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Yugo Lima-Melo

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ABSTRACT

Photosynthesis allows plants to store light energy in organic compounds. Plants have an efficient apparatus to harvest photons from sunlight and use the energy to split water and transport electrons to specific high-energy electron acceptors. A proper balance between light reactions and electron consumption is important to maintain fluent photosynthetic activity during environmental conditions that are constantly changing. At the same time, photosynthetic components are protected through several regulatory mechanisms. The avoidance of damage to photosystem I (PSI) is particularly important because its recovery occurs extremely slowly as compared to that of photosystem II (PSII). Studies on damage, photoinhibition and recovery of PSI are scarcer than those of PSII. In this thesis, the occurrence of photoinhibition of PSI and some of its consequences to the plant metabolism were investigated. *Arabidopsis thaliana* L. plants lacking the PROTON GRADIENT REGULATION 5 protein (*pgr5* mutants) that were treated with excess light were used as a model system for controlled PSI-photoinhibition. This experimental model was validated, and the impact of PSI photoinhibition and recovery on photosynthetic electron transport, primary metabolism, reactive oxygen species (ROS) production and chloroplast retrograde signalling were thoroughly characterised. The results highlight that PSI photoinhibition induces impairment of CO_2 fixation, starch accumulation, and dark respiration. The recovery of PSI function after photoinhibition proved to be dependent on light conditions, being especially deleterious for CO_2 fixation under low irradiances, and supporting the idea that a pool of surplus PSI can be recruited to support photosynthesis under demanding conditions. High light-treated *pgr5* mutants also displayed low occurrence of lipid oxidation associated with attenuated enzymatic oxylipin synthesis and consequent chloroplast regulation of nuclear gene expression. This model also showed that PSI photoinhibition prevents oxidative stress and accumulation of ROS, evidencing a role of PSI inactivation in avoiding over-reduction of downstream redox components.

Keywords: photosynthesis, PSI damage, PGR5, CO_2 assimilation, ROS, chloroplast signalling, P700 oxidoreduction

TIIVISTELMÄ

Fotosynteesissä kasvit muuntavat valoenergiaa kemialliseksi energiaksi, joka varastoituu erilaisiin orgaanisiin yhdisteisiin. Kasvit keräävät tehokkaasti auringon valon fotoneja, hajottavat sen avulla vesimolekyylejä ja kuljettavat elektroneja erityisille vastaanottajamolekyyleille, joiden avulla pystyvät pelkistämään ilmakehän hiilidioksidia. Näiden reaktioiden tasapainottaminen on keskeistä fotosynteettisen aktiivisuuden ylläpitämiseksi jatkuvasti muuttuvissa ympäristöolosuhteissa. Samanaikaisesti on myös suojattava fotosynteettisiä komponentteja ja näistä erityisesti fotosysteemi (PS) I:tä, koska sen palautuminen on hidasta verrattuna PSII:n nopeaan korjauskiertoon. PSI:n vauriota ja palautumista ei ole kuitenkaan tutkittu yhtä paljon kuin PSII:n fotoinhibitiota ja siksi tässä väitöskirjassa kartoitettiin PSI:n fotoinhibition esiintymisen syitä ja sen seurauksia kasvin aineenvaihduntaan. Kokeellisena mallina kontrolloidulle PSI-fotoinhibitiolle käytettiin voimakkaalla valolla käsiteltyjä *Arabidopsis thaliana* L. -kasveja, joista puuttui PROTON GRADIENT REGULATION 5 -proteiini (*pgr5*-mutantti). Malli todettiin toimivaksi ja sen avulla selvitettiin perusteellisesti PSI-fotoinhibition ja siitä palautumisen vaikutuksia fotosynteettiseen elektroninsiirtoon, aineenvaihduntaan, reaktiivisten happilajien muodostumiseen sekä kloroplastin ja tuman väliseen viestintään. Saadut tulokset osoittivat, että PSI:n fotoinhibitio häiritsee vakavasti kasvien aineenvaihduntaa erityisesti heikossa valossa aiheuttaen ongelmia CO_2:n sidontaan, tärkkelyksen kertymiseen ja soluhengitykseen. Lisäksi tutkittiin PSI:n nopeaa fotoinhibitiota ja hidasta palautumista. Tulokset viittaavat siihen, että ylimääräinen PSI, verrattuna PSII:n määrään, ylläpitää fotosynteesiä vaativissa olosuhteissa. Kirkkaalla valolla käsitellyssä *pgr5*-mutantissa lipidien hapettumisen havaittiin vähentyneen ja entsymaattisen oksilipiinisynteesin hidastuneen, minkä seurauksena myös tuman geenienekspression säätely kloroplastissa heikentyi. Malli osoitti myös, että PSI:n fotoinhibitio ei suoraan liity hapettavaan stressiin tai reaktiivisten happilajien kertymiseen, mikä todistaa, että PSI:n inaktivointi suojaa elektroninsiirtoketjun seuraavia komponentteja ylipelkistymiseltä.

Asiasanat: fotosynteesi, PSI:n vaurio, PGR5, hiilensidonta, reaktiiviset happilajit, kloroplastin signalointi, P700:n hapetus-pelkistys

RESUMO

A fotossíntese permite que plantas estoquem energia luminosa em compostos orgânicos. Plantas têm um eficiente aparato para coletar fótons da luz solar e usar a energia para fotolisar moléculas de água e transportar elétrons para aceptores de elétrons específicos. O equilíbrio adequado entre as reações de luz e o consumo de elétrons é importante para manter a fotossíntese regulada durante as condições ambientais sob constante mudança. Ao mesmo tempo, componentes fotossintéticos precisam ser protegidos por vários mecanismos regulatórios. Evitar danos ao fotossistema I (PSI) é particularmente importante porque sua recuperação é extremamente lenta comparada à do fotossistema II (PSII). Estudos sobre danos, fotoinibição e recuperação do PSI são mais escassos do que os do fotossistema II. Nesta tese, investigou-se a ocorrência da fotoinibição do PSI e algumas de suas consequências ao metabolismo vegetal. Plantas de *Arabidopsis thaliana* L. deficientes na proteína PROTON GRADIENT REGULATION 5 (mutantes *pgr5*) tratadas em condições de excesso de luz foram utilizadas como um modelo de indução controlada de fotoinibição do PSI. Este modelo foi validado e o impacto da fotoinibição e recuperação do PSI no transporte de elétrons da fotossíntese, no metabolismo primário, na produção de espécies reativas de oxigênio (EROS) e na sinalização retrógrada do cloroplasto foram caracterizados. Os resultados mostram que a fotoinibição do PSI induz graves consequências ao metabolismo primário das plantas, especialmente sob baixas irradiâncias, incluindo danos à assimilação de CO_2, ao acúmulo de amido e à respiração mitocondrial. A recuperação da atividade do PSI após fotoinibição foi dependente das condições luminosas, sendo especialmente deletéria para a fixação do CO_2 sob baixas irradiâncias, suportando a ideia de que um grupo de PSI pode ser recrutado sob condições específicas. Plantas *pgr5* tratadas com alta luz também apresentaram baixa oxidação lipídica associada a menor síntese enzimática de oxilipinas e consequente regulação cloroplástica da expressão gênica nuclear. Este modelo também mostrou que a fotoinibição do PSI previne estresse oxidativo e acúmulo de EROS, evidenciando um papel da inativação do PSI em evitar a super-redução de componentes aceptores de elétrons.

