Instituto de Bioquímica Vegetal y Fotosíntesis

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Redox regulation of photosynthetic metabolism in chloroplasts of *Arabidopsis thaliana*

Trabajo presentado para optar al título de Doctora en Biología

OJEDA SERVIAN VALLE - 50625440W

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Valle Ojeda Servián

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Directores de la Tesis

CEJUDO FERNANDEZ FRANCISCO JAVIER - 28569988D

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Dr. Francisco Javier Cejudo Fernández
Catedrático de Bioquímica y Biología Molecular

PEREZ RUIZ JUAN MANUEL - 52562658E

Digitally signed by PEREZ RUIZ JUAN MANUEL - 52562658E
DN: c=ES, serialNumber=52562658E, sn=PEREZ RUIZ, givenName=JUAN MANUEL, cn=PEREZ RUIZ JUAN MANUEL - 52562658E
Date: 2019.02.25 10:28:42 +01'00'

Dr. Juan Manuel Pérez Ruiz
Profesor Ayudante Doctor

A mis padres

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INDEX

ABBREVIATIONS

4-POBN	α-(4-Pyridyl N-oxide)-N-tert-butylnitrone
µE	microEinstein
A	Absorbance
A_N	Net CO_2 assimilation rate
ABRC	Arabidopsis Biological Resource Center
ACHT	Atypical Cys/His-rich Thioredoxin
ADP	Adenosine diphosphate
AGI	Arabidopsis Genome Iniciative
AGPase	ADP-glucose pyrophosphorylase
Apx	Ascorbate peroxidase
AsA	Ascorbic Acid
ATP	Adenosine triphosphate
bp	Base pair
ºC	Celsius degree
CaMV 35S	Cauliflower mosaic virus 35S promoter
CBC	Calvin-Benson cycle
cDNA	Complementary DNA
CDSP32	Chloroplastic drought-induced stress protein
CF_1-γ	γ subunit of ATPase
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Chl	Chlorophyll
CI	Confidence interval
C_P	Peroxidatic Cysteine
C_R	Resolving Cysteine
cyt *b_6f*	cytochrome-*b6f* complex
d	Day
dag	Days after germination
DHA	Dehydroascorbic acid
DNA	Deoxyribonucleic acid
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
ε	Molar extinction coefficient
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
EPR	Electron paramagnetic resonance
ETR (II)	Photosynthetic electron transport rate
FAD	Flavin adenine dinucleotide
FBPase	Fructose 1,6-bisphosphatase
Fdx	Ferredoxin

FNR	Fdx-$NADP^+$ reductase
FTR	Ferredoxin-thioredoxin reductase
F_m	Maximal fluorescence
F_v	Variable fluorescence
FW	Fresh weight
g	Gram
g	Standard gravity
Glucose-6P	Glucose 6-phosphate
G6PDH	Glucose 6-phosphate dehydrogenase
GAPDH	3-phosphoglyceraldehyde deshydrogenase
Gpx	Glutathione peroxidase
Grx	Glutaredoxin
GSH	Reduced glutathione
GSSG	Oxidized glutathione
h	Hour
HCF 164	High chlorophyll fluorescence 164 protein
HEPES	4- (2-hydroxyethyl) -1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
Hz	Hertz
KD	Knock-down
kDa	Kilodalton
KO	Knock-out
l	Litre
Lhc	Light harvesting complex
m	Meter
M	Molar
MDHA	Monodehydroascorbate
Mg-CHLI	Magnesium-chelatase I subunit
min	Minute
$MM(PEG)_{24}$	Methyl-maleimide polyethylene glycol $_{24}$
MS	Murashige and Skoog
MSR	Methionine sulfoxide reductase
$NADP^+$	Oxidized Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NADP-MDH	NADP-dependent malate dehydrogenase
NASC	Nottingham Arabidopsis Stock Centre
NEM	*N*-Ethylmaleimide
NP-40	Nonidet P-40
NPQ	Non-photochemical quenching
Nrx	Nucleoredoxins
NTA	Nitrilotriacetic acid
NTR	NADPH-dependent thioredoxin reductase
O_2^-	Superoxide anion

OPPP	Oxidative pentose phosphate pathway
oxi	Oxidized
PAGE	Polyacrylamide gel electrophoresis
PAM	Pulse-amplitude modulation fluorometer
PAR	Photosynthetically active radiation
PC	Plastocyanin
PCR	Polymerase chain reaction
PEP	Plastid-encoded RNA polymerase
PGI	posphoglucose isomerase
P_i	Inorganic phosphate
pmf	Proton motive force
PQ	Plastoquinone
PQH_2	Plastoquinol
PRK	Phosphoribulokinase
Prx	Peroxiredoxin
PSI	Photosystem I
PSII	Photosystem II
PTM	Post-translational modification
PTOX	Plastid terminal oxidase
qE	Energy-dependent quenching
red	Reduced
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative PCR
ROS	Reactive oxygen species
RuBisCo	Ribulose-1,5-bisphosphate carboxylase oxygenase
s	Second
SBPase	Sedoheptulose 1,7-bisphosphatase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE/SEM	Standard error
SOD	Superoxide dismutase
Srx	Sulfiredoxin
Suc	Sucrose
T-DNA	Transfer DNA
TAIR	The Arabidopsis Information Resource
Trx	Thioredoxin
TrxL	Thioredoxin like protein
v/v	Volume/volume
w/v	Weight/volume
Y(II)	Quantum yield of PSII photochemistry
Y(NPQ)	Quantum yield of NPQ

XTT	Tetrazolium dye Na,3′-(1-[phenylaminocarbonyl]-3,4-tetrazolium)-bis-(4-methoxy-6-nitro) benzene sulfonic acid hydrate
W	Watt
wk	week
WT	Wild type

NITROGENOUS BASES

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

AMINO ACIDS

A	Ala	Alanine	L	Leu	Leucine
R	Arg	Arginine	K	Lys	Lysine
N	Asn	Asparagine	M	Met	Methionine
D	Asp	Aspartic acid	F	Phe	Phenylalanine
C	Cys	Cysteine	P	Pro	Proline
E	Glu	Glutamic acid	S	Ser	Serine
Q	Gln	Glutamine	T	Thr	Threonine
G	Gly	Glycine	W	Trp	Tryptophan
H	His	Histidine	Y	Tyr	Tyrosine
I	Ile	Isoleucine	V	Val	Valine

INTRODUCTION

INTRODUCTION

1. The importance of plants in the biosphere

Plants perform oxygenic photosynthesis, a process that involves carbon dioxide (CO_2) fixation and oxygen (O_2) generation, using light energy. This process. Consequently, life on Earth would not be possible without plants as these organisms, among other phototrophs, supply food and maintain the oxygen content in the atmosphere. As sessile organisms, plants are continuously exposed to a variety of environmental changes that affect their development, growth and productivity. The United Nations estimates that by 2050 the world's population will grow by more than two billion people and half will be born in countries that suffer drought, heat waves and extreme weather (United Nations, 2017). Moreover, the Intergovernmental Panel on Climate Change has announced that urgent drastic humanity efforts are required to maintain the global temperature rise below 1.5ºC (IPCC, 2018).

In the past century, the American geneticist Norman Borlaug made important contributions to food production and hunger solution and he led the so-called green revolution, which transformed global agriculture, for which he was recognized with the Nobel Peace Prize in 1970. Climatic changes are expected to have an enormous impact on the Earth's vegetation, thus crop plants will require adaptation to the new environmental conditions. Nowadays, in order to achieve a new green revolution, scientists must focus on understanding the internal basic processes determining the behaviour and productivity of plants. Thus, at present, research in plant science is necessary not only to understand the mechanisms that allow their development and response to environmental stimuli, but to generate new tools useful to develop future strategies of crop adaptation to future climate conditions.

1.1. *Arabidopsis thaliana*

The choice of *Arabidopsis thaliana* as a model organism revolutionized our understanding of plants biology (Provart et al., 2016). Features such as small size, short generation time (6-8 weeks), ease of crossing, high fecundity, small genome

(135 Mbp), availability of large mutant collections and the ability to perform mutant screens have all led to a huge increase in *Arabidopsis* research, illustrating the relevance of this specie as model organism.

The first large collection of T-DNA mutants was created by the *Arabidopsis* research community, taking advantage of feasible genetic manipulation mediated by *Agrobacterium tumefaciens* (Till et al., 2003). Well-organized *Arabidopsis* stock collections were established in the early 1990s, including the Arabidopsis Biological Resource Center (ABRC) and the Nottingham Arabidopsis Stock Centre (NASC). Moreover, open access databases, such as The Arabidopsis Information Resource (TAIR) (Huala et al., 2001) have served to organize sequences, physical maps and register data provided by the Arabidopsis Genome Initiative (The Arabidopsis Genome 2000).

Arabidopsis research is still playing a critical role in the implementation and optimization of experimental protocols. In fact, model systems are not conceived to explain everything but to give researchers a reference for comparison. For this reason, the knowledge acquired in *Arabidopsis* establishes the basis for complementary experiments in other plant species, such as crop plants.

2. Photosynthesis

The appearance of oxygenic photosynthesis and subsequent oxygenation of Earth's atmosphere is one of the most important transition processes in the history of life (Martin et al., 2018). Oxygenic photosynthesis, performed by plants, green algae and cyanobacteria, consists in the use of light energy to assimilate oxidized components, including CO_2 fixation, using water as source of reducing power and releasing oxygen. Thus, photosynthesis is the main source of biomass and oxygen in the planet. In plants, photosynthesis occurs in chloroplasts, an organelle with a double membrane that enclose a fluid compartment called stroma and a membrane system known as thylakoids, which defines an internal space called lumen. Thylakoids can be found stacked, known as thylakoid grana, or can be connected by non-stacked membranes called stroma lamellae. The thylakoid membrane hosts the components of the photosynthetic electron

transport chain, which uses light energy captured in photosystems II (PSII) and I (PSI) to drive electrons provided from water to ferredoxin (Fdx) and $NADP^+$, with the concomitant generation of an electrochemical gradient of protons, which is used to generate ATP. Then, reduced Fdx (Fdx_{red}), NADPH and ATP are used for biosynthetic pathways to generate organic matter in the chloroplast stroma. Besides their crucial function as producers of metabolic precursors that promote plant growth and development, chloroplasts are also involved in plant acclimation to changing environmental conditions. Because plant growth and development are integrated by chloroplast biogenesis and function (Jarvis and López-Juez 2013), the study of these organelles has attracted a high interest in plant science.

2.1. Photophosphorylation

The primary step of photosynthesis, the conversion of sunlight into chemical energy, is driven by a series of reactions that occur in the thylakoid membrane (Figure 1) (Nelson and Ben-Shem, 2004). This conversion is carried out by two separated multi-subunit protein complexes, PSII and PSI. Light is absorbed by the chlorophyll of each antenna complex (Lhc) and photosynthetic reaction centers operate to perform water oxidation and $NADP^+$ reduction, respectively (Nelson and Junge, 2015). The cytochrome-*b_6f* complex (cyt *b_6f*), which is defined as a plastoquinone-plastocyanin oxidoreductase, mediates electron transport between PSII and PSI and generates a proton-motive force (pmf) using a mechanism know as Q cycle (Cramer et al., 2011). The production of ATP is catalysed by ATP synthase (ATPase), which uses the transmembrane pmf generated during the photosynthetic electron transport (Junge and Nelson, 2015). In addition, cyclic phosphorylation, which increases the transmembrane pmf without the production of NADPH (Yamori and Shikanai, 2016), or the non-photochemical quenching (NPQ), which dissipates the excess of absorbed energy as heat (Ruban, 2016), are important processes to maintain an appropriate rate of ATP synthesis and to avoid photoinhibition.

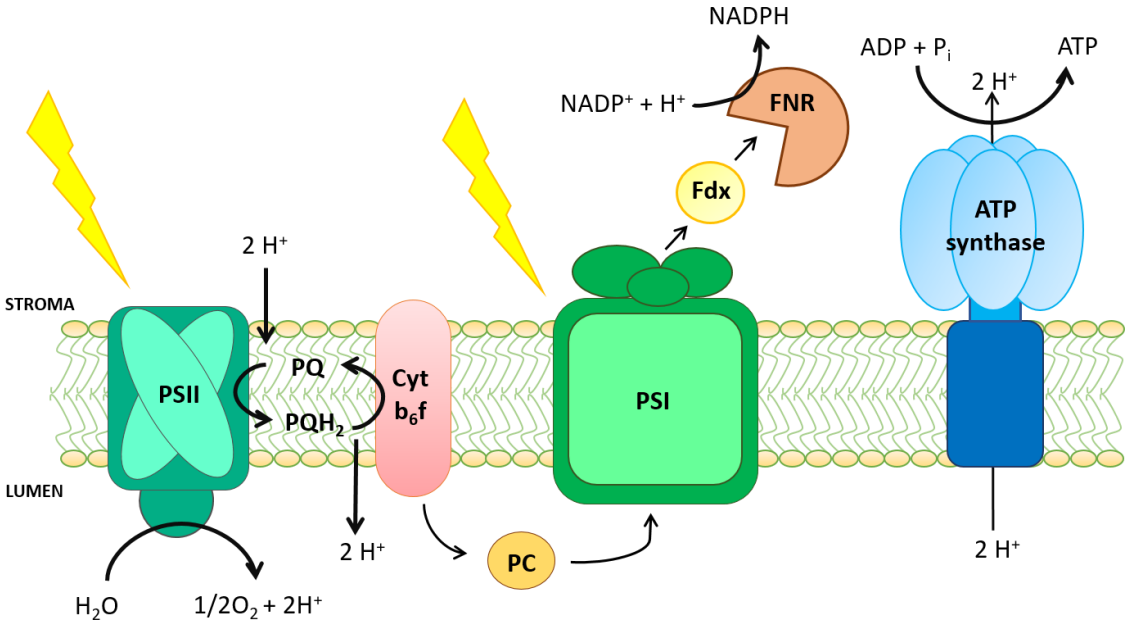

Figure 1. Linear electron transport and photophosphorylation. Photophosphorylation occurs in the thylakoid membranes. Light photons are absorbed by Photosystem II (PSII) and Photosystem I (PSI). PSII performs splitting of H_2O that releases O_2. PSII transfers electrons to PSI via plastoquinone (PQ), cytochrome-*b*$_6$*f* complex (cyt *b*$_6$*f*) and plastocyanin (PC), pumping H^+ ions from thylakoid stroma into the thylakoid lumen and generating a proton-motive force (pmf). ATP synthase uses this H^+ gradient to produce ATP. PSI uses the electrons to reduce Ferredoxin (Fdx) and generate NADPH from $NADP^+$ via Ferredoxin NADPH reductase (FNR).

2.2. Carbon assimilation

The following phase of photosynthesis, initially proposed in Bassham et al. (1950), uses ATP and NADPH to reduce CO_2 and produce sugars (triose-P) by a sequence of reactions known as the Calvin-Benson cycle (CBC). The CBC, which involves eleven different enzymes (Figure 2), can be divided into three main stages. The first stage incorporates CO_2 into an organic molecule in a reaction catalysed by ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo), which is the most abundant soluble protein in the chloroplast, actually the most abundant enzyme in the biosphere (Bracher et al., 2017). Then, in the second stage, called reduction, the 3-phosphoglycerate formed is used to form the triose phosphates by two reactions that consume ATP and NADPH. Finally, the

regeneration stage is composed by several reactions that convert the triose phosphates into ribulose-1,5-bisphosphate, the CO_2 acceptor molecule, to restart the cycle. Thus, there is a net production of triose phosphate, which serves for the synthesis of sucrose, starch, lipids and amino acids.

