucomutase on the fluxes to starch and sucrose in *Arabidopsis thaliana* (L.) Heynh. Planta **182:** 445-454

Nott A, Jung HS, Koussevitzky S, Chory J (2006) Plastid-to-nucleus retrograde signaling. Annu Rev Plant Biol **57:** 739-759

Osteryoung KW, Stokes KD, Rutherford SM, Percival AL, Lee WY (1998) Chloroplast division in higher plants requires members of two functionally divergent gene families with homology to bacterial ftsZ. Plant Cell **10:** 1991-2004

Pena-Ahumada A, Kahmann U, Dietz KJ, Baier M (2006) Regulation of peroxiredoxin expression versus expression of Halliwell-Asada-Cycle enzymes during early seedling development of Arabidopsis thaliana. Photosynth Res **89:** 99-112

Perez-Ruiz JM, Cejudo FJ (2009) A proposed reaction mechanism for rice NADPH thioredoxin reductase C, an enzyme with protein disulfide reductase activity. FEBS Lett **583:** 1399-1402

Perez-Ruiz JM, Gonzalez M, Spinola MC, Sandalio LM, Cejudo FJ (2009) The quaternary structure of NADPH thioredoxin reductase C is redox-sensitive. Mol Plant **2:** 457-467

Perez-Ruiz JM, Spinola MC, Kirchsteiger K, Moreno J, Sahrawy M, Cejudo FJ (2006) Rice NTRC is a high-efficiency redox system for chloroplast protection against oxidative damage. Plant Cell **18:** 2356-2368

Peterson FC, Lytle BL, Sampath S, Vinarov D, Tyler E, Shahan M, Markley JL, Volkman BF (2005) Solution structure of thioredoxin h1 from Arabidopsis thaliana. Protein Sci **14:** 2195-2200

Piippo M, Allahverdiyeva Y, Paakkarinen V, Suoranta U, Battchikova N, Aro E (2006) Chloroplast-mediated regulation of nuclear genes in

Arabidopsis thaliana in the absence of light stress. Physiol Genomics **25:** 142-152

Pilon M, Ravet K, Tapken W (2011) The biogenesis and physiological function of chloroplast superoxide dismutases. Biochimica et Biophysica Acta (BBA) - Bioenergetics **In Press, Corrected Proof:**

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

Pulido P, Spinola MC, Kirchsteiger K, Guinea M, Pascual MB, Sahrawy M, Sandalio LM, Dietz KJ, Gonzalez M, Cejudo FJ (2010) Functional analysis of the pathways for 2-Cys peroxiredoxin reduction in Arabidopsis thaliana chloroplasts. J Exp Bot **61:** 4043-4054

Pyke KA (1999) Plastid division and development. Plant Cell **11:** 549-556

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

Queval G, Issakidis-Bourguet E, Hoeberichts FA, Vandorpe M, Gakiere B, Vanacker H, Miginiac-Maslow M, Van Breusegem F, Noctor G (2007) Conditional oxidative stress responses in the Arabidopsis photorespiratory mutant cat2 demonstrate that redox state is a key modulator of daylength-dependent gene expression, and define photoperiod as a crucial factor in the regulation of H2O2-induced cell death. Plant J **52:** 640-657

Reed JW, Nagatani A, Elich TD, Fagan M, Chory J (1994) Phytochrome A and phytochrome B have overlapping but distinct functions in Arabidopsis development. Plant Physiol **104:** 1139-1149

Reichheld JP, Meyer E, Khafif M, Bonnard G, Meyer Y (2005) AtNTRB is the major mitochondrial thioredoxin reductase in Arabidopsis thaliana. FEBS Lett **579:** 337-342

Reinbothe C, Pollmann S, Reinbothe S (2010) Singlet oxygen signaling links photosynthesis to translation and plant growth. Trends Plant Sci **15:** 499-506

Rey P, Cuine S, Eymery F, Garin J, Court M, Jacquot JP, Rouhier N, Broin M (2005) Analysis of the proteins targeted by CDSP32, a plastidic thioredoxin participating in oxidative stress responses. Plant J **41:** 31-42

Rintamäki E, Martinsuo P, Pursiheimo S, Aro EM (2000) Cooperative regulation of light-harvesting complex II phosphorylation via the plastoquinol and ferredoxin-thioredoxin system in chloroplasts. Proc Natl Acad Sci U S A **97:** 11644-11649

Ritte G, Lloyd JR, Eckermann N, Rottmann A, Kossmann J, Steup M (2002) The starch-related R1 protein is an alpha -glucan, water dikinase. Proc Natl Acad Sci U S A **99:** 7166-7171