Palavras-chave: fotossíntese, dano do PSI, PGR5, assimilação de CO_2, EROS, sinalização cloroplástica, oxirredução do P700.

"Man, he took his time in the sun,
Had a dream to understand
A single grain of sand.
He gave birth to poetry
But one day'll cease to be.
Greet the last light of the library.
We were here!"

Tuomas LJ Holopainen
The Greatest Show on Earth

"We are going to die, and that makes us the lucky ones.
Most people are never going to die because they are never going to be born.
The potential people who could have been here in my place,
but who will in fact never see the light of day,
outnumber the sand grains of *Sahara*.
Certainly those unborn ghosts include greater poets than Keats,
scientists greater than Newton.
We know this because the set of possible people allowed by our DNA so massively
exceeds the set of actual people.
In the teeth of these stupefying odds,
it is you and I,
in our ordinariness, that are here.
We privileged few, who won the lottery of birth against all odds.
How dare we whine at our inevitable return to that prior state
from which the vast majority have never stirred?"

Richard Dawkins
Unweaving the Rainbow

"There is grandeur in this view of life,
with its several powers,
having been originally breathed into a few forms or into one;
and that, whilst this planet has gone cycling
on according to the fixed law of gravity,
from so simple a beginning
endless forms most beautiful and most wonderful have been,
and are being,
evolved."

Charles Darwin
The Origin of Species

LIST OF ORIGINAL PUBLICATIONS

This thesis is composed of the following scientific articles, referred to in the text by their Roman numerals.

I. **Gollan PJ, Lima-Melo Y, Tiwari A, Tikkanen M, Aro E-M** (2017) Interaction between photosynthetic electron transport and chloroplast sinks triggers protection and signalling important for plant productivity. Philosophical Transactions of the Royal Society B 372:20160390

II. **Lima-Melo Y, Gollan PJ, Tikkanen M, Silveira JAG, Aro E-M** (2019) Consequences of photosystem-I damage and repair on photosynthesis and carbon use in *Arabidopsis thaliana*. The Plant Journal 97:1061–1072

III. **Lima-Melo Y, Alencar VTCB, Lobo AKM, Sousa RHV, Tikkanen M, Aro E-M, Silveira JAG, Gollan PJ** (2019) Photoinhibition of photosystem I provides oxidative protection during imbalanced photosynthetic electron transport in *Arabidopsis thaliana* (Manuscript)

Other recent publications related to the topic of this thesis:

Sousa RHV, Carvalho FEL, Ribeiro CW, Passaia G, Cunha JR, **Lima-Melo Y**, Margis-Pinheiro M, Silveira JAG (2015) Peroxisomal APX knockdown triggers antioxidant mechanisms favourable for coping with high photorespiratory H_2O_2 induced by CAT deficiency in rice. Plant, Cell and Environment 38:499-513

Lima-Melo Y, Carvalho FEL, Martins MO, Passaia G, Sousa RHV, Lima Neto MC, Margis-Pinheiro M, Silveira JAG (2016) Mitochondrial GPX1 silencing triggers differential photosynthesis impairment in response to salinity in rice plants. Journal of Integrative Plant Biology 58:737-748

Castro JLS, **Lima-Melo Y**, Carvalho FEL, Feitosa AGS, Lima Neto MC, Caverzan A, Margis-Pinheiro M, Silveira JAG (2018) Ascorbic acid toxicity is related to oxidative stress and enhanced by high light and knockdown of chloroplast ascorbate peroxidases in rice plants. Theoretical and Experimental Plant Physiology 30:41-55

Sousa RHV, Carvalho FEL, **Lima-Melo Y**, Alencar VTCB, Daloso DM, Margis-Pinheiro M, Komatsu S, Silveira JAG (2019) Impairment of peroxisomal APX and CAT activities increases protection of photosynthesis under oxidative stress. Journal of Experimental Botany 70:627-639

Souza PVL, **Lima-Melo Y**, Carvalho FEL, Reichheld J-P, Fernie AR, Silveira JAG, Daloso DM (2018) Function and compensatory mechanisms among the components of the chloroplastic redox network. Critical Reviews in Plant Sciences 38:1-28

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ABBREVIATIONS

$^{\bullet}OH$	hydroxyl radical
$^1O_2$	singlet oxygen
A	net CO_2 assimilation rate
A/C_i	maximum carboxylation efficiency
ADP	adenosine diphosphate
AOX	alternative oxidase
APX	ascorbate peroxidase
ASC	ascorbate
ATP	adenosine triphosphate
bp	nucleotide base pair
CAT	catalase
C_i	internal CO_2 concentration
CSD	Cu/Zn-superoxide dismutase
Col-0	*Arabidopsis thaliana* L. ecotype Columbia-0
Cyt *b6f*	cytochrome *b6f* complex
DAB	3,3-diaminobenzidine
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
E	transpiration rate
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FED	ferredoxin 2
FeS	iron-sulphur
Fd	ferredoxin
Fdm, Fdxm	maximal reduction state of ferredoxin
FL	fluctuating light
Fm	maximum chlorophyll *a* fluorescence
FNR	ferredoxin:$NADP^+$ oxidoreductase
Fo	minimum chlorophyll *a* fluorescence
FSD	Fe-superoxide dismutase
FTR	ferredoxin:thioredoxin reductase
Fv/Fm	maximum efficiency of PSII
GL	growth light (120 or 125 µmol photons m^{-2} s^{-1})
gl1	*Arabidopsis thaliana* L. ecotype Columbia glabra-1
GO	Gene Ontology
GO-BP	Gene Ontology Biological Process
GPX	glutathione peroxidase
GR	glutathione reductase
GRX	glutaredoxin
g_s	stomatal conductance rate
GSH	glutathione