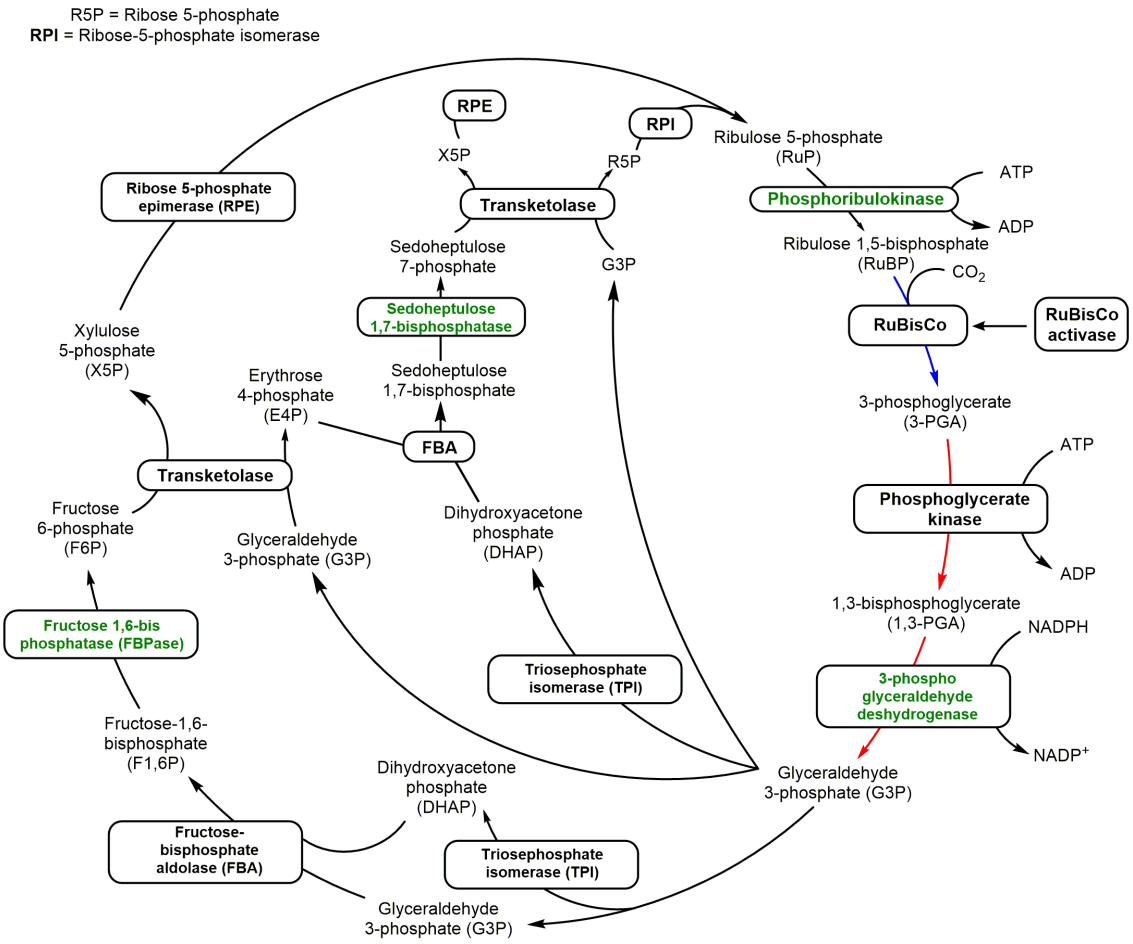

Figure 2. The Calvin-Benson Cycle. The sequence of reactions that allow the fixation of CO_2 is termed the Calvin-Benson Cycle (CBC). The reactions of the CBC can be divided into three stages: carboxylation (blue arrow), reduction (red arrows) and regeneration (black arrows). ATP and NADPH, produced by the electron transport chain, are required for carbon fixation. The enzymes that catalyse the 11 reactions of the CBC are indicated in boxes. In addition, RuBisCo activase is required for RuBisCo activation. Thioredoxin-dependent redox regulated enzymes are highlighted in green (see below, Section 4.3.1. Targets of the FTR/Trx system). Adapted from Michelet et al. (2013).

3. Chloroplast antioxidant systems

Like all aerobic organisms, plant cells generate reactive oxygen species (ROS) in their different compartments, ROS production being particularly important in chloroplasts, peroxisomes, and mitochondria (Foyer and Noctor, 2003). ROS are defined as oxygen-containing molecules which exhibit higher chemical reactivity than O_2, and include singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). Although high ROS levels can provoke damage and, eventually, cell death, ROS are also involved in multiple processes that contribute to the fine tuning of cell metabolism (Waszczak et al., 2018). Indeed, ROS production is essential for redox sensing, signalling and regulation within cell (Mittler, 2017). Therefore, in order to regulate ROS levels, cells are equipped with a complex antioxidant machinery, including enzymatic and non-enzymatic antioxidant systems. These antioxidant systems, that often have overlapping or interacting functions, are located in specific subcellular compartments (Table I).

Antioxidant	Localization	ROS
NON-ENZYMATIC		
Ascorbic Acid (AsA)	apo, cyt, chl, mit, per, vac	$O_2^{\cdot-}$ H_2O_2 $^{\cdot}OH$
Carotenoids	chl and other non-green plastids	$^1O_2$
Reduced Glutathione (GSH)	apo, cyt, chl, mit, per, vac	$^1O_2$ $O_2^{\cdot-}$ H_2O_2 $^{\cdot}OH$
α-Tocopherol	membranes	$^1O_2$
ENZYMATIC		
Ascorbate peroxidase	cyt, chl, mit, per	H_2O_2
Catalase	mit, per	H_2O_2
Dehydroascorbate reductase (DHA reductase)	cyt, chl, mit	Regenerates AsA
Glutathione peroxidases	cyt, chl, er, mit, nuc	H_2O_2
Glutathione reductase	cyt, chl, mit	Regenerates GSH
Guaiacol peroxidase	cyt, er, mit	H_2O_2
Monodehydroascorbate reductase (MDHA reductase)	cyt, chl, mit	Regenerates AsA
Peroxiredoxins	cyt, chl, mit, nuc	H_2O_2
Superoxide dismutase	cyt, chl, mit, per	$O_2^{\cdot-}$

Table 1. Enzymatic and non-enzymatic antioxidants along with their functions and cellular localization. apo, apoplast; cyt, cytosol; chl, chloroplasts; er, endoplasmatic reticulum; mit, mitochondria; vac, vacuole; per, peroxisomes; nuc, nucleus. Adapted from Das and Roychoudhury (2014); Noctor et al. (2018).

Chloroplast ROS production is associated with light-dependent photosynthetic reactions (Dietz et al., 2016). In order to balance ROS levels, chloroplasts contain a battery of antioxidant systems (Figure 3). On one hand, when the light absorption exceeds the capacity of photosynthetic electron transport, charge reactions in PSII can lead to the formation of triplet state of Chl in the reaction centre, which reacts with triplet oxygen ($^3O_2$) generating $^1O_2$ (Fischer et al., 2013), highly reactive and exclusive of chloroplasts. PSII-generated $^1O_2$ is scavenged by non-enzymatic antioxidant systems, such as carotenoids, tocopherol and ascorbate (AsA) (Fischer et al., 2013). On the other hand, chloroplasts produce O_2^- at the stromal side of thylakoid membranes by two mechanisms (Dietz et al., 2016); first, the Mehler reaction, consisting in the transfer of electrons from over-reduced PSI to O_2 and, second, plastid terminal oxidase (PTOX), which oxidases plastoquinone and reduces O_2 to H_2O, and might generate O_2^- in a side reaction under high light or stress conditions. Nevertheless, O_2^- generated in the stroma has received little attention because it is rapidly dismutated into H_2O_2 via stromal superoxide dismutase (SOD) (Smirnoff and Arnaud, 2018). Chloroplasts contain both "prokaryotic" and "eukaryotic" SOD types, Fe-SOD and Cu/Zn-SOD, respectively (Pilon et al., 2011). Therefore, O_2^- dismutation is considered as the major source of H_2O_2 in chloroplasts. The main antioxidant systems to detoxify H_2O_2 in chloroplasts are ascorbate peroxidases and thiol peroxidases (Dietz, 2016), which are described below. The flow of electrons from water at PSII, through the photosynthetic electron transport chain, to PSI, with the consequent generation of O_2^- and H_2O_2, and then back to water due to the action of antioxidant systems, is known as the water-water cycle and allows the dissipation of excess excitation energy and electrons as source of oxidative signals (Asada, 1999).

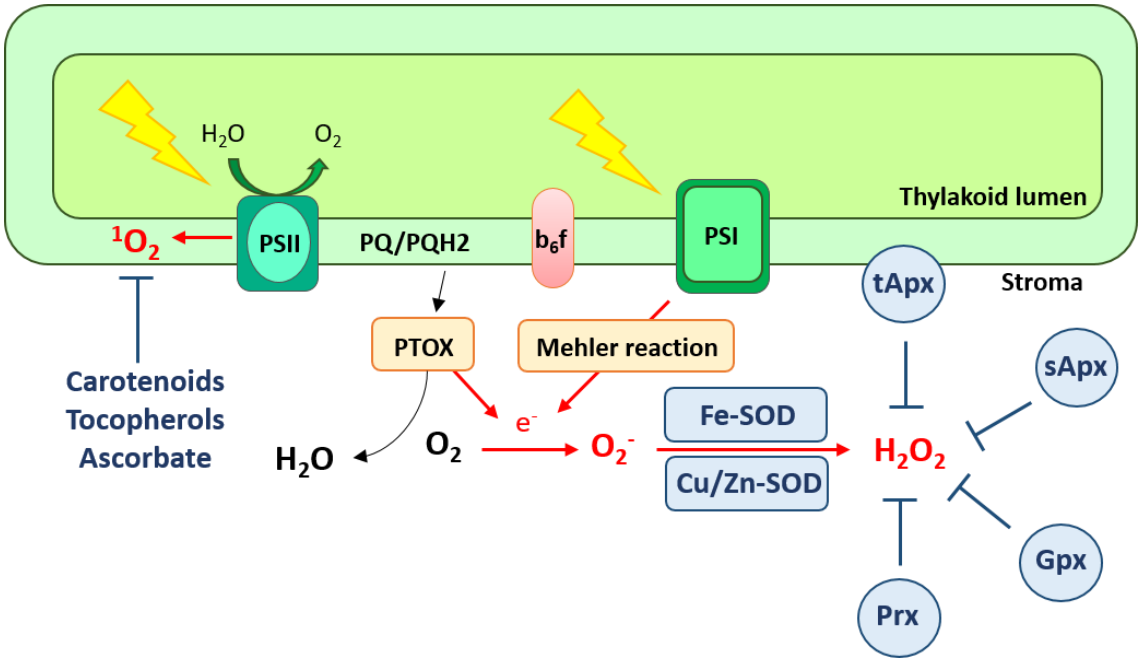

Figure 3. Production and scavenging of ROS in chloroplasts. Singlet oxygen ($^1O_2$) arises within thylakoid membranes mainly by the transfer of energy from the exited Chl of PSII to ground state molecular oxygen. The major $^1O_2$ antioxidant systems are non-enzymatic, such as carotenoids, tocopherol and ascorbate. Superoxide anion (O_2^-) at the stromal side is produced by the transfer of electrons to O_2 by the Mehler reaction and plastid terminal oxidase (PTOX). Dismutation of O_2^- by the two types of superoxide dismutases (SOD), Fe-SOD and Cu/Zn-SOD, produces hydrogen peroxide (H_2O_2). The main antioxidant system for detoxification of H_2O_2 in chloroplasts are ascorbate peroxidases, located at the thylakoid membrane (tAPX) and the stroma (sAPX), and thiol peroxidases, as Peroxiredoxin (Prx) and Glutathione peroxidase (Gpx).

3.1. Ascorbate Peroxidases

Arabidopsis chloroplasts contain two isoforms of ascorbate peroxidases (APXs), thylakoid membrane-bound APX (tAPX) and stromal APX (sAPX) (Maruta et al., 2016). H_2O_2 produced in chloroplasts is detoxified to water by APXs, which use AsA as electron donor, which in turn, is oxidized to monodehydroascorbate (MDHA). Regeneration of AsA from MDHA involves enzymatic reactions mediated by monodehydroascorbate reductase (MDHA reductase), dehydroascorbate reductase (DHA reductase) and glutathione reductase, all of them forming the so-called ascorbate-glutathione cycle (Foyer, 2018). APXs are considered to play an

important role in the reductive detoxification of H_2O_2 during the water-water cycle (Maruta et al., 2016). However, *Arabidopsis* mutants lacking both sAPX and tAPX exhibited no visible symptoms of stress after high light exposure compared with wild-type plants (Giacomelli et al., 2007; Kangasjärvi et al., 2008), suggesting that additional detoxification mechanisms could compensate for the lack of chloroplast APXs.

3.2. Thiol Peroxidases

In proteins, the Cys residue can be found in different oxidation states. These include the reduced thiolic form (-SH) and oxidized states, such as sulfenic (−SOH), sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) forms. Besides, Cys can form intra- or inter-molecular disulphide bonds (Cremers and Jakob, 2013). The redox state of Cys residues in proteins is biologically relevant as it has profound effects on protein conformation and, thereby, in their regulation and functions. Remarkably, Cys is one of the least abundant amino acids in proteins and, when present, it is usually highly conserved (Cremers and Jakob, 2013). Moreover, the content of Cys in proteomes increases with the complexity of the organisms ranging from ~0.4% in some archaea to ~2.2 in mammals (Cremers and Jacob, 2013). All these data support the notion that Cys plays a very relevant function in protein conformation and activity. An example that illustrates this issue is the case of thiol peroxidases, enzymes that reduce H_2O_2 using the reducing power of two Cys thiolic groups, which form part of their catalytic active site and are converted to disulphide after the reaction. In chloroplasts, there are two groups of thiol peroxidases, glutathione peroxidases (Gpx) and peroxiredoxins (Prx) (Dietz, 2016).

3.2.1. Glutathione peroxidases

In the majority of vertebrates, Gpxs possess selenocysteine in their active site. This is the case of mammalian Gpx1, which contains selenocysteine and uses reduced glutathione (GSH) as electron donor to reduce H_2O_2 (Lubos et al., 2011). On the contrary, plant Gpxs carry cysteines, which are reduced by thioredoxin (Trx) instead of GSH (Iqbal et al., 2006; Navrot et al., 2006). The genome of

Arabidopsis thaliana encodes a family of eight Gpxs, termed AtGpx 1–8, with homology to mammalian Gpx isoenzymes and harbouring a Cys in their active site (Bela et al., 2015). It is known that two AtGpxs, Gpx1 and Gpx7, are located in chloroplast (Bela et al., 2015). The characterization of *Arabidopsis* lines altered in plastid Gpxs showed a compromised high-light stress tolerance and increased basal resistance to virulent bacteria, indicating that chloroplast Gpxs contribute to both processes (Chang et al., 2009). Moreover, it has been proposed that Gpxs, as other antioxidant systems, may act as H_2O_2 sensors transferring redox signal to specific target proteins, suggesting a regulatory and signalling role for these enzymes (Passaia and Margis-Pinheiro, 2015).

3.2.2. Peroxiredoxins

Prxs are ubiquitous thiol-dependent peroxidases, present in all kingdoms of life and in, virtually, all types of organisms (Rhee, 2016). These enzymes catalyze the reduction of H_2O_2 by a reaction mechanism which involves two Cys residues in their active site: a peroxidatic Cys (C_P), conserved in all Prxs, and a resolving Cys (C_R), present in some Prx types (Wood et al., 2003a) (Figure 4). The reaction mechanism is based on a nucleophilic attack to H_2O_2 carried out by the thiol group of the C_P, generating the sulfenic acid (C_P-SOH) intermediate, which reacts with the thiol of the C_R (C_R-SH) to form a disulphide that needs to be reduced by an appropriate electron donor for a new catalytic cycle. Prxs are classified into two types, 1-Cys and 2-Cys Prxs, which respectively lack and contain the C_R (Figure 4). 2-Cys Prxs, containing conserved C_P and C_R, are classified in typical, which are homodimeric, and atypical, which are monomeric (Liebthal et al., 2018) (Figure 4). The C_P-SOH of the typical 2-Cys Prx reacts with the C_R-SH of the other subunit, or the same subunit in the case of atypical 2-Cys Prxs, thus forming an inter- or intra-molecular disulphide bridge, respectively. In order to regenerate the active form of the enzyme, an appropriate electron donor is needed, which usually is Thioredoxin (Trx) or Glutaredoxin (Grx), and, in chloroplast, NADPH thioredoxin reductase C (NTRC). In 1-Cys Prxs, lacking the C_R, the C_P-SOH intermediate is reduced by other proteins or small thiol molecules such as GSH or AsA.

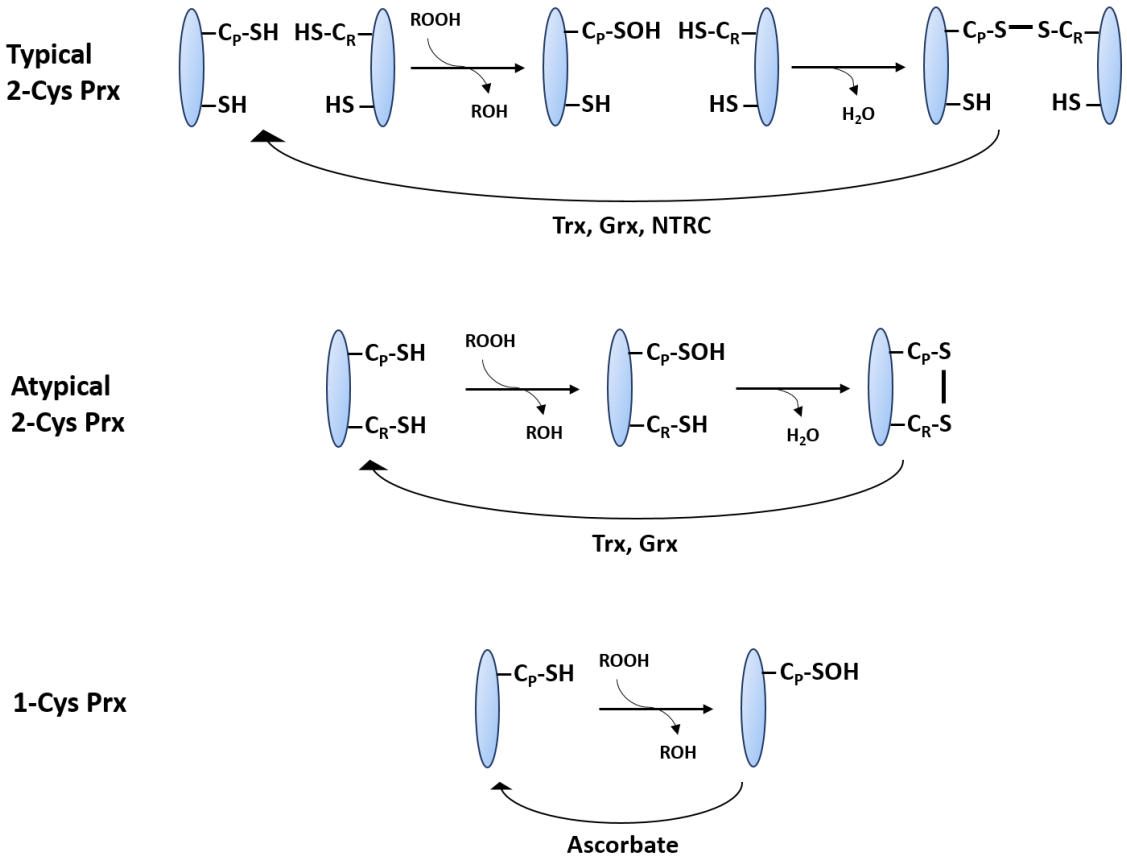

Figure 4. Reaction mechanism of the different types of Peroxiredoxins. The reaction mechanism of Prxs consists of three steps: (i) Reduction of the peroxide (ROOH) and generation of the sulfenic acid form of C_P (C_P-SOH). (ii) Resolution of disulphide by C_R (C_R-SH) and water release. (iii) Regeneration of the thiol by an appropriate electron donor. Typical 2-Cys Prxs are homodimeric, the C_P-SOH from one subunit is resolved by the C_R-SH of the adjacent subunit, forming an inter-molecular disulphide bridge. Atypical 2-Cys Prxs are monomeric, the C_R-SH, located in the same subunit forms an intra-molecular disulphide bridge with C_P-SOH. In the regeneration step, 2-Cys Prxs are reduced by specific redox transmitters such as thioredoxins (Trxs) and glutaredoxins (Grxs), or, in the case of chloroplast, NTRC. For 1-Cys Prx, the C_P-SOH directly reacts with a thiol or another reductant, such as ascorbate.