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol **57:** 675-709

Rouhier N, Jacquot JP (2002) Plant peroxiredoxins: alternative hydroperoxide scavenging enzymes. Photosynth Res **74:** 259-268

Ruggiero A, Masullo M, Ruocco MR, Grimaldi P, Lanzotti MA, Arcari P, Zagari A, Vitagliano L (2009) Structure and stability of a thioredoxin reductase from Sulfolobus solfataricus: a thermostable protein with two functions. Biochim Biophys Acta **1794:** 554-562

Sakamoto W (2006) Protein degradation machineries in plastids. Annu Rev Plant Biol **57:** 599-621

Schmelz EA, Engelberth J, Alborn HT, O'Donnell P, Sammons M, Toshima H, Tumlinson JH,III (2003) Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants. Proc Natl Acad Sci U S A **100:** 10552-10557

Schmitz AJ, Glynn JM, Olson BJSC, Stokes KD, Osteryoung KW (2009) Arabidopsis FtsZ2-1 and FtsZ2-2 are functionally redundant, but FtsZ-based plastid division is not essential for chloroplast partitioning or plant growth and development. Molecular Plant **2:** 1211-1222

Schoefs B, Franck F (2003) Protochlorophyllide reduction: mechanisms and evolutions. Photochem Photobiol **78:** 543-557

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

Schürmann P, Jacquot JP (2000) Plant thioredoxin systems revisited. Annu Rev Plant Physiol Plant Mol Biol **51:** 371-400

Schwarz O, Schurmann P, Strotmann H (1997) Kinetics and thioredoxin specificity of thiol modulation of the chloroplast H+-ATPase. J Biol Chem **272:** 16924-16927

Serrato AJ, Perez-Ruiz JM, Spinola MC, Cejudo FJ (2004) A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes

hypersensitivity to abiotic stress in Arabidopsis thaliana. J Biol Chem **279:** 43821-43827

Slesak I, Libik M, Karpinska B, Karpinski S, Miszalski Z (2007) The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. Acta Biochim Pol **54:** 39-50

Sokolov LN, Dominguez-Solis JR, Allary AL, Buchanan BB, Luan S (2006) A redox-regulated chloroplast protein phosphatase binds to starch diurnally and functions in its accumulation. Proc Natl Acad Sci U S A **103:** 9732-9737

Solymosi K, Schoefs B (2010) Etioplast and etio-chloroplast formation under natural conditions: the dark side of chlorophyll biosynthesis in angiosperms. Photosynth Res **105:** 143-166

Sparla F, Costa A, Lo Schiavo F, Pupillo P, Trost P (2006) Redox regulation of a novel plastid-targeted beta-amylase of Arabidopsis. Plant Physiol **141:** 840-850

Stenbaek A, Hansson A, Wulff RP, Hansson M, Dietz KJ, Jensen PE (2008) NADPH-dependent thioredoxin reductase and 2-Cys peroxiredoxins are needed for the protection of Mg-protoporphyrin monomethyl ester cyclase. FEBS Lett **582:** 2773-2778

Stenbaek A, Jensen PE (2010) Redox regulation of chlorophyll biosynthesis. Phytochemistry **71:** 853-859

Stenbaek A (2010) Regulation of chlorophyll biosynthesis – from light-harvesting to signalling compounds. PhD thesis. University of Copenhagen, Copenhagen

Strand A, Asami T, Alonso J, Ecker JR, Chory J (2003) Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. Nature **421:** 79-83

Stumpp MT, Motohashi K, Hisabori T (1999) Chloroplast thioredoxin mutants without active-site cysteines facilitate the reduction of the regulatory disulphide bridge on the gamma-subunit of chloroplast ATP synthase. Biochem J **341 (Pt 1):** 157-163

Tanaka A, Tanaka R (2006) Chlorophyll metabolism. Curr Opin Plant Biol **9:** 248-255

Telfer A, Bishop SM, Phillips D, Barber J (1994) Isolated photosynthetic reaction center of photosystem II as a sensitizer for the formation of singlet oxygen. Detection and quantum yield determination using a chemical trapping technique. J Biol Chem **269:** 13244-13253

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408:** 796-815

Toledano MB, Delaunay A, Monceau L, Tacnet F (2004) Microbial H2O2 sensors as archetypical redox signaling modules. Trends Biochem Sci **29:** 351-357

Tzin V, Galili G (2010) New insights into the shikimate and aromatic amino acids biosynthesis pathways in plants. Mol Plant **3:** 956-972

Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Roveri A, Schomburg D, Flohe L (1995) Diversity of glutathione peroxidases. Methods Enzymol **252:** 38-53

Veljovic-Jovanovic S, Noctor G, Foyer CH (2002) Are leaf hydrogen peroxide concentrations commonly overestimated? The potential influence of artefactual interference by tissue phenolics and ascorbate. Plant Physiology and Biochemistry **40:** 501-507

Verdoucq L, Vignols F, Jacquot JP, Chartier Y, Meyer Y (1999) In vivo characterization of a thioredoxin h target protein defines a new peroxiredoxin family. J Biol Chem **274:** 19714-19722

Vignols F, Brehelin C, Surdin-Kerjan Y, Thomas D, Meyer Y (2005) A yeast two-hybrid knockout strain to explore thioredoxin-interacting proteins in vivo. Proc Natl Acad Sci U S A **102:** 16729-16734

Vothknecht UC, Westhoff P (2001) Biogenesis and origin of thylakoid membranes. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1541:** 91-101

Waksman G, Krishna TS, Williams CH,Jr, Kuriyan J (1994) Crystal structure of Escherichia coli thioredoxin reductase refined at 2 A resolution. Implications for a large conformational change during catalysis. J Mol Biol **236:** 800-816

Wang Q, Sullivan RW, Kight A, Henry RL, Huang J, Jones AM, Korth KL (2004) Deletion of the chloroplast-localized Thylakoid Formation1 gene product in Arabidopsis leads to deficient thylakoid formation and variegated leaves. Plant Physiol **136:** 3594-3604

Weber A, Flugge UI (2002) Interaction of cytosolic and plastidic nitrogen metabolism in plants. J Exp Bot **53:** 865-874

Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M, Inze D, Van Camp W (1997) Catalase is a sink for H2O2 and is indispensable for stress defence in C3 plants. EMBO J **16:** 4806-4816

Wolosiuk RA, Buchanan BB (1978) Activation of chloroplast NADP-linked glyceraldehyde-3-phosphate dehydrogenase by the ferredoxin/thioredoxin system. Plant Physiology **61:** 669-671

Woodson JD, Chory J (2008) Coordination of gene expression between organellar and nuclear genomes. Nat Rev Genet **9:** 383-395

Yamasato A, Nagata N, Tanaka R, Tanaka A (2005) The N-terminal domain of chlorophyllide a oxygenase confers protein instability in response to chlorophyll B accumulation in Arabidopsis. Plant Cell **17:** 1585-1597

Yang P, Chen H, Liang Y, Shen S (2007) Proteomic analysis of de-etiolated rice seedlings upon exposure to light. Proteomics **7:** 2459-2468

Yang Y, Glynn JM, Olson BJ, Schmitz AJ, Osteryoung KW (2008) Plastid division: across time and space. Curr Opin Plant Biol **11:** 577-584

Yano S, Terashima I (2001) Separate localization of light signal perception for sun or shade type chloroplast and palisade tissue differentiation in Chenopodium album. Plant Cell Physiol **42:** 1303-1310

Yu F, Fu A, Aluru M, Park S, Xu Y, Liu H, Liu X, Foudree A, Nambogga M, Rodermel S (2007) Variegation mutants and mechanisms of chloroplast biogenesis. Plant Cell Environ **30:** 350-365

Yu TS, Kofler H, Hausler RE, Hille D, Flugge UI, Zeeman SC, Smith AM, Kossmann J, Lloyd J, Ritte G, Steup M, Lue WL, Chen J, Weber A (2001) The Arabidopsis sex1 mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. Plant Cell **13:** 1907-1918

Zeeman SC, Kossmann J, Smith AM (2010) Starch: its metabolism, evolution, and biotechnological modification in plants. Annu Rev Plant Biol **61:** 209-234

Zeeman SC, Northrop F, Smith AM, Rees T (1998) A starch-accumulating mutant of Arabidopsis thaliana deficient in a chloroplastic starch-hydrolysing enzyme. Plant J **15:** 357-365

Zeeman SC, Smith SM, Smith AM (2007) The diurnal metabolism of leaf starch. Biochem J **401:** 13-28

Zhang N, Portis AR,Jr (1999) Mechanism of light regulation of Rubisco: a specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f. Proc Natl Acad Sci U S A **96:** 9438-9443

Zheng M, Aslund F, Storz G (1998) Activation of the OxyR transcription factor by reversible disulfide bond formation. Science **279:** 1718-1721