GSSG	glutathione disulphide
H_2O_2	hydrogen peroxide
HL	high light (1000 µmol photons m^{-2} s^{-1})
IRGA	infra-red gas analyser
JA	jasmonic acid
KLAS	kinetic LED-array spectrophotometer
LHCI	light harvesting complex I
LHCII	light harvesting complex II
LOX	lipoxygenase
LOX-C	chloroplast lipoxygenase
MDHAR, MDAR	monodehydroascorbate reductase
MSD	Mn-superoxide dismutase
$NADP^+$	oxidised nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NBT	nitro-blue tetrazolium
NDH	NADH dehydrogenase-like complex
NPQ	non-photochemical quenching
$O_2^{\bullet-}$	superoxide radical
OEC	oxygen evolving process
OPDA	12-oxophytodienoic acid
P680	photosystem II reaction centre
P700	photosystem I reaction centre
PC	plastocyanin
PCm	maximum oxidation state plastocyanin
PGR5	proton gradient regulation 5
PGRL1	PGR5-like 1
Pi	inorganic phosphate
Pm	maximum oxidizable P700
pmf	proton motive force
PPFD	photosynthetic photon flux density
PQ	plastoquinone
PRX	peroxiredoxin
PSI	photosystem I
PSII	photosystem II
PTOX	plastid terminal oxidase
PVDF	polyvinylidene difluoride
qE	energy-dependent quenching
ROS	reactive oxygen species
RuBisCO	ribulose-1,5-biphosphate carboxylase/oxygenase
sAPX	stromal ascorbate peroxidase
SDS-PAGE	Na-dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	superoxide dismutase
tAPX	thylakoidal ascorbate peroxidase

TBARS	thiobarbituric acid reactive substances
TRX	thioredoxin
TRX_{ox}	oxidised thioredoxin
TRX_{red}	reduced thioredoxin
WUE	water use efficiency
ΔpH	proton gradient
ΔΨ	membrane potential

1. INTRODUCTION

1.1. Photosynthesis

Photosynthesis is vital for life as we know it, being the main source of organic compounds on Earth. Water-splitting photosynthesis also releases oxygen (O_2), making this process crucial for all aerobic life. Photosynthesis is intrinsically associated with plant productivity (Raines, 2011) through biomass yield and allocation of assimilated carbon. Therefore, efficient photosynthesis is essential to maintain the growth and productivity of crops (Sun et al., 2009; Foyer et al., 2017). Several studies have provided evidence to support an increase in photosynthetic capacity as a viable route to increase the yield of crop plants (Long et al., 2015; Caemmerer and Furbank, 2016; Kromdijk et al., 2016; Simkin et al., 2017; Salesse-Smith et al., 2018). The importance of these studies for the development of higher-yielding crop varieties is also related to the panorama of increasing food and fuel demands by the growing world population (Fischer and Edmeades, 2010; Ray et al., 2012; Long et al., 2015; Simkin et al., 2017).

Photosynthesis is the process prevailing in plants and algae to convert light energy into chemical energy, which is stored as carbohydrates molecules synthesised from carbon dioxide (CO_2) and water. In plants, photosynthesis encompasses two steps: photochemistry and carbon assimilation/fixation. In the first step, chlorophyll and other photosynthetic pigments of the cell absorb light energy to produce the energy-carrier molecules ATP and NADPH. In the second step, ATP and NADPH generated from the photochemical phase are used to reduce CO_2 molecules to produce carbohydrates and their derivative products. Both steps are detailed in the following sections.

1.1.1. The photosynthetic electron transport chain

The photochemical phase of photosynthesis, also known as "light-dependent reactions" or simply "light reactions", allows the synthesis of ATP and NADPH molecules by using energy from light. This step involves a linear electron flux, or a cyclic electron flux under specific conditions (explained in section 1.3), through a succession of redox cofactors, most of which are housed

in integral or peripheral protein complexes of the thylakoid membrane (Figure 1). First, the photons are harvested by light-harvesting pigment-protein antennae. The harvested photons excite chlorophyll pools and other accessory pigments, which transfer the energy to reaction centres in photosystem I and II (PSI and PSII, respectively). Light-harvesting complex I (LHCI) delivers excitation specifically to PSI, while light-harvesting complex II (LHCII) serves both PSII and PSI. After being excited, each photosystem reaction centre induces separation of electric charge, producing a strong electron donor and a strong electron acceptor (Govindjee et al., 2017).

After photon-induced excitation through the LHCII, the PSII reaction centre chlorophyll P680 transfers electrons through a series of PSII cofactors to a plastoquinone (PQ) pool, filling the electron hole at P680 with electrons extracted from molecules of water through an oxygen-evolving complex (OEC), which also liberates O_2 and protons into the thylakoid lumen (Vinyard et al. 2013). Reduced PQ transfers electrons to the cytochrome *b6f* complex (cyt *b6f*) and becomes oxidised and available to be reduced by PSII again. The reduced cyt *b6f* donates electrons to a soluble electron carrier located in the thylakoid lumen named plastocyanin (PC). Similar to PSII, the PSI reaction centre (P700) chlorophyll is excited by light through both LHCI and LHCII (Grieco et al., 2012; Wientjes et al., 2013; Grieco et al., 2015; Rantala and Tikkanen, 2018). In the case of PSI, the electrons are donated to the stromal ferredoxin (Fd) and replaced by electrons provided by the PC pool. Considering the linear electron flow, the Fd pool transfers electrons to ferredoxin-$NADP^+$ reductase, which finally allows the regeneration of $NADP^+$ to NADPH (Vinyard et al., 2013; Ruban, 2015; Govindjee et al., 2017). The Fd pool can also donate electrons to ferredoxin-thioredoxin reductase (FTR), which allows the maintenance of the ferredoxin-dependent thioredoxin system. This system is also important for the CO_2 assimilation step by activating essential enzymes of the Calvin-Benson-Bassham (CBB) cycle (Buchanan, 2016; Nikkanen et al., 2016).

In addition to the reduction of $NADP^+$, the electron flow through the thylakoid membrane is essential for the synthesis of ATP to feed the reactions of CO_2 fixation (explained in the following section). The synthesis of ATP during the light reactions is possible because of the formation of a transmembrane proton motive force (*pmf*), which is made up of the proton gradient (ΔpH) across the thylakoid membrane and the membrane potential ($\Delta\Psi$). In the linear

electron flow, the most important steps in which protons are concentrated in the thylakoid lumen in relation to the stromal side of the membrane are the splitting reaction of H_2O occurring in the PSII by its water-oxidising complex, and the electron transfer from the PQ pool to the cyt *b6f*. The chloroplastic ATP synthase makes use of the *pmf* to translocate protons from the lumen to the stroma, using the derived energy to produce ATP from ADP and inorganic phosphate.