In plants, the first Prx identified was a 1-Cys Prx from barley (Aalen et al., 1994; Stacy et al., 1996). Afterwards, it was reported that in *Arabidopsis thaliana* Prxs are encoded by 10 genes (Horling et al., 2002). Chloroplasts are the organelles with a higher content of Prxs, which include two almost identical typical 2-Cys Prxs (A and B), and atypical Prxs Q and IIE (Liebthal et al., 2018). Genetic analyses

performed in *Arabidopsis* have shown that knock-down (Pulido et al., 2010) and knock-out (Awad et al., 2015) 2-Cys Prxs mutants show a slight retarded growth phenotype, as compared to the wild type plants. Likewise, single mutants with decreased levels of Prx Q (Lamkemeyer et al., 2006) or Prx IIE (Romero-Puertas et al., 2007) also display an almost wild-type phenotype. Overall, these results suggest that the absence of a particular type of Prx may be compensated for by the remaining Prxs of the organelle.

In plants, 2-Cys Prx are one of the most abundant proteins in the chloroplast stroma (Peltier et al., 2006). During the catalytic cycle of 2-Cys Prxs, the sulfenic form (C_P–SOH) is susceptible to further oxidation to the sulfinic form (C_P-SO_2H) (Yang et al., 2002) (Figure 5). This process, known as overoxidation, lead to the inactivation of the peroxidase function of the enzyme and the formation of high molecular weight oligomers that display chaperone activity (Jang et al., 2004). With the exception of some cyanobacteria (Pascual et al., 2010), sensitivity to overoxidation occurs in 2-Cys Prxs from eukaryotic organisms (Wood et al., 2003b), suggesting that this feature is a gain-of-function of eukaryotic organisms, which allows local increase of the concentration of H_2O_2 for signalling purposes, this is the so-called floodgate hypothesis (Wood et al., 2003b). As overoxidation of the enzyme at high H_2O_2 concentrations would allow the excess H_2O_2 to function as a redox signal, 2-Cys Prxs are considered as primary H_2O_2 sensors (Puerto-Galán et al., 2013). The overoxidation of 2-Cys Prxs is not irreversible, as it was initially proposed. An ATP-dependent reaction catalysed by sulfiredoxin (Srx) is able to convert the C_P-SO_2H into the C_P-SOH form (Biteau et al., 2003; Woo et al., 2003) (Figure 5). In plants, Srx is encoded by a single gene (Liu et al., 2006) and the protein shows dual localisation in chloroplast and mitochondria (Iglesias-Baena et al., 2011). The study of *Arabidopsis* mutants lacking Srx (Lia et al., 2006) established the implication of the enzyme in the response to photoxidative stress (Rey et al., 2007). Moreover, the coupling between circadian oscillation and 2-Cys Prx overoxidation has been shown in mammalian (O´Neil and Reddy, 2011) and algal (O'Neill et al., 2011) cells. However, 2-Cys Prx overoxidation in plant chloroplasts seems to respond to light rather than to circadian oscillations (Puerto-Galán et al., 2015; Cerveau et al., 2016). In addition, it should be taken into account that the inactivation of 2-Cys Prxs by overoxidation would provoke

lower oxidation of their targets, also leading to a rise in H_2O_2 levels (Veal et al., 2018).

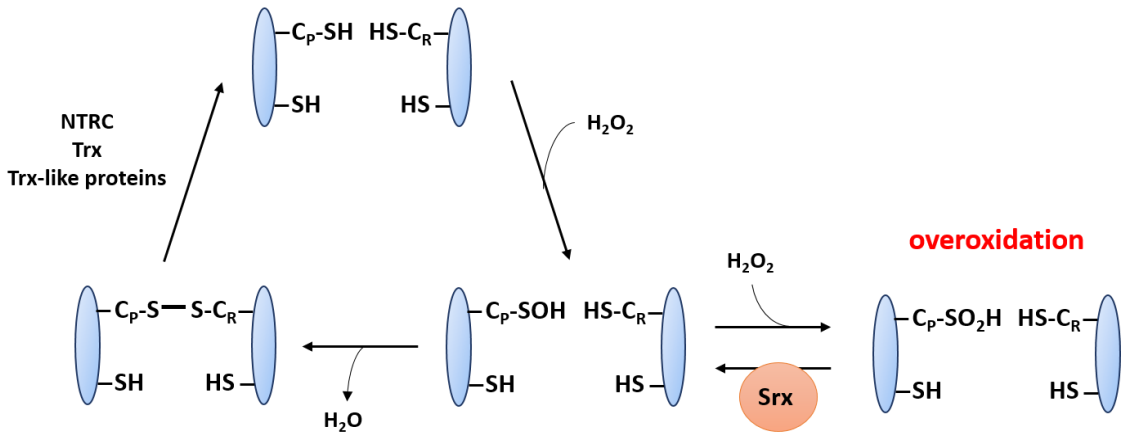

Figure 5. The mechanism of overoxidation of typical 2-Cys Prx in chloroplast. The thiol group of the peroxidatic Cys (C_P) of one subunit is oxidized to sulfenic (C_P-SOH) after H_2O_2 reduction. The resolving Cys (C_R) of the other subunit reacts with the C_P-SOH to generate the inter-molecular disulphide bridge. The action of NTRC, thioredoxins (Trx) or Trx-like proteins regenerate the reduced form of the enzyme in chloroplast. Alternatively, the sulfenic intermediate may be overoxidized to sulfinic (C_P-SO_2H), which can be retro-reduced by sulfiredoxin (Srx).

4. Chloroplast redox regulation

It is well-known that plants have the ability to adapt their metabolism to unpredictable changes in light availability. In this context, thiol based redox regulation plays a central role in modulating metabolic pathways, being a key universal strategy that allows cells to adapt to changing environmental conditions (Balsera et al., 2014). Redox regulation depends on the extraordinary properties of the thiol group of Cys and is defined as a reversible post-translational modification (PTM), consisting in a disulphide-dithiol interchange. The reduction of disulphide bridges in redox-regulated proteins is mainly controlled by the protein disulphide reductase activity of Trx and Grx (Meyer et al., 2012).

4.1. Thioredoxins

The first Trx, an enzyme with protein disulphide reductase activity, was identified in *Escherichia coli* (Laurent et al., 1964). Trxs are low molecular weight proteins (12-14 kDa), with a characteristic tridimensional structure, the so-called Trx fold, which consists of β-sheets surrounded by α-helices, having a canonical catalytic motif with the sequence WCGPC located on a highly conserved fold at the periphery of the protein (Collet and Messens 2010). The catalytic mechanism of Trxs consists in the transfer of electrons from the thiolic groups of the two Cys in their active site to the disulphide in the substrate protein. After a catalytic cycle, the disulphide of the target protein is reduced and the active site Cys of the Trx form a disulphide bond (Collet and Messens 2010) (Figure 6). For a new catalytic cycle, this disulphide needs to be reduced in a reaction that depends on NADPH through a specific reductase named NADPH Trx reductase (NTR) (Figure 6). This redox couple, known as the NTR/Trx system, is universally distributed in all types of organisms, including bacteria, fungi, animals and plants (Meyer et al., 2012). Similar to Trxs, Grxs mediate the reduction of disulphide bridges in redox-regulated proteins, however, these enzymes are reduced by GSH releasing oxidized glutathione (GSSG), which is itself reduced by glutathione reductase (Meyer et al., 2012). Contrary to Trxs, relatively few studies have been dedicated to study the functions of chloroplast Grxs.

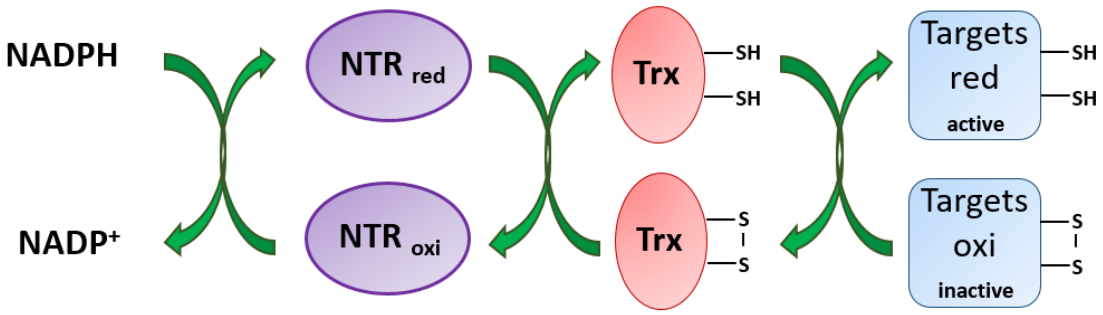

Figure 6. Electron flow from NAPDH to oxidized proteins via the NTR/Trx system. Oxidized target proteins are reduced by Trx. As a result, active site Trx Cys residues form a disulphide bond. Trx regeneration relies on NADPH with the participation of an NADPH-dependent Trx reductase (NTR). red, reduced; oxi, oxidized.

Redox regulation exerts a relevant role in plants. It was proposed that chloroplast acquisition by plant cells resulted in an increased production of ROS, and this led to the increase of Cys residues, expanding the redox protein network (Woehle et al., 2017). Indeed, the number of Trxs present in heterotrophic organisms such as *E. coli* (2), *S. cerevisiae* (3) or humans (2) is small compared to autotrophic organisms. For instance, the *Arabidopsis* genome encodes more than 20 Trxs isoforms, which are distributed in different cell compartments (Meyer et al., 2012; Geigenberger et al., 2017; Nikkanen et al., 2017; Thormählen et al., 2018). Among them, *h*-type Trxs constitute a heterogeneous group divided into three subtypes (I, II and III). Trxs *h* of type I (Trx *h*1, *h*3, *h*4 and *h*5), type II (*h*2, *h*7 and *h*8) and type III (*h*9, *h*10 and atypical CxxS1-2) are located mainly in the cytosol, but also in the nucleus, mitochondria and endomembrane systems (Hägglund et al., 2016). Trxs *o*1 and *o*2 are located in mitochondria (Laloi et al., 2001) and nucleus (Martí et al., 2009). Besides, Nucleoredoxins, Nrx1 and Nrx2, have dual nuclear and cytosolic location (Marchal et al., 2014). On other hand, chloroplasts contain a diverse set of Trxs, accounting for half the total Trx isoforms in *Arabidopsis* cells. The ten chloroplast Trx isoforms are divided into five types *f*1-2, *m*1-4, *x*, *y*1-2 and *z* and their functional roles on chloroplast metabolism has been well analyzed (Meyer et al., 2012; Geigenberger et al., 2017; Nikkanen et al., 2017; Thormählen et al., 2018). There are also atypical Trxs in chloroplasts, the function of them being less well known. These include the 6 isoforms of the atypical Cys/His-rich Trx (ACHT) family (Dangoor et al., 2009), the chloroplastic drought-induced stress protein of 32 kDa (CDSP32) (Rey et al., 1998), the transmembrane high chlorophyll fluorescence 164 protein (HCF164) (Motohashi and Hisabori, 2006) and the two isoforms of Thioredoxin-like proteins (TrxL), which contain non-canonical WCRKC redox sites (Cain et al., 2009; Chibani et al., 2009).

4.2. Thioredoxin reductase systems in plants

Plants harbour different Trx reductase systems that reside in cell compartments (Jacquot et al., 2009). Similar to heterotrophic organisms, plants possess flavin-containing NTRs, NTRA (Laloi et al., 2001) and NTRB (Jacquot et

al., 1994), which use NADPH as source of reducing power and are predominantly localized in the mitochondria and cytosol, respectively. In addition, chloroplasts harbour a second type of Trx reductase, an iron-sulfur protein named Fdx Trx reductase (FTR), which uses Fdx as source of reducing power (Buchanan et al., 1967; Wolosiuk and Buchanan 1977; and reviewed by Buchanan 2016). The FTR/Trx system connects light availability to redox regulation of chloroplast metabolism (Figure 7). Thus, under illumination, photosynthesis consists in the transport of electrons through the photosystems to reduce Fdx. Then, the reduced Fdx [2Fe-2S]$^{2+}$ cluster acts as electron-donor, through the FTR [4Fe–4S] cluster, to reduce the disulphide bridge of a Trx, which acts as electron-acceptor (Schürmann and Buchanan 2008). Once reduced, Trxs interact with conserved disulphides of target enzymes, which modify their enzyme activity upon reduction. While the molecular basis of the light-dependent reduction of redox-regulated chloroplast enzymes has been well described, how these enzymes become rapidly oxidized upon darkness remains unknown. In this context, the participation of H_2O_2 in this oxidative deactivation has been proposed (Kaiser, 1979; Tanaka et al., 1982).

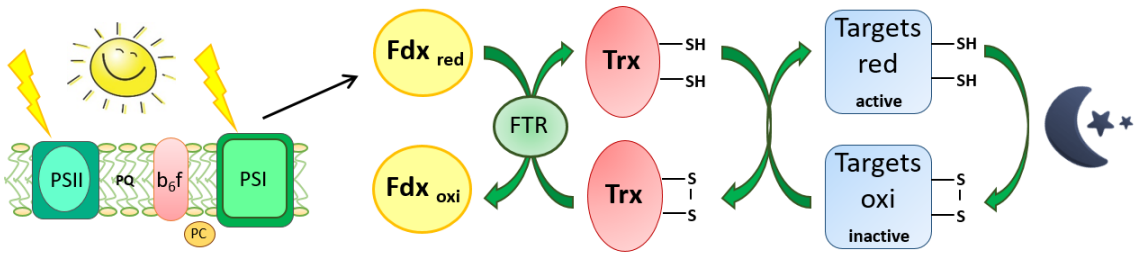

Figure 7. FTR/Trx system in chloroplasts. During the night, chloroplast biosynthetic enzymes are oxidized and inactive. During the day, the light signal is transmitted sequentially through the chloroplast electron transport chain to reduce Fdx. In turn, Fdx_{red} transfers electrons to reduce Trxs via an Fdx Trx reductase (FTR). Target proteins are subsequently reduced by Trx, restoring enzyme activity. red, reduced; oxi, oxidized.

The classical notion that chloroplast redox regulation relies exclusively on reduced Fdx (Fdx $_{red}$), was modified by the discovery of NTRC by our group (Serrato et al., 2002; Serrato et al., 2004). This enzyme is a new type of NTR,

exclusively found in oxygenic photosynthetic organisms, including plants, algae and some cyanobacteria (Pascual et al., 2010; Nájera et al., 2017). In plants, this novel enzyme is located in all types of plastids (Kirchsteiger et al., 2012), being relatively abundant in the chloroplast stroma (Serrato et al., 2004). NTRC is a bimodular enzyme, composed of a Trx-like domain fused to the C-terminus of an NTR domain (Serrato et al., 2004). Remarkably, it was reported that NTRC efficiently reduces 2-Cys Prx, thus integrating both NTR and Trx activities in a single polypeptide (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007) (Figure 8). The active form of NTRC is a homodimer arranged in a head-to-tail conformation, which uses NADPH as source of reducing power (Pérez-Ruiz and Cejudo 2009; Bernal-Bayard et al., 2012) (Figure 8). Therefore, NADPH electrons are transferred via the FAD cofactor and the disulphide at the NTR domain of one of the subunits to the disulphide of the Trx domain of the other subunit. It is noteworthy that the NADPH/NADP$^+$ ratio in the chloroplast might determine the reductant capacity of NTRC and, consequently, the redox regulation of its targets (Spinola et al., 2008).