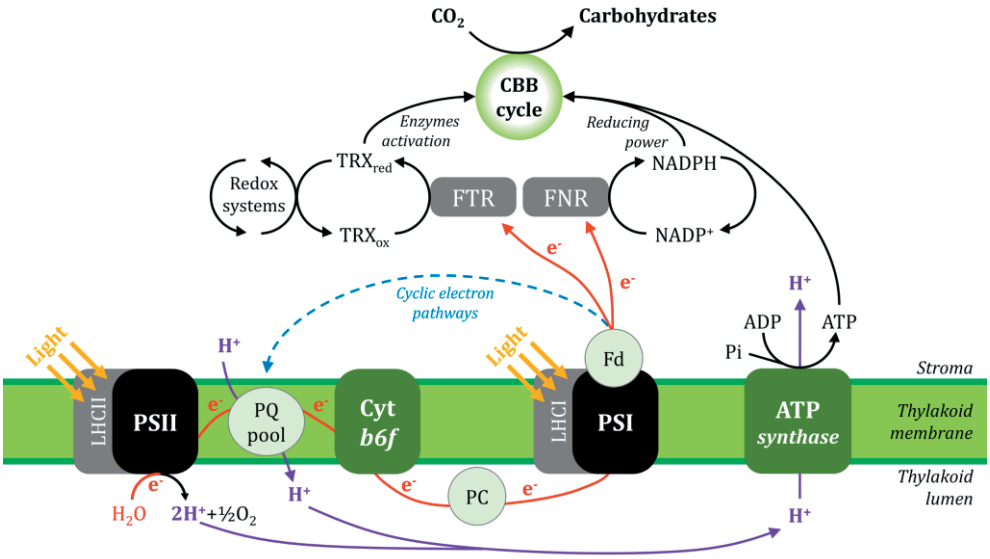

Figure 1. A simplified scheme of the photosynthetic electron transport chain in the thylakoid membrane and its interaction with CO_2 assimilation in the Calvin-Benson-Bassham cycle. Linear electron (e^-) transport is shown in red lines and cyclic electron transport is represented in a blue dashed line. The proton (H^+) fluxes are represented in purple lines. ADP = adenosine diphosphate; ATP = adenosine triphosphate; CBB cycle = Calvin-Benson-Bassham cycle; Cyt *b6f* = cytochrome *b6f*; Fd = ferredoxin; FNR = ferredoxin:$NADP^+$ oxidoreductase; FTR = ferredoxin:thioredoxin reductase; LHCI = light-harvesting complex I; LHCII = light-harvesting complex II; $NADP^+$ = oxidised nicotinamide adenine dinucleotide phosphate; NADPH = reduced nicotinamide adenine dinucleotide phosphate; PC = plastocyanin; Pi = inorganic phosphate; PQ = plastoquinone; PSI = photosystem I; PSII = photosystem II; TRX_{ox} = oxidised thioredoxin; TRX_{red} = reduced thioredoxin.

1.1.2. CO_2 assimilation and the Calvin-Benson-Bassham cycle

The diffusion of CO_2 into plant leaves is regulated by stomata. Stomatal resistance and aperture are the major limiting factors for CO_2 uptake by plants and thus for photosynthesis and plant growth (Lawson and Blatt, 2014; Wang et al., 2014). Stomatal regulation is very sensitive to the environment, mainly in response to changes in light and relative humidity, and involves highly coordinated and dynamic signalling processes (Daloso et al., 2017; Devireddy et al., 2018). After passing through stomata, CO_2 molecules concentrate in the intercellular air space, before passing across the cell wall, plasmalemma, cytosol, and chloroplast envelope and finally reaching the stroma, where they are available to be used as a substrate for the CBB cycle (Evans and von Caemmerer, 1996; Evans et al., 2009).

ATP and NADPH molecules synthesised at the photochemical phase are used to reduce CO_2 into phosphate trioses (Benson et al., 1950) through three steps of the CBB cycle: (1) CO_2 fixation, which is catalysed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO); (2) reduction of 3-phosphoglycerate to glyceraldehyde-3-phosphate; and (3) regeneration of ribulose-1,5-bisphosphate, which is a substrate for RuBisCO in addition to CO_2, from triose phosphate sugars. The glyceraldehyde-3-phosphate molecules generated during the second step of the cycle can be used to directly provide energy via glycolysis or serve as a substrate for synthesis of other carbohydrates with different functions, including stored energy (e.g. starch), sources of energy that are transported throughout plant tissues (e.g. sucrose), structural carbohydrates (e.g. cellulose), and signalling compounds (Paul and Foyer, 2001; Kölling et al., 2015; Wingler, 2018). For each molecule of glyceraldehyde-3-phosphate, three molecules of CO_2 are assimilated and nine molecules of ATP plus six of NADPH are consumed during each round of the cycle (Benson et al., 1950; Raines, 2003).

1.2. Photo-oxidative stress, photoinhibition and photoprotection

Although light energy is vital for photosynthesizing organisms, this same energy can also damage the photosynthetic apparatus in a condition named photo-oxidative stress. This condition occurs when the electron

pressure in the photosynthetic electron transport chain exceeds the capacity of electron consumption by electron sink pathways and regulation mechanisms provide insufficient protection (photoprotection is discussed in section 1.3). As a result, transient or sustained production of reactive oxygen species (ROS) develops, leading to photo-oxidation processes. Photo-oxidative conditions are usually triggered by changes in environmental conditions and lead to a phenomenon known as "photoinhibition", which is characterised as the inactivation of the photosystems (Powles, 1984; Aro et al., 1993; Gururani et al., 2015).

Photoinhibition negatively affects photosynthetic capacity and thus is deleterious for plant growth and crop yield (Takahashi and Murata, 2008; Kato et al., 2012; Simkin et al., 2017). Among the photoinhibitory conditions, light intensity is especially important since it is directly related to photon incidence on leaves. For example, high electron pressure conditions, like high light intensity and fluctuating light conditions, induce damage to the photosynthetic apparatus, leading to a photoinhibitory condition (Powles, 1984; Aro et al., 1993; Gururani et al., 2015). In addition, photoinhibition is exacerbated by other environmental stresses (e.g. low and high temperatures, drought, salinity) through the limitation of the photosynthetic fixation of CO_2 (Takahashi and Murata, 2008). The following sections will approach the harmful and signalling properties of ROS, the occurrence of photoinhibition and mechanisms of photoprotection.

1.2.1. Reactive oxygen species as both harmful and beneficial components

ROS, including singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$), are reactive derivatives of molecular oxygen that are capable of oxidation of various cellular components and can cause oxidative destruction in the cell (Mittler, 2002; Apel and Hirt, 2004; Munns, 2005; Czarnocka and Karpiński, 2018; Mhamdi and Van Breusegem, 2018). More precisely, the term "ROS" has been defined as any oxygen derivative that is more reactive than an oxygen molecule (O_2) (Foyer and Noctor, 2009; Mittler, 2017; Mhamdi and Van Breusegem, 2018). Formation of ROS occurs when electrons or excitation are transferred to molecular oxygen (O_2), which takes place constantly as a by-product of

metabolic pathways in almost all cells (Mhamdi and Van Breusegem, 2018). However, excessive ROS concentrations cause oxidative stress, which implicates ROS in the impairment of metabolic homeostasis through oxidative damage to lipids, proteins, and nucleic acids because of the high affinity between ROS and these molecules (reviewed in: Sharma et al., 2012; Soares et al., 2018).

Increased generation of ROS occurs when metabolic pathways are mismatched, which is usually associated with biotic and abiotic stress conditions. This occurs, for example, when photosynthetic electron carriers become highly reduced (Sonoike and Terashima, 1994; Terashima et al., 1994; Grieco et al., 2012; Suorsa et al., 2012; Takagi et al., 2016b). Although ROS are produced in all compartments within the cell, chloroplasts, mitochondria, and peroxisomes are recognised as the metabolic ROS powerhouses of leaf cells (Foyer and Noctor, 2003; Noctor and Foyer, 2016).

While ROS are harmful under high concentrations, these chemical compounds also act as signalling molecules, regulating important biological processes in both animal and plant cells (Dat et al., 2000; Mittler, 2002; Halliwell, 2006). Although ROS-dependent signalling is still poorly understood, studies have shown its importance for several biological processes including cellular proliferation and differentiation, plant development, as well as for activation of responses to stresses and metabolic defence pathways (Suzuki et al., 2012; Exposito-Rodriguez et al., 2017; Mittler, 2017; Locato et al., 2018; Mhamdi and Van Breusegem, 2018; Noctor et al., 2018). Because it has a relatively long half-life compared to other ROS and the ability to cross membranes via aquaporins (Bienert et al., 2007), H_2O_2 has special importance for signalling and stress-sensing events, being among the most studied ROS-signalling molecules (Marinho et al., 2014; Černý et al., 2018; Smirnoff and Arnaud, 2019). Specifically, H_2O_2 can drive redox changes leading to (in)activation of signalling networks (Exposito-Rodriguez et al., 2017; Noctor et al., 2018).

The precise control of different ROS concentrations in cells is critical for metabolic homeostasis. Accordingly, aerobic organisms have developed several non-enzymatic and enzymatic ROS-scavenging systems to prevent oxidative damage and to control the concentration of these species in cells (Dat et al., 2000). Enzymatic and non-enzymatic ROS-scavenging systems are present in

all cellular compartments, demonstrating the importance of the control of the ROS concentrations for cell homeostasis (Mittler et al., 2004; Sharma et al., 2012; Souza et al., 2018), Together, both systems, which are interdependent, are part of a complex metabolic network which involves, for example, more than 150 genes in *Arabidopsis thaliana* (Mittler et al., 2004; Souza et al., 2018).

Among the non-enzymatic components, the redox balance between the reduced and oxidised forms of ascorbate (reduced ascorbate/dehydroascorbate; ASC/DHA) and glutathione (reduced glutathione/glutathione disulphide; GSH/GSSG) are probably the most studied systems in terms of antioxidant metabolism. Tocopherols, flavonoids, anthocyanins, and carotenoids also make part of the non-enzymatic scavengers. These molecules are antioxidants of low molecular weight that work as redox buffers, interacting with ROS and acting as a molecular interface to modulate proper acclimation responses or programmed cell death. The enzymatic ROS-scavenging system includes several isoforms of superoxide dismutases (SOD), catalases (CAT), peroxiredoxins (PRX), ascorbate peroxidases (APX), monodehydroascorbate reductases (MDAR), dehydroascorbate reductases (DHAR), glutathione peroxidases (GPX), glutathione reductases (GR), glutaredoxins (GRX) and other peripheral enzymes. These enzymes are important not only for scavenging excessive ROS but also for regulating the redox balance of ascorbate and glutathione (Souza et al., 2018).

Additionally, subsequent products of reactions involving ROS are central to photosynthesis signalling and regulation (Pintó-Marijuan and Munné-Bosch, 2014; Mullineaux et al., 2018). For example, $^1O_2$ generated in the thylakoid electron transport chain can be primarily quenched by carotenoids and α-tocopherol, generating products that can act as molecular signals (Ramel et al., 2012a; Ramel et al., 2012b; Shumbe et al., 2014). Similarly, oxidation products of lipids, such as oxylipins, have been shown to act as signalling compounds (Mosblech et al., 2009; López et al., 2011; Satoh et al., 2014). Lipid oxidation is associated with the metabolism of jasmonates, which are essential phytohormones involved with regulation of plant development and environmental adaptation (Mosblech et al., 2009; Chini et al., 2016).

1.2.2. Photosystem II and its photoinhibition

PSII is a dimer complex and each monomer is composed of 20 to 23 subunits, depending on the organism (Bezouwen et al., 2017; Su et al., 2017). Most of these subunits are membrane-intrinsic proteins, including the PSII reaction centre core proteins D1 (PsbA) and D2 (PsbD) and inner antennae proteins CP43 (PsbB) and CP47 (PsbC), which bind several chlorophylls (Shen, 2015; Bezouwen et al., 2017; Su et al., 2017). The light-harvesting complex II (LHCII) contains three major trimeric light-harvesting chlorophyll a/b-binding proteins (LHCB1, LHCB2 and LHCB3), while three minor monomeric LHCB pigment-proteins are associated with PSII (LHCB4, LHCB5 and LHCB6) (Lu, 2016; Bezouwen et al., 2017). The PSII core also binds to the OEC proteins (PsbO, PsbP, PsbQ), which are located at the lumenal side (Bricker et al., 2012; Su et al., 2017). Several other subunits are involved with PSII complex assembly, stability, and repair (Nixon et al., 2010; Nickelsen and Rengstl, 2013; Järvi et al., 2015; Lu, 2016).

PSII is particularly susceptible to photoinhibition because of the very strong oxidative potential of its reaction centre, which is required to oxidise water (Ruban, 2015), making PSII a significant source of ROS in plants (Noctor et al., 2018). For example, $^1O_2$ production can occur when active PSII absorbs excitation through its surrounding chlorophylls, and the pool of PQ is highly reduced (Krieger-Liszkay, 2005; Zavafer et al., 2017). ROS around PSII can also be generated from two-electron oxidations of water or one-electron reductions of O_2 on the PSII electron donor and acceptor sides of the OEC, respectively (Kale et al., 2017). These conditions lead to the formation of triplet chlorophylls in the PSII reaction centre (P680) by charge recombination, which readily react with O_2, producing $^1O_2$ (Zavafer et al., 2017; Vass et al., 1992; Telfer et al., 1994).

The ROS generated around PSII can cause PSII photoinhibition mainly by oxidising the D1 and D2 proteins at the PSII reaction centre (Aro et al., 1993; Kale et al., 2017). The damaged PSII proteins, mainly D1, are replaced by newly synthesized versions after PSII complex disassembly and degradation in an event called the PSII repair cycle (Aro et al., 1993; Kato et al., 2012; Li et al., 2018). The PSII repair rate depends on light, although it is saturated at low light intensities (Tyystjärvi and Aro, 1996; Allakhverdiev and Murata, 2004). Also, exposure to environmental stresses (such as high light, salt, cold, moderate heat

and oxidative stress) inhibits the PSII turnover as a consequence of the inhibition of the *de novo* D1 protein synthesis at translation level, which also characterises a photoinhibitory condition (Allakhverdiev and Murata, 2004; Takahashi and Murata, 2008).