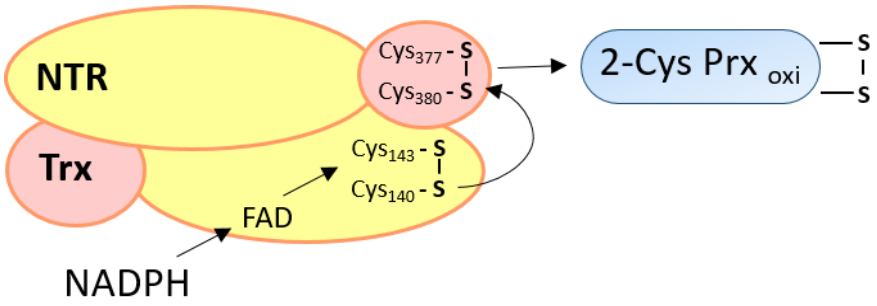

Figure 8. Proposed reaction mechanism for NTRC. The minimal catalytic form of NTRC is a homodimer. Each subunit of NTRC contains an NTR and a Trx domain. In the reaction mechanism, the electron transfer pathway involves NADPH, the FAD cofactor and the disulphide of the NTR domain of one of the subunits. Then electrons are transferred to the disulphide of the Trx domain of the other subunit, which interacts with the 2-Cys Prx.

Chloroplasts thus contain two redox systems, the classic FTR/Trx, which relies on photo-reduced Fdx and connects redox regulation of target enzymes to light, and the more recently discovered NTRC, which relies on NADPH (Figure 9). Plants absorb light energy during the day, hence increasing the levels of Fdx_{red} due to the photosynthetic electron transport chain. During the night, when photosynthetic electron transport chain ceases, the level of Fdx_{red} decreases; however, NADPH is still produced from glucose 6 phosphate (glucose-6P) by the oxidative pentose phosphate pathway (OPPP). Under these conditions, NTRC may become the most relevant redox system in the chloroplast (Cejudo et al., 2012).

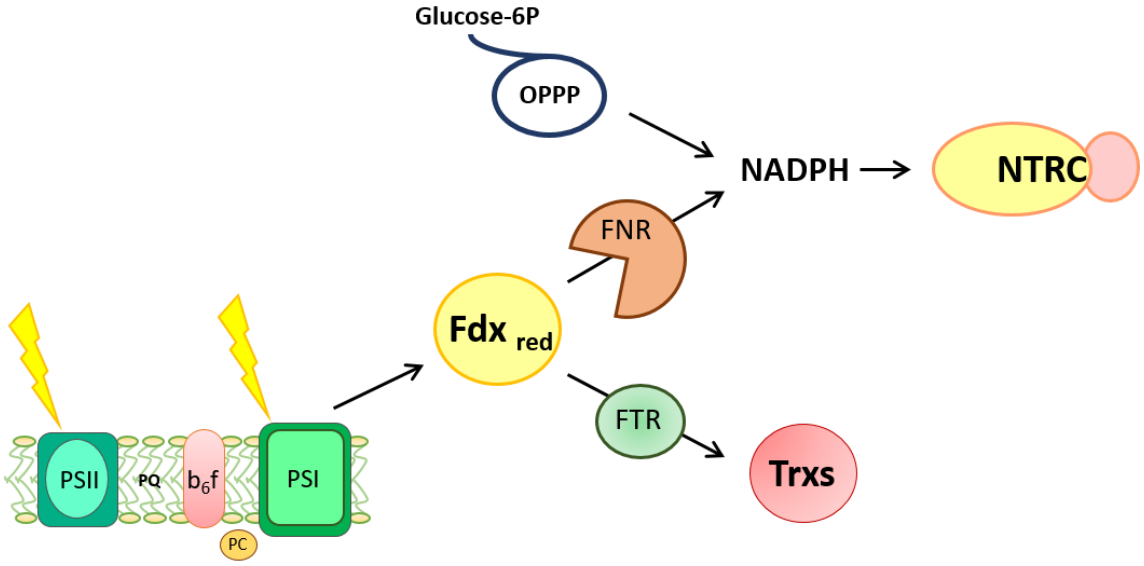

Figure 9. Chloroplast redox regulation: FTR/Trx and NTRC systems. Under light conditions, reduced ferredoxin (Fdx_{red}) serves as the source of reducing power for the FTR/Trx system. Besides, NADPH is generated by Ferredoxin NADPH reductase (FNR), which uses the electrons from Fdx_{red} to reduce $NADP^+$. Thus, NADPH can be used as source of reducing power through the NTRC system. In addition, NADPH is also produced from glucose 6-phosphate (Glucose-6P) by the oxidative pentose phosphate pathway (OPPP). Therefore, the NTRC redox system can be operative during the day and during the night.

4.3. Targets of the chloroplast thioredoxin systems

As stated above, chloroplast redox regulation is performed by two systems, FTR/Trx and NTRC, which maintain the redox status of a large set of enzymes and allow cell adjustments to fluctuations in environmental conditions (Geigenberger et al., 2017; Thormählen et al., 2018). During the last few years, the knowledge of redox-regulated processes in chloroplasts has increased considerably.

4.3.1 Targets of the FTR/Trx system

Initial biochemical experiments (reviewed by Buchanan, 2016) showed that four of the CBC enzymes are regulated by light via Trxs (Michelet et al., 2013) (Figure 2): phosphoribulokinase (PRK) (Wolosiuk and Buchanan, 1978), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Wolosiuk and Buchanan, 1978), fructose-1,6-bisphosphatase (FBPase) (Wolosiuk and Buchanan, 1977) and sedoheptulose-1,7-bisphosphatase (SBPase) (Breazeale et al., 1978). Subsequently, the first identified plant Trxs, Trx *f* and *m,* were proposed to regulate FBPase and NADP-dependent malate dehydrogenase (NADP-MDH), respectively (Jacquot et al., 1978). The availability of the *Arabidopsis* genome allowed the identification of additional chloroplast Trxs in higher plants, uncovering the complexity of redox regulation in this organelle. Pioneering *in vitro* studies were carried out in order to determine the targets for each Trx isoform (Collin et al., 2003; Collin et al., 2004). Additionally, proteomics analyses have identified a large number of putative targets of Trxs (Montrichard et al., 2009). Identified proteins participate in metabolic processes such as starch metabolism, chlorophyll biosynthesis, cyclic electron transport or chloroplast protein import, besides antioxidant processes and plastid gene expression (Geigenberger et al., 2017). These findings thus extend the relevance of chloroplast redox regulation far beyond the CBC. Moreover, while past research was focused on biochemical studies, the study of *Arabidopsis thaliana* loss of function mutants has enabled to increase the knowledge of this complex thiol redox network in plants. Surprisingly, under normal light conditions routinely used for growth in chambers, mutant lines deficient in a particular chloroplast Trx

type showed either wild type or weak mutant phenotypes (Courteille et al., 2013; Thormählen et al., 2013; Wang et al., 2013; Pulido et al., 2010; Laugier et al., 2013; Yoshida et al., 2015; Naranjo et al., 2016a).

Trxs *f* and *m* have been predominantly associated to the regulation of metabolic pathways. The original *in vitro* observation that Trx *f* is involved in regulation of CBC enzymes (Jacquot et al., 1978; Collin et al., 2003) was supported by the analysis of *Arabidopsis* plants devoid of Trxs *f*1 and *f*2, which present altered levels of reduction of these enzymes in the light (Yoshida et al., 2015; Naranjo et al., 2016). In this regard, it was later shown that *m* type Trxs, have also a relevant function in the light-dependent redox regulation of CBC enzymes (Okegawa and Motohashi, 2015). Trxs *m*1, *m*2 and *m*4, but not *m*3, were originally reported to activate NADP-MDH *in vitro* (Jacquot et al., 1978; Collin et al., 2003) and the study of *Arabidopsis trxm1* and *trxm2* single mutants confirmed the participation of these enzymes in this process (Thormählen et al., 2017). Notably, plants deficient in Trx *m*3, the less abundant *m*-type Trx (Okegawa and Motohashi, 2015), showed unaffected chloroplast performance but impairment in symplastic protein transport, which affects meristem development (Benitez-Alonso et al., 2009). Other enzymes not belonging to the CBC, such as the redox-regulated γ-subunit of the ATP synthase (CF_1-γ) (McKinney et al., 1978), are also regulated by Trxs *f* and *m* (Okegawa and Motohashi, 2015). Besides, it has been reported that deficiency in Trx *m*4 impairs the cyclic electron transport (Courteille et al., 2013) whereas altered biogenesis of PSII was observed in *Arabidopsis* lines silenced simultaneously in the three Trx *m* genes (Wang et al., 2013). Moreover, there are evidences that both Trxs, *f* and *m*, regulate the chlorophyll biosynthetic enzyme Mg-chelatase (Mg-CHL) (Luo et al., 2012) and Trx *f* controls ADP-glucose pyrophosphorylase (AGPase), involved in starch biosynthesis (Geigenberger et al., 2005; Thormählen et al., 2013; Thormählen et al., 2015). Although further *in vivo* experimental confirmation is needed, there are other metabolic processes that are expected to be regulated by Trxs *f* and *m*, such as lipid biosynthesis (Sasaki et al., 1997; Yoshiki et al., 2006), the shikimate pathway (Entus et al., 2002) and protein import processes (Bartsch et al., 2008). Finally, it should be taken into account that a few Trx-regulated chloroplast enzymes are inactive in

its reduced form. For instance, the glucose-6P dehydrogenase, a key enzyme of the OPPP, is reductively inactivated by Trx *f* (Née et al., 2009).

Trx *x* and *y* functions are mainly related with enzymes involved in oxidative stress response. Thus, Trx *x* shows better efficiency reducing 2-Cys Prxs *in vitro* than other chloroplast Trxs (Collin et al., 2003; Collin et al., 2004). However, the redox balance of 2-Cys Prxs is not impaired in plants devoid of Trx *x* (Pulido et al., 2010). In addition to Trx *x*, chloroplast atypical Trxs such as CDSP32 (Broin et al., 2002), ACHT (Dangoor et al., 2012; Eliyahu et al., 2015) and TrxL2 (Yoshida et al., 2018) have been shown to reduce 2-Cys Prxs *in vitro*. On the other hand, Trxs *y* are efficient electron donors for Prx Q (Collin et al., 2004) and Gpx (Navrot et al., 2006). Besides, Trxs *y* are associated with regulation of methionine sulfoxide reductases (MSRs), which regenerate methionine from methionine sulfoxide (Vieira Dos Santos et al., 2007). This function of Trxs *y* was confirmed by the analysis of *Arabidopsis* plants lacking Trx *y*1 and *y*2, which showed attenuated protein repair mechanisms in leaves due to impaired redox activation of MSR (Laugier et al., 2013).

Finally, the notion that the deficiency of a Trx type leads to no or minor effects on plant phenotype is not valid for the type *z*. The analysis of plants lacking Trx *z*, which show an albino phenotype and severely impaired growth, indicated that this enzyme participates in plastid transcription by regulating, via a yet unknown mechanism, the plastid-encoded RNA polymerase (PEP) (Arsova et al., 2010). However, this effect seems to be independent of redox activity (Wimmelbacher and Bornke, 2014). Furthermore, *in vitro* biochemical analyses suggest that Trx *z* might also be involved in stress response, supporting the activity of Prx Q, Gpx and MSR proteins (Chibani et al., 2011). Finally, the mechanism of reduction for Trx *z* generates controversy since Trx *z* was reported to be reduced by FTR in poplar (Chibani et al., 2011) and by other Trx types in *Arabidopsis* (Bohrer et al., 2012).

4.3.1 Targets of the NTRC system

The relevance of redox regulation performed by NTRC was initially revealed by the analysis of an *Arabidopsis* line deficient in this redox system (Serrato et al., 2004). The *ntrc* mutant, knock-out for NTRC, presents a pleiotropic phenotype consisting in retarded growth and pale-green leaves with less chlorophyll content than the wild-type (Serrato et al., 2004). The *ntrc* phenotype is dependent on light availability, being more pronounced under short-day conditions (Lepistö et al., 2009), and fluctuating light intensities (Thormählen et al., 2017). Besides, the *ntrc* mutant shows decreased CO_2 fixation activity, especially at low light intensities, and alteration of chloroplast structure with an irregular distribution of mesophyll cells (Pérez-Ruiz et al., 2006; Lepistö et al., 2009). In addition, plants lacking NTRC show increased sensitivity to different abiotic stresses, such as salinity (Serrato et al., 2004), prolonged darkness (Pérez-Ruiz et al., 2006) or heat (Chae et al., 2013), and biotic stress (Ishiga et al., 2012). Furthermore, *ntrc* plants show high NPQ levels concomitant with a decreased photosynthetic performance, which means that NTRC is required for an efficient light energy utilization (Naranjo et al., 2016b). Overall, these phenotypes suggest that NTRC has an important physiological role in the context of chloroplast redox regulation.

Bimolecular fluorescence complementation (BiFC) and isothermal titration calorimetry (ITC) have shown that both NTRC and Trx *x* interact with 2-Cys Prx *in vivo* and *in vitro*, respectively (Bernal-Bayard et al., 2014). However, as stated above, NTRC is the most efficient reductant of 2-Cys Prx, showing higher catalytic efficiency than CDSP32 or Trx *x* (Pérez-Ruiz et al., 2006, Bernal-Bayard et al., 2014). This notion was also confirmed by the *in vivo* analysis of the 2-Cys Prxs redox state, which was severely impaired in *ntrc* but not in *trxx* plants (Pulido et al., 2010). Moreover, plants simultaneously devoid of NTRC and Srx, which catayzes the reversion of the over-oxidized form of 2-Cys Prx, essentially showed an *ntrc* phenotype, indicating that the activity of NTRC, rather than Srx, controls the function of 2-Cys Prx (Puerto-Galán et al., 2015). Overall, these evidences establish that the redox balance of 2-Cys Prx and the signalling activities of the enzyme mainly depends on NTRC (Figure 5).

In addition to its role in antioxidant metabolism, further studies have shown that NTRC, complementary to the FTR/Trx system, regulates metabolic processes. The characteristic pale-green phenotype of the *ntrc* mutant indicates that the chlorophyll biosynthesis pathway requires the activity of NTRC (Serrato et al., 2004). Thus, it has been reported that NTRC is involved in the regulation of tetrapyrrole biosynthesis enzymes, such as the glutamyltransfer RNA reductase GluTR1, CHLM (Richter et al., 2013) and the Mg-CHLI (Pérez-Ruiz et al., 2014). Moreover, the *ntrc* mutant shows decreased starch content, thus the participation of NTRC in the redox regulation of AGPase and, consequently, in starch synthesis in response to sugars in the dark has been also reported (Michalska et al., 2009; Lepistö et al., 2013). In addition, it was shown that NTRC interacts *in vivo* with FBPase, PRK and CF_1-γ, being all of them well-established Trx targets (Nikkanen et al., 2016). Finally, *in vitro* experiments in *Chlamydomonas reinhardtii* suggest that NTRC could also participate in the regulation of chloroplast gene translation (Schwarz et al., 2012).

4.4. Interaction between FTR/Trx and NTRC redox systems

Redox regulation affects most of the processes that occur in the chloroplast, being a regulatory mechanism essential for the rapid adaptation of plants performance to ever changing light conditions. The co-existence of two thiol-based redox systems in the chloroplast, controlling common targets, is intriguing and raises the question of whether these systems show specificity for their targets or have overlapping regulatory effects (Fig. 10). Therefore, a key question concerns the functional relationship between NTRC and the FTR/Trx system in chloroplasts. This issue has been addressed through the analysis of *Arabidopsis* mutants combining the deficiency of NTRC and Trx or FTR (Thormählen et al., 2015; Yoshida and Hisabori 2016; Da et al., 2017). Altogether, the results obtained confirm a coordinated action between NTRC and the other plastidial Trxs. However, further studies are needed to determine the specific elements that link redox regulation mediated by these systems.