1.2.3. Photosystem I and its photoinhibition

In plants, the PSI-LHCI supercomplex comprises the PSI core (composed of the membrane-embedded subunits PsaA, PsaB, PsaF, PsaG, PsaH, PsaI, PsaJ, PsaK, PsaL, and the stromal subunits PsaC, PsaD and PsaE) and the peripheral light-harvesting complex I (LHCI) dimers (LHCA1/4 and LHACA2/3) (Qin et al., 2015; Suga et al., 2016; Mazor et al., 2017). Under normal light and normal CBB cycle functioning, electrons are transported from plastocyanin (PC) to Fd through PSI by cofactors P700 and A_0 chlorophylls, phylloquinone A_1, and the iron-sulphur (FeS) centres F_X, F_A, and F_B (Figure 2) (Amunts et al., 2007; Kozuleva and Ivanov, 2016). P700, A_0, A_1 and F_x are held by subunits PsaA and/or PsaB, which form the central heterodimer of PSI and are bound to the majority of the components of the PSI core and antenna (Golbeck, 1992; Ben-Shem et al., 2003; Amunts and Nelson, 2009; Qin et al., 2015; Mazor et al., 2017). The PSI subunit PsaC binds the FeS centres F_A, and F_B and, together with PsaD and PsaE, has a central role for reduction of Fd (Golbeck, 1992; Cashman et al., 2014; Marco et al., 2018). While PsaC establishes close protein contact required for fast electron transfer between the iron-sulfur clusters of PSI and Fd, PsaD and PsaE are responsible for the electrostatic guidance of Fd into the PSI binding pocket (Busch and Hippler, 2011; Marco et al., 2018). There is a consensus that PsaD protein has a central role in the docking of Fd (Barth et al., 1998; Pierre et al., 2002; Cashman et al., 2014; Kapoor et al., 2018). The specific functions of the other PSI subunits are less known, but many of them have been shown to be involved with maintenance and stabilisation of the PSI complex structure (Chitnis, 2001; Jensen et al., 2007; Qin et al., 2015; Mazor et al., 2017).

PSI photoinhibition, similar to that of PSII, is associated with the generation of ROS when electron carriers at the photosynthetic transport chain become highly reduced (Sonoike and Terashima, 1994; Terashima et al., 1994; Grieco et al., 2012; Suorsa et al., 2012; Takagi et al., 2016b). This phenomenon has been reported, for example, under low temperatures (Inoue et al., 1986;

Terashima et al., 1994; Tjus et al., 1998) and under high and fluctuating light (Munekage et al., 2008; Suorsa et al., 2012; Kono and Terashima, 2016; Tiwari et al., 2016). Such over-reduction promotes the generation of ROS when the electron-accepting capacity of the PSI acceptors are saturated and molecular oxygen functions as an alternative acceptor. Reduction of O_2 is thought to occur at the acceptor side of PSI or at the phylloquinone A_1 site, in both cases producing $O_2^{\bullet-}$ (Mehler, 1951; Asada et al., 1974; Takagi et al., 2016b). $O_2^{\bullet-}$ produced can react with FeS centres, generating $^{\bullet}OH$ via the Fenton reaction, which can also attack PSI components and induce its photoinhibition (Inoue et al., 1986; Takahashi and Asada, 1988; Sonoike et al., 1995). Recent findings have shown that not only $O_2^{\bullet-}$ and $^{\bullet}OH$, but $^1O_2$ produced from the reaction between P700 triplet-state (^{3}P700) and O_2, also causes PSI photoinhibition (Takagi et al., 2016b). Photoinhibition of PSI is usually associated with the degradation of PSI core proteins subunits like PsaA and mainly PsaB (Sonoike et al., 1995; Sonoike, 1996; Sonoike et al., 1997; Kudoh and Sonoike, 2002). Degradation of PSI subunits has been recently argued to be a consequence, and not the first step, of PSI damage (Takagi et al., 2016b). Although the knowledge on PSI photoinhibition is expanding, its exact molecular mechanism is still unknown. Studies usually tend to unify the understanding of the mechanism of PSI photoinhibition in higher plants, but this phenomenon can occur differently in different species (Kono and Terashima, 2016; Takagi et al., 2016b; Huang et al., 2017; Yang et al., 2017; Huang et al., 2018). Thus, the relation among PSI photoinhibition, ROS production in PSI and oxidative stress should be interpreted with caution.

Little is still known about PSI recovery from photoinhibition and the consequences on primary metabolism. Also, only a few studies explore aspects of the recovery phase after PSI photoinhibition. Recent studies have shown that PSI photoinhibition severely affects net carbon assimilation, photoprotection, and plant growth (Brestic et al., 2015; Zivcak et al., 2015; Yamori and Shikanai, 2016). However, while PSI is highly resistant to photoinhibition in comparison to PSII (Barth et al., 2001; Huang et al., 2010), PSII recovery occurs faster than PSI (Li et al., 2004; Zhang and Scheller, 2004; Zhang et al., 2011; Tikkanen and Grebe, 2018). For this reason, PSI photoinhibition is believed to have more severe consequences on plant metabolism than PSII photoinhibition under

environmental stresses (Sonoike, 2011; Takagi et al., 2016b; Huang et al., 2017).