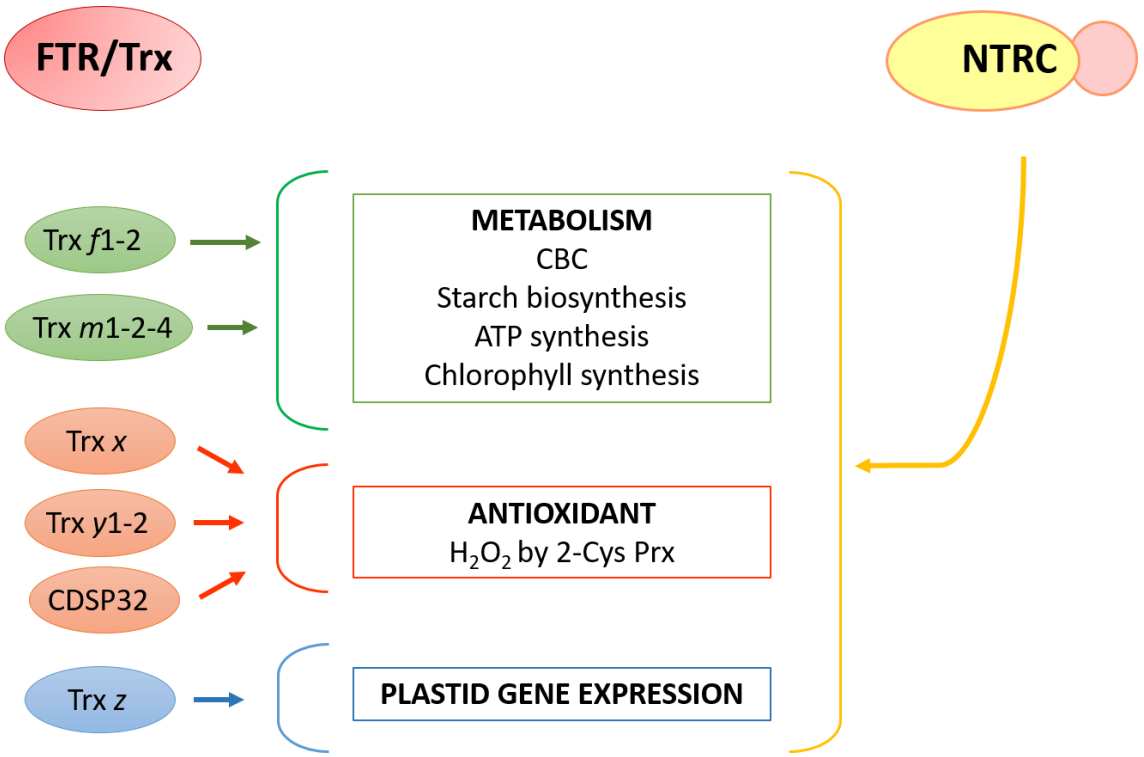

Figure 10. Summary of the functions of FTR/Trx and NTRC systems in chloroplast redox regulation. There are two thiol-based redox systems in chloroplasts: FTR/Trx and NTRC. The scheme represents chloroplast redox processes, which are controlled by NTRC and different Trx isoforms.

OBJECTIVES

OBJECTIVES

The aim of this thesis was to explore the molecular basis of redox regulation of chloroplast in response to light availability. To this end, the following specific objectives were carried out:

1. To elucidate the functional relationship between the two redox systems FTR/Trx and NTRC of the chloroplast
2. To unravel the role of 2-Cys Prxs in chloroplast redox homeostasis.
3. To investigate the mechanism of chloroplast enzyme oxidation in the dark.

These objectives were addressed by a combination of genetic, biochemical and physiological approaches using *Arabidopsis thaliana* as model system.

SUMMARY OF RESULTS

SUMMARY OF RESULTS

Redox regulation based on disulphide-dithiol exchange is a universal regulatory mechanism that allows the rapid adaptation of cell metabolism to the environment. In heterotrophic organisms, redox regulation relies in a two-component system, NTR/Trx, each of these enzymes being encoded by a low number of genes, at most three. In clear contrast, redox regulation in plant chloroplasts shows a high complexity. Besides the large number of chloroplast-localized Trxs, up to 20 in *Arabidopsis*, this organelle harbours two redox systems: the FTR/Trxs, based on photo-reduced Fd, and NTRC, based on NADPH. In this thesis, we have addressed the functional relationship between these two redox systems for chloroplast enzyme regulation and the impact of these systems on plant development and response to light availability. The results of this thesis have been published in different research articles. Thus, the content of this dissertation is structured in three sections (I, II and III), which contain the different articles. The summary of the results of these sections is as follows:

Section I. Functional interaction between the FTR/Trx and NTRC redox systems

Chapter 1 **Ojeda, V.**, Pérez-Ruiz, J.M., González, M., Nájera, V.A., Sahrawy, M., Serrato, A.J., Geigenberger, P., Cejudo, F.J. (2017). NADPH thioredoxin reductase C and thioredoxins act concertedly in seedling development. Plant Physiology 174 (3): 1436-1448. doi: 10.1104/pp.17.00481

Chapter 2 **Ojeda, V.**, Nájera, V.A., González, M., Pérez-Ruiz, J.M., Cejudo, F.J. (2017). Photosynthetic activity of cotyledons is critical during post-germinative growth and seedling establishment. Plant Signaling & Behavior. 12 (9): e-1347244. doi: 10.1080/15592324.2017

To address the functional relationship between the two redox pathways of chloroplasts, NTRC and FTR/Trx, we performed a combination of genetic, biochemical and physiological approaches using Arabidopsis as model system. Given the large number of Trxs in Arabidopsis chloroplasts, in this work we focused on Trxs *x* and *f*, which were chosen as representatives of antioxidant and metabolic functions, respectively. Previous work of the group established that the *trxx* (Pulido et al., 2010) and *trxf1f2* (Naranjo et al., 2016a) mutants show phenotypes similar to the wild type when grown under long-day photoperiod, whereas the *ntrc* mutant shows growth-retard and pale-green phenotype (Serrato et al., 2004). Thus, to analyse whether or not there is functional relationship between NTRC and Trxs *x* or *f*, in this study we have generated *Arabidopsis* mutants combining the deficiencies of NTRC and Trx *x* (the *ntrc-trxx* double mutant) and NTRC and Trxs *f* (the *ntrc-trxf1f2* triple mutant).

Remarkably, the *ntrc-trxx* and, to a higher extent, the *ntrc-trxf1f2* mutant showed altered chloroplast structure and impaired photosynthetic performance, leading to a very severe growth inhibition phenotype. Moreover, these mutations also cause a severe impairment in the light-dependent redox regulation of FBPase, a well-known redox-regulated enzyme of the CBC. Interestingly, *in vitro* experiments showed that FBPase is efficiently reduced by Trxs *f*1 and *f*2, and at lower efficiency by Trx *x*, but not by NTRC. Consequently, the deficiency of NTRC affects the redox state of FBPase indirectly. Finally, the *ntrc-trxx* and, to a higher extent, the *ntrc-trxf1f2* seedlings showed delayed formation of the first true leaves and impaired root growth, indicating that chloroplast redox regulation plays a relevant function in seedling establishment.

Altogether, our results indicate that NTRC is essential for the activity of functionally unrelated Trxs, thus playing a key role in chloroplast redox regulation, which is critical during early stages of plant development.

Section II. The NTRC/2-Cys Prx system modulates the activity of chloroplast Trxs

Chapter 3 Pérez-Ruiz, J.M, Naranjo, B., **Ojeda, V.**, Guinea, M., Cejudo, F.J. (2017). NTRC-dependent redox balance of 2-Cys peroxiredoxins is needed for optimal function of the photosynthetic apparatus. Proceedings of the National Academy of Sciences of the US. 114 (45): 12069-12074. doi: 10.1073/pnas.1706003114

Chapter 4 **Ojeda, V.**, Pérez-Ruiz, J.M., Cejudo, F.J. (2018) The NADPH-dependent thioredoxin reductase C-2-Cys peroxiredoxin redox system modulates the activity of thioredoxin x in Arabidopsis chloroplasts. Plant and Cell Physiology. 59 (10): 2155-2164. doi: 10.1093/pcp/pcy134

The pleiotropic effect of the lack of NTRC on plant growth indicates that this enzyme performs a central role in chloroplast redox regulation, however, the molecular basis of the function of NTRC remains unknown. The source of reducing power for NTRC is NADPH, which allows the efficient reduction of 2-Cys Prxs (Perez-Ruiz et al., 2006), hence suggesting an antioxidant function for NTRC. In addition, it has been shown the participation of NTRC in the redox regulation of processes previously known to be regulated by Trxs. These evidences suggest that both antioxidant and regulatory functions might be interconnected. In order to study this issue, we analysed Arabidopsis lines combining mutations in NTRC and 2-Cys Prxs (the *ntrc-Δ2cp* mutant). Surprisingly, the *ntrc-Δ2cp* mutant, despite lacking NTRC and having severely decreased levels of antioxidant 2-Cys Prxs, displayed a growth phenotype similar to wild type plants. These results indicate that the deficiency of 2-Cys Prxs exerts a suppressor effect of the *ntrc* phenotype. In line with these results, the dramatic growth inhibition phenotype of the *ntrc-trxx* and the *ntrc-trxf1f2* mutants, were also supressed by decreased contents of 2-Cys Prxs. Thus, the phenotypes of the mutants *ntrc-Δ2cp, ntrc-trxx-Δ2cp* and *ntrc-trxf1f2-Δ2cp* show a significant recovery of growth rate, photosynthetic performance and light-

dependent reduction of Trx-regulated enzymes. On the contrary, transgenic plants overexpressing 2-Cys Prxs in the *ntrc* background showed an aggravated growth retardation phenotype.

The characterization of this suppressor effect led us to propose a novel model for chloroplast redox regulation. This model establishes that NTRC controls the redox balance of 2-Cys Prxs, which maintains the reducing capacity of the pool of Trxs and, consequently, proper regulation of targets enzymes. Thus, we propose that the activities of the FTR/Trx and NTRC redox systems are integrated by the redox balance of the 2-Cys Prxs, which controls the redox regulatory network of the chloroplast.

Section III. The role of 2-Cys Prxs in the oxidation of chloroplast enzymes in the dark

Chapter 5 **Ojeda, V.**, Pérez-Ruiz, J.M., Cejudo, F.J. (2018). 2-Cys peroxiredoxins participate in the oxidation of chloroplast enzymes in the dark. Molecular Plant. 11(11):1377-1388. doi: 10.1016/j.molp.2018.09.005

After the discovery of the light-dependent reduction of enzymes of the CBC, extensive biochemical and genetic analyses have led to a comprehensive knowledge of the molecular basis of this regulatory mechanism. While it is equally known that these enzymes become rapidly oxidized upon darkness, the mechanism of oxidative deactivation is yet unknown. We have proposed that under illumination 2-Cys Prxs transfer oxidative equivalents to Trxs, hence playing a key role in maintaining the reducing capacity of the pool of Trxs. This finding prompted us to analyse the possibility that 2-Cys Prxs could participate in enzyme oxidation in the dark. To address this possibility, we have focused on well-known redox regulated enzymes of the CBC, such as FBPase, GAPDH, and the γ-subunit of ATPase. In addition, Arabidopsis lines with altered contents of the NTRC/2-Cys Prx system were generated. These lines include the double mutant, *2cpab,* devoid of the two 2-Cys

Prxs present in *Arabidopsis*, as well as transgenic plants that overexpress NTRC in a wild type background. Interestingly, these lines show delayed oxidation of redox-regulated chloroplast enzymes in light-to-dark transitions, indicating that both the absence of 2-Cys Prxs or high levels of NTRC negatively affect chloroplast enzyme oxidation upon darkness. To study the involvement of other thiol-dependent peroxidases in this oxidation process, we generated mutants combining decreased contents of Prx IIE or Prx Q with the lack of 2-Cys Prxs. Both, the *2cpab-prxIIE* and *2cpab-prxQ* mutants displayed a rate of enzyme oxidation in light-to-dark transitions which was similar to that observed in the *2cpab* mutant, indicating that neither Prx Q nor Prx IIE participate in dark-mediated oxidation. Moreover, we developed a biochemical approach to reconstitute the oxidative pathway *in vitro*. These assays indicated that reducing equivalents are transferred from reduced FBPase to H_2O_2 via Trx *f*1 and 2-Cys Prxs, thus indicating the participation of Trxs as intermediates in enzyme oxidation in the dark.

Overall, our results allow to establish the significant participation of 2-Cys Prxs in the short-term oxidation of chloroplast enzymes in dark, uncovering cover the key role of H_2O_2 as the final sink of the electrons.

SECTION I

Functional interaction between the FTR/Trx and NTRC redox systems

CHAPTER 1

NADPH thioredoxin reductase C and thioredoxins act concertedly in seedling development

Ojeda, V., Pérez-Ruiz, J.M., González, M., Nájera, V.A., Sahrawy, M., Serrato, A.J., Geigenberger, P., Cejudo, F.J. (2017). NADPH thioredoxin reductase C and thioredoxins act concertedly in seedling development. Plant Physiology 174 (3): 1436-1448. doi: 10.1104/pp.17.00481

CHAPTER 2

Photosynthetic activity of cotyledons is critical during post-germinative growth and seedling establishment

Ojeda, V., Nájera, V.A., González, M., Pérez-Ruiz, J.M., Cejudo, F.J. (2017). Photosynthetic activity of cotyledons is critical during post-germinative growth and seedling establishment. Plant Signaling & Behavior. 12 (9): e-1347244. doi: 10.1080/15592324.2017

SECTION II

The NTRC/2-Cys Prx system modulates the activity of chloroplast Trxs

CHAPTER 3

NTRC-dependent redox balance of 2-Cys peroxiredoxins is needed for optimal function of the photosynthetic apparatus

Pérez-Ruiz, J.M, Naranjo, B., **Ojeda, V.**, Guinea, M., Cejudo, F.J. (2017). NTRC-dependent redox balance of 2-Cys peroxiredoxins is needed for optimal function of the photosynthetic apparatus. Proceedings of the National Academy of Sciences of the US. 114 (45): 12069-12074. doi: 10.1073/pnas.1706003114

CHAPTER 4

The NADPH-dependent thioredoxin reductase C-2-Cys peroxiredoxin redox system modulates the activity of thioredoxin *x* in *Arabidopsis chloroplasts*

Ojeda, V., Pérez-Ruiz, J.M., Cejudo, F.J. (2018) The NADPH-dependent thioredoxin reductase C-2-Cys peroxiredoxin redox system modulates the activity of thioredoxin x in Arabidopsis chloroplasts. Plant and Cell Physiology. 59 (10): 2155-2164. doi: 10.1093/pcp/pcy134

SECTION III

The role of 2-Cys Prxs in the oxidation of chloroplast enzymes in the dark

CHAPTER 5

2-Cys peroxiredoxins participate in the oxidation of chloroplast enzymes in the dark

Ojeda, V., Pérez-Ruiz, J.M., Cejudo, F.J. (2018). 2-Cys peroxiredoxins participate in the oxidation of chloroplast enzymes in the dark. Molecular Plant. 11(11):1377-1388. doi: 10.1016/j.molp.2018.09.005

ANNEX

ANNEX

Background

Light is the most important environmental stimulus for plant development, exerting an important effect on the regulation of a large number on enzymes in order to optimize metabolism in response to light availability. The process of photosynthesis, which takes place in chloroplasts, allows plants to use light energy to fix CO_2 and synthesize carbohydrates. However, as photosynthesis involves the transport of electrons in the presence of oxygen, it inevitably generates ROS, such as $^1O_2$, O_2^-, H_2O_2 and $^{\cdot}OH$ (Dietz et al., 2016). The redox changes of photosynthetic electron transport components occur immediately in response to variations of incident light intensities. For example, isolated chloroplasts release H_2O_2 immediately upon high light exposition (Mubarakshina et al., 2010). Part of the H_2O_2 produced inside the chloroplasts can diffuse out of the organelle, escaping the effective antioxidant systems, and acting outside the chloroplast (Mubarakshina et al., 2010). Thus, the ROS signalling network in light acclimation is triggered from distinct subcellular compartments, activating light responses on variable time scales, and inducing specific response patterns (Dietz, 2015). These ROS, which might produce damage in the cell components, have also an important signalling function, being necessary for multiple metabolic, physiological and developmental processes (Waszczak et al., 2018).

2-Cys Prxs are a ubiquitous family of thiol-dependent peroxidases that show a very efficient H_2O_2 scavenging activity (Liebthal et al., 2018). In plant chloroplasts, NTRC is the most efficient reductant of these enzymes (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007). The results of this thesis (sections II and III) show the relevant role of 2-Cys Prxs in chloroplast redox homeostasis and suggest that H_2O_2 may exert a key function to control the redox state of chloroplast enzymes in response to light availability. Thus, an important aspect of this work was to determine the levels of H_2O_2. Different methodologies have been used to measure the levels of H_2O_2, although it is technically challenging in plant tissue extracts (Noctor et al., 2016). In fact, there is no a general agreement on the production rate and concentration of H_2O_2 in different compartments of plant cells (Queval et al.,

2008). Spin trapping assays with 4-POBN coupled to electron paramagnetic resonance (EPR) (Janzen et al., 1978) allow to indirectly determine levels of H_2O_2. This approach is based on the Fenton reaction between H_2O_2 generated in plant tissues and the ferrous FeEDTA complex, which releases $^{\cdot}OH$. This radical reacts with 4-POBN/ethanol and forms a stable organic specie, which can be detected by EPR spectroscopy (Michelet and Krieger-Liszkay, 2012). It should be noted that this measurement provides information about changes in the content of H_2O_2 of the whole cell, instead of the chloroplast subcellular compartment. In our attempt to establish the role that H_2O_2 plays in the context of redox homeostasis of the chloroplast, an internship was performed in the laboratory of Dr. Anja Krieger-Liszkay at the Institute for Integrative Biology of the Cell, Commissariat à l'Energie Atomique et aux Energies Alternatives in Saclay (France). The objective of this internship was to take advantage of the methodologies available in this laboratory to analyse the content of H_2O_2 in the different *Arabidopsis* mutants affected in the NTRC/2-Cys Prx system. The mutants analysed during this stay are: *Δ2cp* (Pulido et al., 2010), knock down for 2-Cys Prx A and knock out for 2-Cys Prx B; *2cpab* (Chapter 5), knock out for both 2-Cys Prx A and B; *ntrc* (Serrato et al., 2004), knock out for NTRC; and *ntrc-Δ2cp* (Chapter 3), combining decreased level of 2-Cys Prxs with the lack of NTRC. Moreover, assays to measure activities of antioxidant enzymes superoxide dismutase (SOD), guaiacol peroxidase and catalase were carried out with the aim of testing whether or not other enzymatic antioxidant systems are altered in *Arabidopsis* lines affected in NTRC/2-Cys Prx system.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana wild-type (ecotype Columbia) and mutant plants were routinely grown in soil in growth chambers under short-day (8h of light/16h of darkness) at 22 °C and 20 °C during light and dark periods, respectively, and light intensity of 125 μE m^{-2} s^{-1}.

Spin-Trapping Electron Paramagnetic Resonance Measurements

Spin-trapping assays with α-(4-Pyridyl *N*-oxide)-*N*-*tert*-butylnitrone (4-POBN) (Sigma-Aldrich) were carried out using leaf pieces from the different lines, essentially as in Michelet and Krieger-Liszkay (2012). Leaf pieces (approx. 25 mg) were manual vacuum-infiltrated with the spin-trap buffer (50 mM 4-POBN, 50 μM Fe-EDTA and 4% ethanol, in 10 mM NaH_2PO_4/Na_2HPO_4 pH 7) and incubated during 30 min in the dark or under growth light (125 μE m^{-2} s^{-1})

EPR spectra were recorded at room temperature in a standard quartz flat cell using an E-Scan spectrometer (Bruker, Rheinstetten, Germany). The following parameters were used: microwave frequency, 9.73 GHz; modulation frequency, 86 kHz; modulation amplitude, 1G; microwave power, 4.45 mW; receiver gain, 5 x 10^2; time constant, 40.96 ms; number of scans, 4. Signals were normalized to leaf weight.

Enzyme activity assays

Leaves from *Arabidopsis* lines grown in short-day conditions were ground in liquid nitrogen before homogenization in extraction buffer (1.5 mM $MnCl_2$, 1 mM EGTA, 1 mM EDTA and 15 mM NaCl in 50 mM HEPES, pH 7.2). The samples were filtered through miracloth, kept in ice 5 min and centrifuged at 5000 *g* at 4°C for 5 min. The supernatants (crude extracts) were transferred to a new eppendorf tube. The protein concentrations were determined following an Amido Black procedure described in Schaffner and Weissmann (1973). Superoxide dismutase (SOD), guaiacol peroxidase and catalase activities were measured essentially as in Mollins

et al. (2013). The activity per mg of protein was normalized giving 100% value to the corresponding one in the wild-type.

In brief, SOD activity was determined spectrophotometrically using xanthine/xanthine oxidase as superoxide (O_2^-) generating system and measuring the O_2^- production based on reduction of the tetrazolium dye Na,3′-(1-[phenylaminocarbonyl]-3,4-tetrazolium)-bis-(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) at 470 nm (ε470 = 24.2 $mM^{-1}cm^{-1}$). A stock solution of xanthine (500 μM) was prepared in water by adding 1 M NaOH until it dissolved. The final assay contained 50 μM xanthine, 100 μM XTT and 0.2 $U \cdot mL^{-1}$ xanthine oxidase from bovine milk in 20 mM HEPES pH 7.0. The kinetics of O_2^- production were measured as an increase in absorbance at 470 nm, and the SOD activity was determined by following the inhibition of the O_2^- production after the addition of the crude extract. Guaiacol peroxidase activity was determined spectrophotometrically by measuring the oxidation of guiacol to tetraguaiacol at 470 nm (ε_{470} = 26.6 $mM^{-1}cm^{-1}$). The reaction mixture contained 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.5, 3 mM H_2O_2. 0.01% (v/v) guaiacol and crude extract. Catalase activity was measured polarographically at 20 °C with a Clark-type electrode in 50 mM HEPES pH 8.0, in the presence of 1 mM H_2O_2 as substrate and crude extract. The final concentration of crude extract in all the assays was 10 $\mu g \cdot mL^{-1}$.

Results and Discussion

In plant cells, chloroplasts are among the most important organelles in terms of ROS generation (Foyer and Noctor, 2003). Chloroplast ROS production is dependent of light intensity and photoperiod. Thus, the generation of ROS is higher in leaves from *Arabidopsis* (Lepistö et al., 2013) and tobacco (Michelet and Krieger-Liszkay, 2012) plants grown under short-day than under long-day photoperiod. Therefore, we chose short-day growth conditions to perform the analysis of the content of H_2O_2 and antioxidant enzyme activities in mutant plants with alterations in the NTRC/2-Cys Prx system. *Arabidopsis* NTRC deficient plants show a characteristic phenotype, consisting in lower chlorophyll content and growth retard as compared to the wild-type (Serrato et al., 2004), which is extremely severe under short-day conditions (Lepistö et al., 2009) (Figure 1). Indeed, under short-day conditions, leaves of the mutant *ntrc* present a heterogeneous phenotype, exhibiting green-old and yellowish-young leaves, which, show lower or higher NPQ levels, respectively (Naranjo et al., 2016b). The *ntrc* mutant grown under short-day conditions presents smaller and fewer chloroplasts than the wild-type (Lepistö et al., 2009). Moreover, chloroplast ultrastructure in the *ntrc* mutant ranges from those with wild-type appearance to those with different degrees of morphological alterations (Lepistö and Rintamäki, 2012; Lepistö et al., 2012). On the other hand, mutant plants altered in 2-Cys Prxs contents show a less severe phenotype than the *ntrc* mutant (Figure 1). While the *Δ2cp* mutant is similar to wild-type plants (Pulido et al., 2010), the *2cpab* mutant shows a slight growth inhibition phenotype (Chapter 5, Figure S2). Furthermore, the mutant *ntrc-Δ2cp*, containing decreased levels of 2-Cys Prx and lacking NTRC, resembles the wild-type phenotype, thus presenting a suppressed *ntrc* phenotype, (Chapter 3, Figure S2).

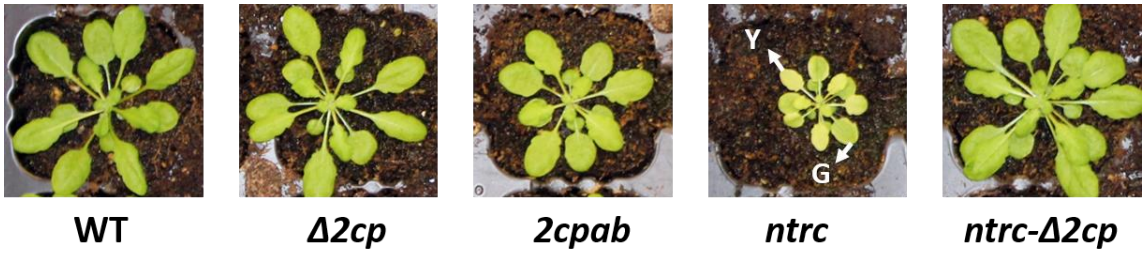

Figure 1. The phenotype of *Arabidopsis* mutants affected in the NTRC/2-Cys Prx system. Plants of wild-type and *Δ2cp*, *2cpab*, *ntrc* and *ntrc-Δ2cp* mutant lines grown under short-day conditions (8h of light/16h of darkness, light intensity of 125 μE m^{-2} s^{-1}) during 40 days. Arrows refer to green (G) and yellowish (Y) leaves.

Detection of EPR signals from H_2O_2-derived ·OH shows that the release of H_2O_2 occurs immediately after high light exposition in isolated thylakoids from tobacco (Heyno et al., 2009), isolated thylakoids and isolated chloroplasts from spinach (Mubarakshina et al., 2010), tobacco (Michelet and Krieger-Liszkay, 2012) and *Arabidopsis* (Lepistö et al., 2013) leaves. Hence, we took advantage of this technique to examine the content of ·OH in leaves from *Arabidopsis* wild-type, *Δ2cp*, *2cpab*, *ntrc* (green and yellowish leaves), and *ntrc-Δ2cp* mutants, grown under short-day conditions (Figure 2). At the end of the night, plants deficient in 2-Cys Prxs present similar contents of ·OH compared to the wild-type, whereas *ntrc* adult leaves show lower content and the *ntrc* young leaves present a higher content of ·OH. These results confirm the heterogeneous leaf phenotype of the *ntrc* mutant and the physiological relevance of the protein in the different developmental stages of the plant. Previously, determination of H_2O_2 by potassium iodide assays in *ntrc* plants after a prolonged dark treatment showed a content of H_2O_2 slightly higher than that of the wild-type, although upon reillumination a substantial increase in the accumulation of H_2O_2 was observed (Perez-Ruiz et al., 2006). At the end of the day, lines deficient in 2-Cys Prxs show higher contents of ·OH than the wild-type. Likewise, a slightly increase is observed in *ntrc* young leaves, whereas *ntrc* adult leaves show similar content of ·OH than the wild-type. In line with these results, previous reports have described that the *ntrc* mutant, without any distinction between the different types of leaves, presents higher contents of ·OH than the wild-type (Lepistö et al., 2013; Naranjo et al., 2016). Interestingly, *ntrc* young leaves show lower contents of ·OH at the end of day compared to the end of the night, whereas

the rest of the lines present higher contents of ·OH during the day. In any case, it should be noted that differences in the contents of ·OH between the night and the day are only significant in *2cpab* mutant. This result is in agreement with a previous report of H_2O_2 content in *2cpab* determined by homovanillic acid fluorescence assay (Awad et al., 2015).

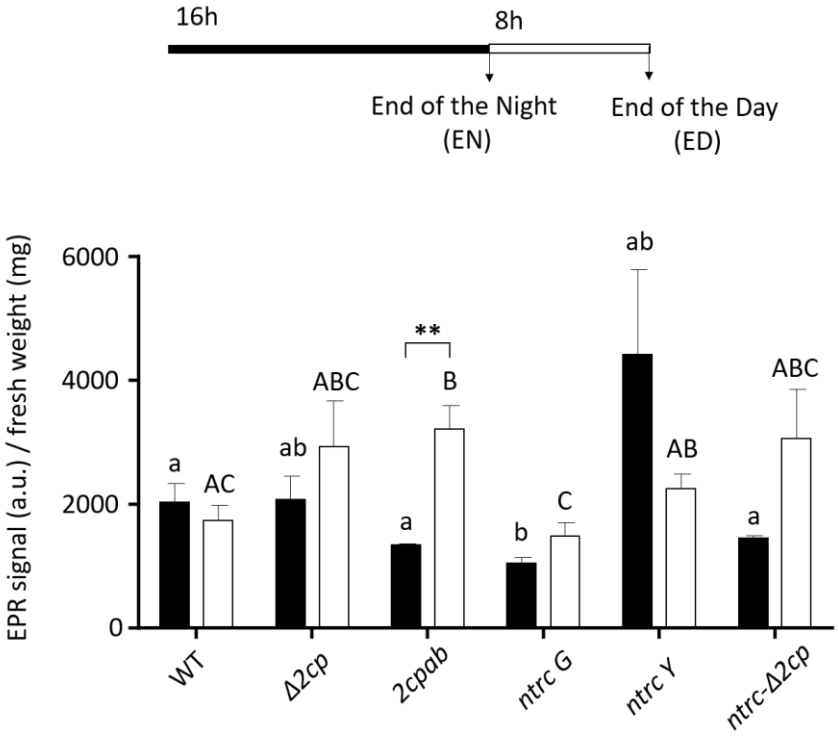

Figure 2. H_2O_2-derived ·OH production based on EPR signals in wild-type and *Arabidopsis* mutants deficient in NTRC and 2-Cys Prxs. The contents of ·OH, indirectly measured by spin trapping assays with 4-POBN/ethanol in the presence of Fe-EDTA, was determined in detached leaves from wild-type and the indicated mutants grown under short-day conditions. Samples were taken at the end of the night (EN) (black bars) or at the end of the day (ED) (white bars). Data are represented as average values of at least four biological replicates ± standard error (SE). Letters indicate significant differences between lines in the same photoperiod time, EN (lower case letters) or ED (upper case letters), with the Student´s *t* test at a 95% confidence interval. Besides, statistical significance (**P*<0.05 and ***P*<0.01) determined with the Student´s *t* test show the comparison between EN and ED values for each line.

Plants with decreased levels of 2-Cys Prxs, *Δ2cp* and *ntrc-Δ2cp*, show a growth phenotype similar to the wild type plants. To test if other chloroplast antioxidant systems might compensate for the deficit of 2-Cys Prxs, the expression levels of additional chloroplast peroxidases were determined in these lines (Chapter 3, Figure S8) and no differences were observed compared with wild-type. Moreover, to test if non-enzymatic antioxidant systems, such as AsA and GSH, are altered in the whole cell, the levels of these molecules were also determined (Chapter 3, Table S1) and no differences were observed. Several reports have shown that H_2O_2 generated in different organelles, such as chloroplast, diffuse to the cytosol and other organelles by its permeability through biological membranes (Mubarakshina et al., 2010; Exposito-Rodriguez et al., 2017; Sousa et al., 2019). Consequently, there might be a coordinated function of H_2O_2 signaling networks in the whole cell, although details of how such interactions work are still unknown (Waszczak et al., 2018). Overall, our results suggest that the suppressor phenotype of *ntrc-Δ2cp* is not due to a compensation of additional antioxidant systems. Nevertheless, plants deficient in 2-Cys Prxs, such as *Δ2cp, 2cpab, ntrc-Δ2cp*, show higher levels of H_2O_2 in leaves during the day compared with wild-type (Figure 2). Thus, in order to test if other enzymatic antioxidant systems are altered in these lines, we analyzed enzymatic activities that affect H_2O_2 levels such as SOD, which generates H_2O_2, and guaiacol peroxidase and catalase, which detoxify H_2O_2 (Figure 3). The SOD activity was lower in plants deficient in 2-Cys Prxs, especially in *Δ2cp* and *ntrc-Δ2cp* lines, compared with wild-type, whereas the *ntrc* mutant presents a subtle increase of SOD activity. The guaiacol peroxidase activity, measured as the oxidation of guaiacol to tetraguaiacol, was significantly increased in the *ntrc* mutant, which could be a consequence of the higher content of H_2O_2 in this line (Figure 2, Lepistö et al., 2013; Naranjo et al., 2016b). Catalase activity is similar in all the studied lines. Therefore, these results show that compensatory effects of enzymatic antioxidants in plants devoid of 2-Cys Prx could be excluded.

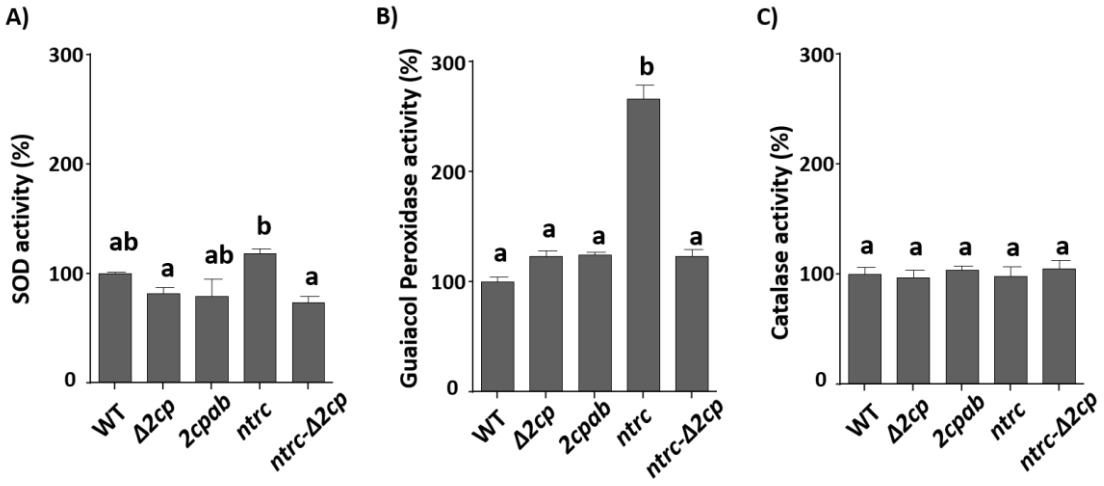

Figure 3. Antioxidant enzyme activities of total protein extracts from leaves of wild-type and *Arabidopsis* mutants deficient in NTRC and 2-Cys Prxs. SOD (A), catalase (B) and guaiacol peroxidase (C) activities were determined in crude extracts from wild-type and the indicated mutants grown under short-day conditions. All activities were normalized to the corresponding value in the wild-type, which was arbitrarily considered 100%. Data are represented as average values of three biological replicates ± standard error (SE). Letters indicate significant differences between lines with the Student´s *t* test (A) and by Tukey test (B, C) at 99% confidence interval.