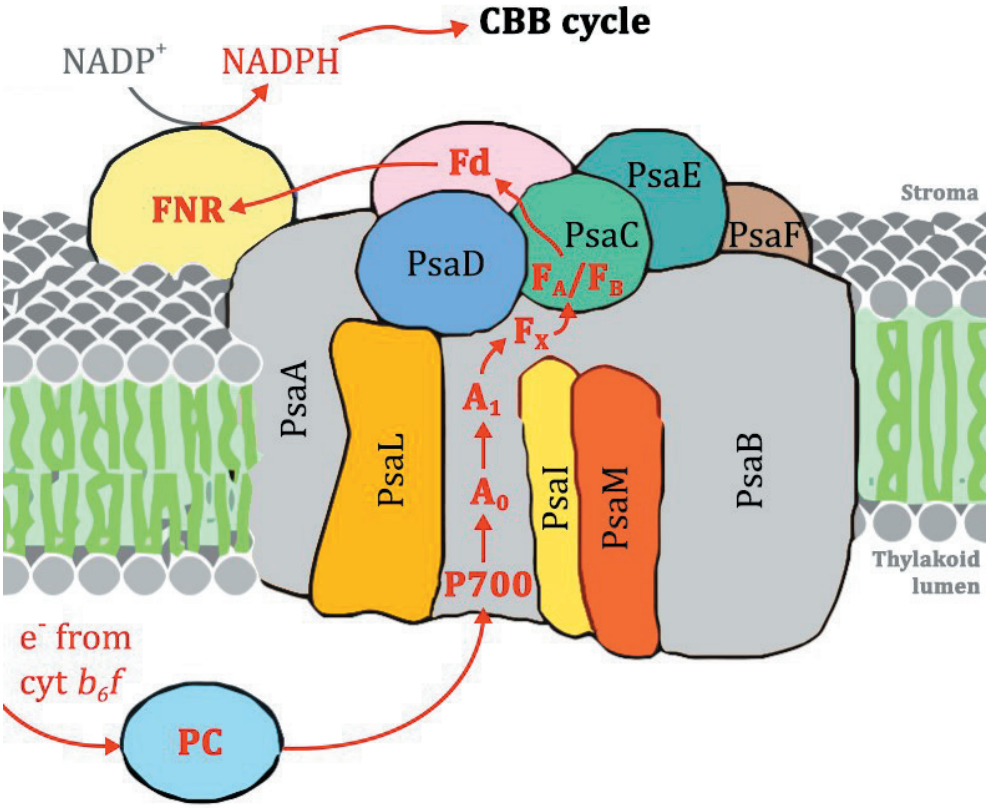

Figure 2. A simplified scheme of the arrangement of the main cofactors and subunits involved in linear electron transport through PSI. CBB cycle = Calvin-Benson-Bassham cycle; Cyt *b6f* = cytochrome *b6f*; Fd = ferredoxin; FNR = ferredoxin:NADP$^+$ oxidoreductase; NADP$^+$ = oxidised nicotinamide adenine dinucleotide phosphate; NADPH = reduced nicotinamide adenine dinucleotide phosphate; PC = plastocyanin. Adapted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map image map00195 (Kanehisa and Goto, 2000) with kind permission.

1.3 Photoprotection

Plants have developed several photoprotective mechanisms to avoid photoinhibition of both photosystems or repair photodamage, which include protection of the photosynthetic apparatus and plant metabolism by regulating light absorption, dissipating absorbed light, balancing photosynthetic electron

transport, effectively consuming the excess of electrons produced from light absorption, and scavenging ROS (Demmig-Adams and Adams, 1992; Takahashi and Badger, 2011; Cazzaniga et al., 2013). The front-line photoprotective mechanism is naturally the avoidance of excessive light absorption, which means physically blocking light from reaching chloroplasts. This includes, for example, the avoidance of light exposure by leaf and chloroplast movement (Kasahara et al., 2002) or by light and/or ultraviolet radiation screening through specific molecules (e.g. phenolic compounds) at the leaf epidermis (Booij-James et al., 2000; Holub et al., 2019).

In case excess light is not avoided, absorbed energy can be dissipated via nonphotochemical quenching (NPQ) of chlorophyll excitation (Ruban et al., 2007). NPQ is a multi-component process, mainly related to its major component, the energy-dependent quenching (qE), which is a consequence of conformational changes within the LHCII proteins that cause the formation of energy traps (Ruban, 2016). The LHCII antenna rearrangement is dependent on protonation of antenna components, mainly the PsbS subunit of PSII, which is involved in the triggering of the dissipation of excess excitation energy as heat (Ruban, 2016; Sacharz et al., 2017). In addition, qE has been shown to be associated with the xanthophyll cycle, in which epoxy groups from xanthophylls (e.g. violaxanthin and antheraxanthin) are enzymatically removed to create zeaxanthin that carries out energy dissipation as heat within LHCII antenna proteins (Goss and Jakob, 2010; Ruban et al., 2012; Sacharz et al., 2017).

Balancing the electron flow through the photosynthetic electron transport in conditions of excessive light absorption is also important to avoid photodamage. Several mechanisms, functioning at different levels of photosynthetic energy conversion, are involved in this balance (reviewed in Tikkanen and Aro 2014). Examples of these mechanisms are (1) the control of the proton gradient between the thylakoid lumen and stroma (ΔpH), which is mostly dependent on the activities of the water-splitting complex in PSII, of cyt *b6f*, and of ATP synthase; (2) the excitation balance between PSII and PSI via LHCII phosphorylation; (3) PSII inactivation; and (4) cyclic electron flow. These mechanisms are interconnected and have an important role in the regulation of plant metabolism to acclimate to diverse environmental changes (Tikkanen and Aro, 2014).

Another important mechanism to avoid or alleviate photoinhibition consists of increasing the capacity for electron consumption by strengthening transitory electron sinks (Padmasree et al., 2002; Alric and Johnson, 2017; Wada et al., 2018). The strongest sink is naturally CO_2 assimilation in the CBB cycle, which uses reducing power produced in the thylakoid electron transport chain for the synthesis of carbohydrates, meaning that this pathway also contributes to avoidance of photoinhibition caused by excessive electron pressure. For example, starch synthesis can serve as a transient sink to allocate excess energy, such as under high light conditions (Paul and Foyer, 2001). In accordance, the excessive accumulation of starch has long been speculated as a negative regulator of photosynthetic activity (Paul and Foyer, 2001; Adams et al., 2013). However, while some studies explore the consequences of PSII photoinhibition in carbohydrate metabolism or source-sink regulation, these subjects are neglected in terms of PSI photoinhibition (Adams et al., 2013). Although the CBB and carbohydrate metabolism probably account for the strongest photosynthetic electron sink, alternative electron transport pathways have been proven to protect plants from photoinhibition (reviewed in Alric and Johnson 2017). The most studied pathways known to be involved in photoinhibition avoidance by electron consumption in plants are photorespiration, mitochondrial respiration (including the alternative oxidase (AOX) pathway), the Mehler reaction within the water-water cycle, and chlororespiration by the plastid terminal oxidase (PTOX).

As previously explained, plants possess a complex antioxidant system which controls their levels of ROS. In case all above-mentioned photoprotective mechanisms are not able to alleviate the electron pressure in the transport chain, ROS can be produced in large quantity and lead to oxidative destruction of cellular components (as detailed in section 1.2.1). Thus, the reinforcement of the ROS-scavenging system is also considered an important photoprotective mechanism (Demmig-Adams and Adams, 1992; Takahashi and Badger, 2011).

1.3.1. Mechanisms for PSI photoprotection

As mentioned above, PSI photoinhibition is harmful to plant fitness because of the slow recovery of PSI, differently from PSII (Takagi et al., 2016b; Huang et al., 2017). This highlights the importance of protecting this

photosystem, which indeed features some specific photoprotective mechanisms. PSI fitness is essentially dependent on the balance between the redox states of its donor side (PC pool) and its acceptor side (Fd pool) (detailed in section 1.2.3).