GENERAL DISCUSSION

GENERAL DISCUSSION

Plants are sessile organisms and, as such, have to respond rapidly to environmental changes, especially to different light availability conditions. Chloroplast redox regulation, based on disulphide-dithiol interchange, is responsible for controlling the activity of a large number of proteins involved in different metabolic pathways. These organelles contain two different redox systems, FTR/Trx and NTRC, however, the interaction between them remains unknown. The central objective of this thesis is to unravel the molecular basis of the functional interaction between these two redox systems. To that end, we have used a combination of genetic, biochemical and physiological approaches in the model plant *Arabidopsis thaliana.*

1. NTRC acts in concert with the FTR/Trx system to sustain chloroplast performance

The classical view of chloroplast redox regulation is based on the activity of the FTR/Trx system, which relies on reducing power from Fdx_{red} in the presence of light. Compared with heterotrophic organisms, which contain at most three Trx isoforms, chloroplast harbours a complex network formed by up to 20 Trxs and Trx-like proteins. In order to determine the specific function of each chloroplast Trx and to identify putative Trx targets, *in vitro* experiments (Collin et al., 2003; Collin et al., 2004) and mass spectrometry analysis in conjunction with trap-techniques (Montrichard et al., 2009) have been reported. As a result of these studies, it has generally been assumed that Trx of the types *m* and *f* are involved in redox regulation of metabolic pathways whereas those of types *y* and *x* have antioxidant function. Most of these studies are based on biochemical analyses, however, the possibility to perform *in vivo* functional studies with *Arabidopsis thaliana* loss-of-function mutants have enabled a great increase of the knowledge of the complex thiol redox network in plant chloroplasts. Remarkably, *Arabidopsis* mutants deficient in a single type of Trx show minor phenotypic effect, as compared to the wild type plants. This is the case of *Arabidopsis* mutants devoid of Trx *f*1 (Thormählen et al., 2013) or both Trxs *f*, 1 and 2 (Yoshida et al., 2015; Naranjo et al.,

2016a), single mutants deficient in Trxs *m*1, *m*2 or *m*4, (Courteille et al., 2013) or lines with simultaneous silencing of these three *TRX m* genes (Wang et al., 2013), the knock out mutant for Trx *x* (Pulido et al., 2010) and for Trxs *y* (Laugier et al., 2013). These results suggest either the functional redundancy between the different types of Trxs or that additional chloroplast redox systems might compensate for the deficiency of these Trxs. On the contrary, the deficiency of Trx *z* generates a severe albino phenotype associated to impaired chloroplast transcription, thus compromising chloroplast biogenesis (Arsova et al., 2010). However, it is not clear whether the role of Trx *z* in the expression of plastid encoded genes is redox-dependent (Wimmelbacher and Bornke, 2014).

The discovery of NTRC, which integrates both NTR and Trx activities in a single protein and shows high affinity for NADPH (Serrato et al., 2004), modified the classical view of chloroplast redox regulation based exclusively on Fdx_{red}. While the deficiency of different types of chloroplast Trxs has a little effect on plant growth, plants devoid of NTRC show a clear phenotype of growth inhibition and a characteristic pale-green leaves phenotype (Serrato et al., 2004). Besides, the *ntrc* mutant shows a drastic impairment on photosynthetic performance, including higher NPQ, lower Fv/Fm and ETR (Carrillo et al., 2016; Naranjo et al., 2016b), and lower rate of CO_2 fixation (Pérez-Ruiz et al., 2006) than the wild-type plants. These results show that NTRC has an important physiological relevance in chloroplast redox regulation. Further studies indicated the participation of NTRC in different processes, which were previously shown to be regulated by Trxs, such as chlorophyll (Richter et al., 2013; Pérez-Ruiz et al., 2014) or starch (Michalska et al., 2009; Lepistö et al. 2013) biosynthesis, besides the antioxidant function as the main reductant of 2-Cys Prx (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007). Altogether, these results suggest overlapping functions of the FTR/Trx and NTRC redox systems in plant chloroplasts. Therefore, a central issue in understanding chloroplast redox regulation is to determine if an interaction between these two redox systems exists.

In the work shown in the first section of this thesis, we addressed this issue by performing a comparative analysis of Arabidopsis mutants combining the deficiencies of NTRC and two Trxs, *x* or *f*, with unrelated functions. Severe effects on

the chloroplast structure (Chapter 1, Fig. 5; Chapter 2, Fig. 1) and lower content of photosynthetic components (Chapter 1, Fig. 6) have been observed in both *ntrc-trxx* and *ntrc-trxf1f2* mutants. It should be noted that these mutants show a large number of plastoglobules, which are indicative of oxidative stress (Austin et al., 2006), suggesting that impairment of chloroplast redox regulation affects the plant response to stress. Consequently, these mutations resulted in a very dramatic growth inhibition phenotype, much more severe than that observed in the *ntrc* mutant, which indicates that this effect is more than additive (Chapter 1, Fig. 1). Simultaneous deficiencies in both redox systems generate a severe effect on the plant, which is in agreement with the clear decrease of light energy utilization efficiency (Chapter 1, Fig. 4). In line with this notion, it has been reported that silencing the expression of genes encoding Trxs *m*1, *m*2 *and m*4 in the *ntrc* background results in a similar dwarf phenotype (Da et al., 2017). Finally, an *Arabidopsis* double mutant impaired in the catalytic subunit of FTR and NTRC displays a lethal phenotype (Yoshida and Hisabori, 2016). Altogether, these results show the concerted action of the FTR/Trx and NTRC in chloroplast redox regulation.

Although most of the studies in photosynthesis redox regulation have focused on the adult phase of development, a critical stage in plant life occurs after germination, when seedling must reach autotrophy before the seed storage is consumed (Kircher and Schopfer, 2012). We observed a high mortality of *ntrc-trxx* and *ntrc-trxf1f2* lines grown on soil, coinciding with the time of true-leaves appearance (Chapter 1, Fig. 7). Although a low number of these individuals reach the adult stage, they are able to complete the life cycle and produce seeds. These results show the relevance of an appropriate redox regulation of photosynthesis of cotyledon chloroplasts, which has a deep effect on plant development. This notion was supported by root growth assays in synthetic medium, as mutant seedlings deficient in both FTR/Trx and NTRC redox systems showed impaired root growth, which was recovered by addition of sucrose (Chapter 1, Fig. 8), indicating that sucrose produced by the photosynthetic activity of cotyledons is crucial for seedling establishment.

A possibility to explain the concerted action of the NTRC and the FTR/Trxs redox systems is that both modulate the activity of common redox-regulated targets.

In support of this idea, it was previously reported that NTRC interacts *in vivo* with PRK, FBPase and the γ-subunit of the ATP synthase, all these enzymes being well-established Trx targets (Nikkanen et al., 2016). We have addressed this issue by analysing the redox state of FBPase, a CBC enzyme mainly regulated by Trxs *f* (Michelet et al., 2013), in plants deficient in FTR/Trxs and NTRC redox systems. As expected, *Arabidopsis* mutant deficient in Trxs *f* showed diminished light-dependent reduction of FBPase (Chapter 1, Fig. 2), as previously reported (Yoshida et al., 2015; Naranjo et al., 2016a). However, the level of light-dependent reduction of FBPase in the *trxx* mutant was intriguingly similar to that observed in the *trxf1f2* mutant (Chapter 1, Fig. 2), despite the fact that CBC enzymes are not considered as targets of Trx *x* (Collin et al., 2003). The level of the light-dependent reduction of FBPase in the *ntrc* mutant was even more affected than in the *trxx* and *trxf1f2* mutants, while reduction of FBPase was essentially undetectable in *ntrc-trxx* and *ntrc-trxf1f2* mutants (Chapter 1, Fig. 2). Hence, these results support the notion that both types of Trxs (*f* and *x*) and NTRC act concertedly in the redox regulation of FBPase. That is, the deficiency of one of the two redox systems may be compensated by the other, while simultaneous deficiency of both systems drastically affects the redox regulation of FBPase. To test this possibility, we analysed the effect of Trxs *x*, *f* and NTRC on the redox state of FBPase *in vitro* with the corresponding purified proteins. While Trx *f*1 and Trx *f*2 show a high efficiency for FBPase reduction *in vitro*, Trx *x* shows lower efficiency and NTRC was unable to reduce the enzyme (Chapter 1, Fig. 3). Therefore, NTRC is important for light-dependent FBPase reduction and for the activity of functionally unrelated Trxs, as shown by the *in vivo* analyses, yet the effect of NTRC is exerted indirectly, as shown by the *in vitro* results.

2. The NTRC/2-Cys Prx system modulates the activity of the chloroplast Trxs

2-Cys Prxs are thiol peroxidases, which reduce H_2O_2 to water (Liebthal et al., 2018) and are one of the most abundant proteins of the chloroplast stroma (Peltier et al., 2006). Although 2-Cys Prxs are reduced by the different chloroplast Trxs, the most efficient reductant of 2-Cys Prxs is NTRC and, based on these results, it was

initially proposed an antioxidant function for this enzyme (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007). In the work shown in the second section of this thesis, we have studied the implication of 2-Cys Prxs in chloroplast redox regulation by analysing the genetic interaction of the three elements: NTRC, Trxs and 2-Cys Prxs.

Arabidopsis chloroplasts contain two almost identical 2-Cys Prxs, A and B. The *Arabidopsis Δ2cp* mutant, knock down for 2-Cys Prx A and knock out for 2-Cys Prx B, contains approx. 5% of the contents of 2-Cys Prxs of the wild type, which does not significantly affect plant growth (Pulido et al., 2010). Interestingly, the *ntrc-Δ2cp* triple mutant, combining deficiencies of NTRC and 2-Cys Prxs, recovers the wild-type phenotype, thus the phenotype of the *ntrc* mutant is suppressed by low levels of 2-Cys Prxs (Chapter 3, Fig. 1). This suppressor effect depends on the dose of 2-Cys Prx, isoforms A and B having indistinguishable effect (Chapter 3, Fig. 1). The suppression of the *ntrc* phenotype is higher as the contents of 2-Cys Prxs decreases; thus, lines *ntrc-2cpb, ntrc-2cpaGK,* and *ntrc-Δ2cp*, which have lower contents of 2-Cys Prxs, show a phenotype more similar to the wild type than the line *ntrc-2cpa,* which has a higher dose of 2-Cys Prx (Chapter 3, Fig. 1). Moreover, transgenic lines overexpressing 2-Cys Prxs, A or B, in the *ntrc* background, but not in wild-type background, show an aggravation of the *ntrc* phenotype (Chapter 3, Fig. S3). This result indicates that increasing contents of 2-Cys Prxs in the absence of NTRC becomes detrimental for plants. In line with these phenotypes, the light-dependent reduction of Trx *f* is altered in *ntrc* but restored in the *ntrc-Δ2cp* mutant (Chapter 3, Fig. 3). Likewise, the redox state of FBPase and PRK, redox regulated CBC enzymes, is also altered in the *ntrc* mutant and restored in the suppressed line (Chapter 3, Fig. 3). In addition, the *ntrc-Δ2cp* suppressed line recovers wild type levels of chlorophyll biosynthesis enzymes (Richter et al., 2018), indicating that the suppressor effect caused by decreased levels of 2-Cys Prxs is not exclusive of CBC enzymes. It was previously established that the *in vivo* redox state of 2-Cys Prx is imbalanced in the absence of NTRC, which provokes the accumulation of the oxidized form of the enzyme (Kirchsteiger et al., 2009; Pulido et al., 2010; Puerto-Galán et al., 2015). Moreover, the reduction of 2-Cys Prxs observed in the *ntrc* mutant occurs in the light, but not in darkness (Chapter 3, Fig. 3), suggesting that 2-Cys Prxs use reducing power from the pool of Trxs in absence of NTRC. Based on these results, we

hypothesized that NTRC may regulate, via 2-Cys Prxs, the reducing capacity of Trxs and, consequently, the redox balance of its chloroplast targets. This proposal provides an explanation for the effect of NTRC on such a large variety of chloroplast processes that are regulated by Trxs. For instance, the redox state of FBPase, which is not reduced *in vitro* by NTRC (Chapter 1, Fig. 3), is altered in mutants lacking NTRC (Chapter 1, Fig. 2; Chapter 3, Fig. 3) and restored in the *ntrc-Δ2cp* mutant (Chapter 3, Fig. 3). No changes were observed in the non-enzymatic, AsA or GSH (Chapter 1, Table S1), or enzymatic antioxidants systems, guaiacol peroxidase, catalase or SOD (Annex, Figure 3), nor in the expression levels of other chloroplast peroxidases (Chapter 3, Figure S8). Thus, these results, support the notion that the suppressor effect is due to a decrease of the withdrawal of electrons from the pool of Trxs, instead of compensation by additional antioxidant mechanisms. Therefore, we propose that the NTRC/2-Cys Prx system modulates the activity of chloroplast Trxs, hence the redox regulation of chloroplast enzymes.

Arabidopsis mutants simultaneously lacking NTRC and Trxs *x* or *f* show a very severe growth inhibition phenotype (Section I). Surprisingly, this dramatic effect is also supressed by decreased 2-Cys Prxs contents. The *Arabidopsis* quadruple *ntrc-trxx-Δ2cp* (Chapter 4) and quintuple *ntrc-trxf1f2-Δ2cp* (Chapter 3) mutants show a significant recovery of growth rate (Chapter 3, Fig. 4; Chapter 4, Fig. 1), photosynthetic efficiency (Chapter 3, Fig. 4; Chapter 4, Fig. 3 and Table 1) and light-dependent reduction of chloroplast enzymes (Chapter 3, Fig. 4; Chapter 4, Fig. 5). Likewise, the effect of the dose of 2-Cys Prxs is confirmed by the aggravation of the growth phenotypes in transgenic plants which overexpress 2-Cys Prx A in the *ntrc-trxx-Δ2cp* (Chapter 4, Fig. 4) or *ntrc-trxf1f2-Δ2cp* (Chapter 3, Fig. S3) backgrounds. It is noteworthy that the regulatory action of the NTRC/2-Cys Prx system acts over functionally unrelated Trxs such as those of the *x*- and *f*- types. Besides, the fact that tetrapyrrole biosynthesis is severely affected in *Arabidopsis* mutants combining the deficiencies of NTRC and Trxs *m* (Da et al., 2017), suggests that the NTRC/2-Cys Prxs system might also affect the function of *m*- type Trxs. Altogether, these results show that the severe growth phenotype observed in these mutants, deficient in both NTRC and FTR/Trx redox systems, is caused by an imbalanced chloroplast redox network.

Our results suggest that 2-Cys Prxs could act as a sink for electrons from different chloroplast Trxs, which are able to transfer reducing equivalents to 2-Cys Prxs (Broin et al, 2002; Collin et al., 2003; Collin et al., 2004; Dangoor et al., 2012; Eliyahu et al., 2015; Hochmal et al., 2016), though much less efficiently than NTRC (Pérez-Ruiz et al., 2006; Muthuramalingam et al., 2009; Bernal-Bayard et al., 2014). The regulation of 2-Cys Prxs via NTRC might determine the amount of reducing equivalents that are taken from the pool of Trxs, hence affecting the redox state of Trx targets. According to this idea, we have proposed a model where the activities of the FTR/Trxs and NTRC redox systems are integrated by the redox balance of the 2-Cys Prxs, which controls the redox regulatory network of the chloroplast (Figure 1).

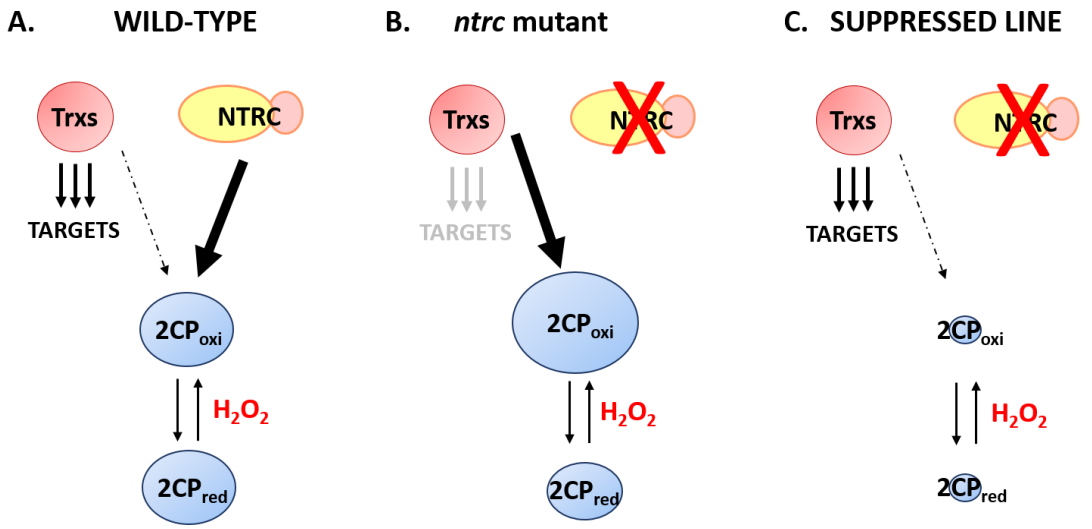

Figure 1. Low contents of 2-Cys Prxs suppress the *ntrc* phenotype. (A) In wild-type plants, NTRC maintains the redox state of 2-Cys Prxs (2CP). Chloroplast Trxs reduce 2-Cys Prxs with lower efficiency than NTRC, which allow the light-dependent reduction of Trx targets. **(B)** In *ntrc* mutant, the lack of NTRC alters the redox balance of 2-Cys Prxs, which act as sink of electrons from the pool of Trxs. Consequently, the light-dependent reduction of Trx targets is impaired in the *ntrc* mutant. **(C)** In the suppressed line, levels of 2-Cys Prxs decrease in NTRC-deficient plants. Thus, the drainage of reducing equivalents from the pool of Trxs is lower and the light-dependent reduction of Trx targets is restored. red, reduced; oxi, oxidized.

3. 2-Cys Prxs participate in chloroplast enzyme oxidation in the dark

It is well known since the beginning of redox biology studies in chloroplast that most redox-regulated enzymes of this organelle are reduced during the day and oxidized during the night. While the mechanism of reduction during the day has received extensive attention and the molecular basis of this regulatory mechanism is well known, the mechanism of enzyme oxidation in the dark remains unknown. During the day, photochemical reactions generate Fdx_{red} so that the "electron pressure" of the FTR/Trx system is high and, consequently, downstream targets are reduced and active. During the night, however, the entrance of reducing power is relieved, and these enzymes become deactivated by oxidation (Shürmann and Buchanan, 2008). The important role of 2-Cys Prxs in light-dependent redox regulation of chloroplast enzymes has been discussed in the previous sections. The participation of 2-Cys Prxs implies the transfer of reducing equivalents from thiols to H_2O_2 via this thiol peroxidase and, thus, it could provide an explanation for the issue of how chloroplast enzymes are oxidized in the dark. Indeed, it was previously proposed the implication of H_2O_2 in the oxidation of the chloroplast thiol enzymes, yet by a direct mechanism (Kaiser, 1979; Tanaka et al., 1982). In the work shown in the third section of this thesis, we have analysed the participation of the NTRC/2-Cys Prx system in this process.

First, we generated a new double mutant of *Arabidopsis*, here termed *2cpab,* knock out for the two isoforms of 2-Cys Prxs, A and B. The *2cpab* mutant shows a slight growth inhibition phenotype and lower chlorophyll levels as compared to wild-type (Chapter 5, Fig. S2), confirming the phenotype reported by other authors for plants lacking 2-Cys Prxs, which showed lower photosynthetic efficiency and sensitivity to high light (Awad et al., 2015). Based on our proposal that 2-Cys Prxs play an important role maintaining an "electron pressure" on the FTR/Trx system in the light, we tested the possibility that 2-Cys Prxs might be involved in oxidation by relieving this "electron pressure" in the dark. To address this possibility, we have studied the dark-dependent oxidation of well-established redox regulated CBC enzymes, such as FBPase and GAPDH, and an additional chloroplast redox-regulated protein as γ-subunit of ATPase (CF_1-γ), involved in energy production. These

enzymes are mainly reduced in light-adapted leaves and become rapidly oxidized in the dark, so that after 5 min for the FBPase and GAPDH and 15 min for CF_1-γ, oxidation was complete. Interestingly, the short-term oxidation of these enzymes is delayed in the *2cpab* mutant (Chapter 5, Fig. 1). Indeed, CF_1-γ showed a remarkable difference with FBPase and GAPDH, remaining predominantly reduced after the darkness treatment (Chapter 5, Fig. 1). These results indicate different sensitivity of these enzymes to the chloroplast redox condition. Moreover, the delay of enzyme oxidation in the *2cpab* mutant uncovers the participation of 2-Cys Prxs in the mechanism of short-term enzyme oxidation in the dark. Second, given the important function of NTRC controlling the redox balance of 2-Cys Prxs, alteration of the content of NTRC might affect the rate of oxidation of chloroplast Trxs targets in the dark. To test the impact of NTRC levels on enzyme oxidation in the dark, we generated *Arabidopsis* transgenic plants that overexpress the *NTRC* gene in the wild-type background. These transgenic plants mimic the phenotype of the *2cpab* mutant in terms of growth (Chapter 5, Fig. 2). Previous reports showed that short-day-grown *NTRC* overexpressing transgenic plants in the *ntrc* background present an increase in rosette growth (Toivola et al, 2013; Nikkanen et al., 2016), although these discrepancies might be due to the hight light intensity (600 $\mu E\ m^{-2}\ s^{-1}$) used in this study (Toivola et al., 2013). Indeed, it is known that light intensity exerts a strong effect of the growth phenotype on the *ntrc* mutant (Pérez-Ruiz et al. 2006; Lepistö et al., 2009; Thormählen et al., 2017). Remarkably, the short-term oxidation of FBPase, GAPDH and CF_1-γ in darkness is also delayed the transgenic plants that overexpress *NTRC* (Chapter 5, Fig. 3), showing that high levels of NTRC delay chloroplast enzyme oxidation upon darkness, as observed in the *2cpab* mutant. Overall, these results suggest that the function of NTRC in chloroplast enzyme oxidation could be exerted through the redox balance of 2-Cys Prxs. In this regard, it has previously observed that plants overexpressing NTRC show incomplete oxidation of FBPase, PRK and CF_1-γ in the dark (Nikkanen et al., 2016); thus, our results provide an explanation for these findings.

The fact that chloroplast enzyme oxidation in darkness, despite being delayed, still occurs in the *2cpab* mutant indicates that additional mechanisms are involved in this process. Because 2-Cys Prxs are thiol peroxidases, which use reducing power of thiols to reduce the H_2O_2, other chloroplast thiol peroxidases could be candidates

to participate in the mechanism of enzyme oxidation in the dark. Thiol peroxidases are classified into two subgroups Prx (Liebthal et al., 2018) and Gpx (Bela et al., 2015). The genome of *Arabidopsis thaliana* encodes a family of eight Gpxs and ten Prxs. Of them, two Gpxs, Gpx1 and Gpx7, and four Prxs, 2-Cys A, 2-Cys Prx B, Prx Q and Prx IIE, are targeted to the chloroplast (Bela et al., 2015; Liebthal et al., 2018). To study the involvement of these thiol peroxidases in the oxidation process, we analyzed the dark oxidation of chloroplast enzymes, FBPase and CF_1-γ, in plants deficient in Prx IIE and Prx Q. First, single mutants with severely decreased contents of Prx IIE (Romero-Puertas et al., 2007) or Prx Q (Lamkemeyer et al., 2006), which showed growth (Chapter 5, Fig. 4) and photosynthetic performance (Chapter 5, Fig. 5) similar to the wild-type, showed no alteration in the dark-dependent oxidation of the enzymes tested in this study (Chapter 5, Fig. 6). Second, we generated *Arabidospis* mutants, *2cpab-prxIIE* and *2cpb-prxQ*, simultaneously deficient in 2-Cys Prxs and Prx IIE or Prx Q, respectively. These mutants showed a similar growth phenotype (Chapter 5, Fig. 4) and photosynthetic performance (Chapter 5, Fig. 5) than the *2cpab* mutant. Besides, these mutants displayed a rate of enzyme oxidation in light-dark transitions similar to that observed in the *2cpab* mutant (Chapter 5, Fig. 6). These results suggest a minor contribution, if any, of Prx IIE and Prx Q to the short-term dark-dependent oxidation of the chloroplast enzymes analyzed. Nonetheless, in addition to Prxs, chloroplast also contain Trx-dependent thiol peroxidases Gpx1 and Gpx7, which could be involved in short-term oxidation of chloroplast enzymes in dark. The participation of these Gpxs as an additional mechanism should be tested in the future.

Overall, our results uncover the important role of 2-Cys Prxs in the oxidative deactivation of chloroplast enzymes upon darkness. The next question arising was whether 2-Cys Prxs act directly or indirectly, via Trxs, on reduced enzymes. We set up a biochemical approach, using pre-reduced FBPase, Trx *f*1 or *f*2 and 2-Cys Prxs, to reconstitute the oxidative pathway *in vitro*. We determined redox state of FBPase by alkylation with *N*-ethylmaleimide (NEM) and SDS–PAGE analysis and the rate of H_2O_2 consumption by a ferrous ion oxidation assay. The *in vitro* assays showed neither oxidation of reduced FBPase nor consumption of H_2O_2 in presence of 2-Cys Prx, while addition of Trx *f*1 or *f2* provoked the oxidation of FBPase and increased consumption of H_2O_2 (Chapter 5. Fig. 7). These results indicate the participation of

2-Cys Prxs, via Trxs *f,* in the process of enzyme oxidation in the dark. Interestingly, this is in contrast with the pathway of thiol oxidation proposed in human cells in which Trx is not needed (Stöcker et al., 2017). Our results show that Trxs *f* enable the oxidation of FBPase *in vitro*, however, other chloroplast Trxs might also participate in this deactivation process. Indeed, recent reports support this possibility. It has been shown that 2-Cys Prxs act as Trx oxidase (Vaseghi et al., 2018) and that TrxL2, an atypical chloroplast Trx with a non-canonical WCRKC redox site (Cain et al., 2009; Chibani et al., 2009), displays oxidative activity in combination with 2-Cys Prxs (Yoshida et al., 2018). Furthermore, in previous reports it was shown that 2-Cys Prxs drive the oxidation of Trxs, such as ACHT1 in response to moderate light intensity (Dangoor et al., 2012) or ACHT4 for oxidation of the small subunit of AGPase (Eliyahu et al., 2015). Altogether, these results confirm the importance of 2-Cys Prxs in enzyme oxidative deactivation and show the participation of Trxs as intermediates.

4. The NTRC/2-Cys Prx system integrates chloroplast redox regulation in response to light availability

Chloroplasts present electron transport activity, coupled with the production of electrochemical gradients across the thylakoid membrane. Under these conditions, H_2O_2 is produced via the dismutation of O_2^- (Smirnoff and Arnaud, 2018). The main antioxidant systems that remove H_2O_2 in chloroplasts are ascorbate and thiol peroxidases such as 2-Cys Prxs (Dietz, 2016). The results presented in this thesis support a relevant role of 2-Cys Prxs integrating disulfide-dithiol exchange of redox-regulated enzymes. Despite this relevant function of 2-Cys Prxs in chloroplast redox regulation, the *Arabidopsis* mutant deficient in both 2-Cys Prx A and B is viable (Chapter 5, Fig. S2; Awad et al., 2015). Similarly, a yeast strain lacking all eight thiol peroxidases is also viable (Fomenko et al., 2011), suggesting that additional antioxidant systems might operate in the absence of 2-Cys Prxs in both organisms. However, the fact that the *2cpab-prxQ* and *2cpab-prxIIE* mutants present similar phenotype compared to the *2cpab* mutant indicate that Prx IIE and Prx Q do not play this role (Chapter 5). Conversely, the *ntrc* mutant and plants lacking both redox system, *ntrc-trxx* and *ntrc-trxf1f2,* show an extremely severe growth inhibition

phenotype that is recovered when levels of 2-Cys Prxs decrease (Chapter 3, Fig. 4 and Chapter 4, Fig. 1). Thus, altered redox regulation of 2-Cys Prx generates a dramatic growth inhibition phenotype whereas the absence of 2-Cys Prxs has a minor effect. These results emphasize the relevant physiological role of NTRC, an NADPH-dependent enzyme, which mainly regulates 2-Cys Prxs. Thus, we hypothesize that NADPH, which can be produced in light from Fdx_{red} or in dark from the OPPP, maintains the redox balance of the NTRC/2-Cys Prx system.

After many years of research in chloroplast redox regulation focusing on the activation of chloroplast enzymes in the light, the participation of 2-Cys Prxs in the dark deactivation has been finally reported (Chapter 5; Vaseghi et al., 2018; Yoshida et al., 2018). In agreement with this proposal, 2-Cys Prxs are able to transfer oxidizing equivalents from redox regulated enzymes in human cells (Stöcker et al., 2017). These results also highlight the role of H_2O_2 to maintain the redox homeostasis of biological systems, and the role of peroxidases as mediators of this dishulphide-thiol interchange (Stöcker, 2018). Consequently, the function of H_2O_2 as a sink of reducing equivalents constitutes a universal strategy in terms of metabolic regulation. The results obtained in this thesis and the evidences discussed in this section, have allowed us to propose a model of the role that the NTRC/2-Cys Prx system plays in the chloroplast redox regulation (Fig. 2). This model integrates the redox exchange of Trx and redox-regulated targets with H_2O_2 via the action of 2-Cys Prxs. The action of 2-Cys Prx is dependent of NTRC and subsequently of NADPH. Thus, although we cannot discard the idea that the NTRC protein has additional functions, the main role of NTRC is to regulate the 2-Cys Prxs in response to light availability.

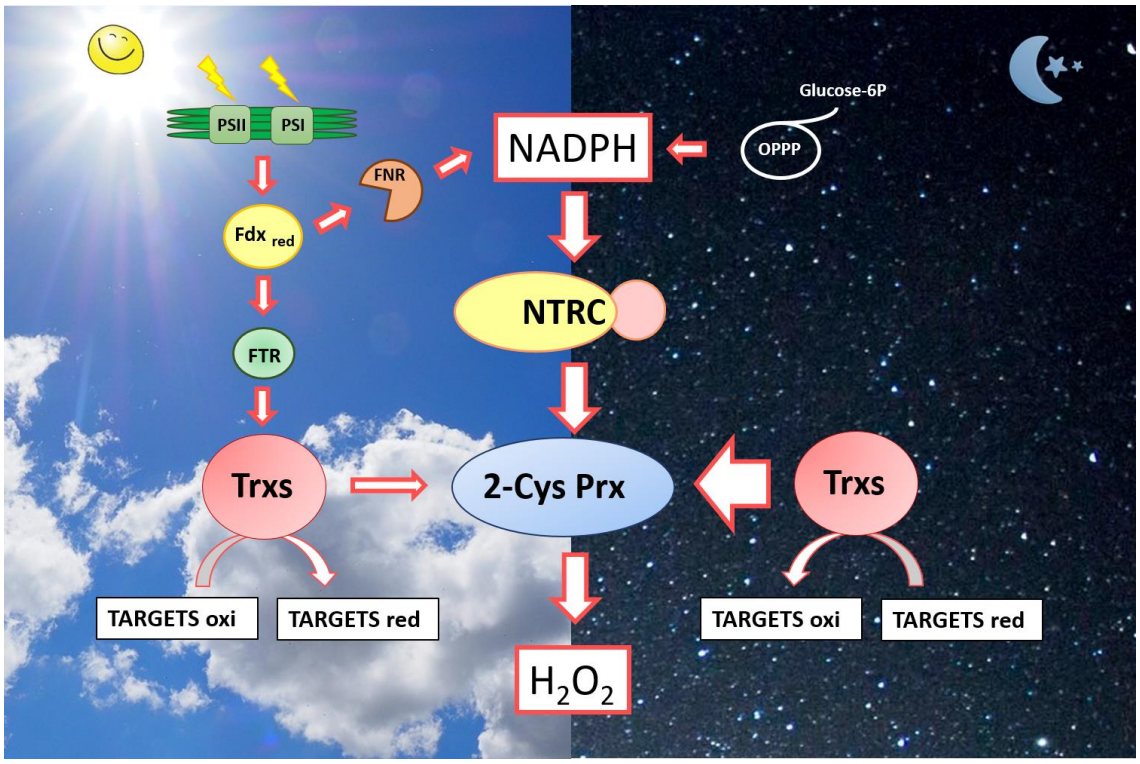

Figure 2. The NTRC/2-Cys Prx system integrates chloroplast redox regulation. The redox balance of 2-Cys Prxs is maintained by NTRC using NADPH, which is formed via Ferredoxin NADPH reductase (FNR) from reduced ferredoxin (Fdx_{red}) in the light or from the oxidative pentose phosphate pathway (OPPP) in the dark. Although chloroplast Trxs are able to transfer reducing power to 2-Cys Prxs, the rate is lower than that of NTRC. During the day, the photosynthetic electron transport chain produces Fdx_{red}. The FTR/Trx system transfers reducing equivalents of Fdx_{red} to redox-regulated enzymes, which consequently become reduced and activated. During the dark, the input of reducing equivalents via Fdx_{red} ceases and 2-Cys Prxs mediate the oxidation of reduced stromal targets transferring electrons from Trxs to H_2O_2, which acts as final sink of electrons. red, reduced; oxi, oxidized.

CONCLUSIONS

CONCLUSIONS

1. While the lack of Trxs *f* or *x* have minor phenotypic effects in *Arabidopsis*, these deficiencies in the *ntrc* mutant background result in decreased photosynthetic performance and severe growth inhibition, indicating that NTRC is needed for the proper function of Trxs *x* and *f*.

2. The light-dependent reduction of FBPase is more impaired in plants lacking NTRC than in plants lacking Trxs *f* or *x*. Contrary to these Trxs, NTRC is unable to reduce FBPase in vitro, indicating that the effect of NTRC on chloroplast redox regulation is exerted by an indirect mechanism.

3. The high mortality of the *ntrc-trxx* and *ntrc-trxf1f2* mutants at the seedling stage uncovers that chloroplast redox regulation plays an essential role during early plant development.

4. Based on the suppressor effect exerted by decreased contents of 2-Cys Prxs on the *ntrc* phenotype, we propose a new model for chloroplast redox regulation. According to this model, the redox balance of 2-Cys Prxs, which is maintained by NTRC, modulates the activity of the FTR/Trx redox system.

5. 2-Cys Prxs participate in the short term oxidation of chloroplast redox-regulated enzymes in the dark, through an indirect mechanism that involves Trxs.

6. The relevant role of the NTRC/2-Cys Prxs system in chloroplast redox homeostasis implies that hydrogen peroxide exerts a key role in plant adaptation to light and darkness.

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