A key mechanism for PSI protection at the PSI donor side is the establishment of a proton gradient across the thylakoid membrane (ΔpH), which slows the rate of electron transfer from PSII to PSI through acidification of the thylakoid lumen (Joliot and Johnson, 2011; Tikkanen et al., 2015; Shikanai, 2016). The downregulation of electron transfer from PSII to PSI by acidification of the thylakoid lumen is achieved through two different mechanisms. One of them is the activation of the thermal dissipation of excessively absorbed light energy from PSII antennae (usually monitored through the NPQ component qE), which is dependent on xanthophyll quenching and on the interaction between the PsbS protein and the LHCII, as detailed in section 1.3 (reviewed in Ruban 2016). The other mechanism, also known as "photosynthetic control", is the downregulation of cyt *b6f* complex activity (Stiehl and Witt, 1969; Tikhonov, 2014).

The control of electron flow through the cyt *b6f* complex is especially important for protecting PSI from over-reduction under high electron pressure conditions like fluctuating light and high light (Suorsa et al., 2012; Kono et al., 2014; Tikkanen et al., 2015; Takagi and Miyake, 2018). A ΔpH is generated as a consequence of the photosynthetic electron flow, both linear and cyclic, which generates a proton motive force (*pmf*) and allows the production of ATP by ATP synthase (section 1.1.1). The thylakoid lumen acidification is therefore coupled with two important mechanisms, the electron flow and the activity of ATP synthase.

In cyclic electron flow (CEF), electrons are recycled around photosystem I by re-routing them from Fd to PQ. As a result, ΔpH is formed across the thylakoid membrane, leading to the production of ATP without concomitant production of NADPH, thus increasing the ATP:NADPH ratio within the chloroplast (Yamori and Shikanai, 2016). At least two routes for CEF are widely accepted: the PGR pathway, involving PGR5 (PROTON GRADIENT REGULATION 5) and PGRL1 (PGR5-like1); and the NADH dehydrogenase-like complex (NDH)-mediated pathway (Burrows et al., 1998; Munekage et al., 2002; Munekage et al., 2004; Shikanai, 2007; Suorsa, 2015). However, although

PGR5 has been proven to control ΔpH across the thylakoid membrane, the direct involvement of PGR5 in electron transport to PQ, and therefore the existence of a PGR5/PGRL1-dependent pathway, is currently under debate (Nandha et al., 2007; Suorsa et al., 2012; Tikkanen et al., 2012b; Takagi and Miyake, 2018).

Arabidopsis PGR5 is the product of the gene At2g05620. Mature PGR5 is a 10-kDa protein located in the chloroplast thylakoid membrane, sharing high homology with correspondent PGR5 proteins in other photosynthetic organisms (e.g. rice, soybean, algae and cyanobacteria) (Munekage et al., 2002; Okegawa et al., 2007). Several studies have shown that PGR5 is indeed necessary for lumen acidification (Munekage et al., 2002), and, in accordance, for protecting PSI functionality by downregulating the electron flow through the cyt *b6f* complex (Tikkanen et al., 2012a; Tikkanen et al., 2015; Mosebach et al., 2017; Takagi and Miyake, 2018). As a consequence, PGR5 has been reported as an important modulator of the linear electron flow, and this has been attributed as its major role in plants (DalCorso et al., 2008; Suorsa et al., 2016; Takagi and Miyake, 2018). Thus, PGR5 has been shown to be vital for plant viability during environmental acclimation (Suorsa, 2015; Yamori and Shikanai, 2016) although its exact molecular function is unknown.

PSI photoinhibition has been shown to occur in Arabidopsis and rice *pgr5* mutants under high luminosity and fluctuating light conditions because of the excessive accumulation of electrons in the photosynthetic electron chain (Munekage et al., 2002; Kono et al., 2014; Kono and Terashima, 2016; Yamori et al., 2016). The difference between the WT and the *pgr5* mutant in controlling the electron flow through the cyt *b6f* for PSI photoprotection is illustrated in Figure 3.

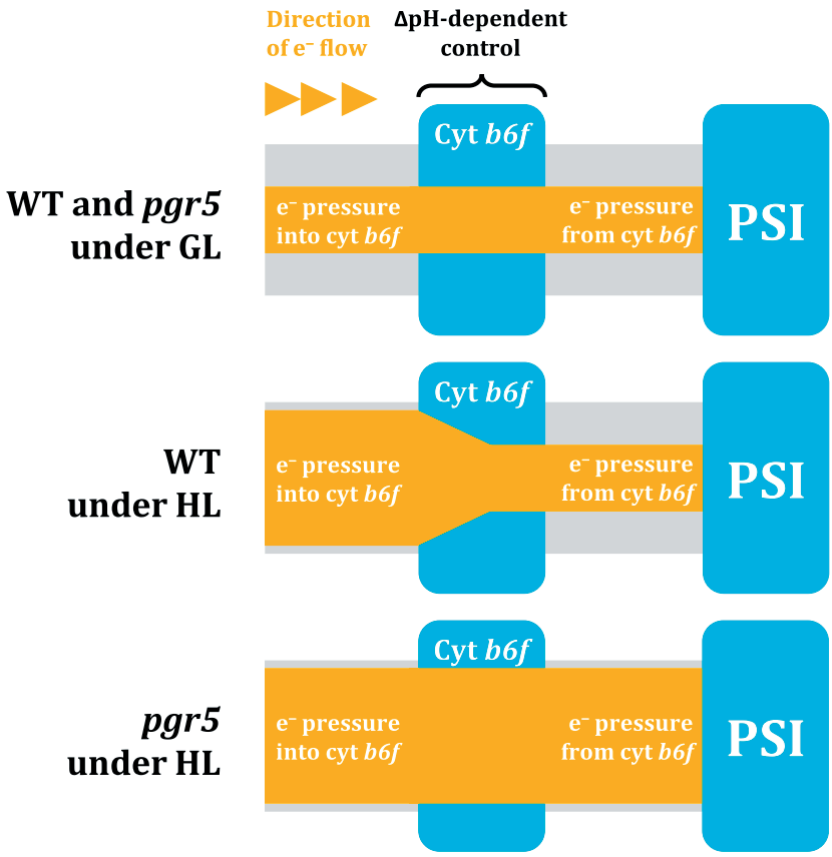

Figure 3. Simplified scheme of the ΔpH-dependent control of the electron pressure through the cytochrome *b6f* (cyt *b6f*) complex in wild-type plants (WT) and the *pgr5* mutant immediately upon transition from growth light (GL) to high light (HL). Under GL, the ΔpH-dependent control of the cyt *b6f* is not engaged. In WT under HL, electron flow through the cyt *b6f* is controlled because the activity of PGR5 protein ensures ΔpH formation across the thylakoid membrane, thus protecting PSI from photoinhibition. However, the *pgr5* mutant is unable to control electron flow through the cyt *b6f* and thus the high electron pressure at PSI induces PSI photoinhibition.

In addition to the regulation of electron flow at the PSI donor side, the CBB cycle and alternative reduction pathways work as electron sinks to alleviate the electron pressure in the electron transport chain and avoid PSI photoinhibition. Several PSI-acceptor-side mechanisms have been proposed to specifically avoid PSI over-reduction, like increases in the electron sink of photosynthesis (i.e. CO_2 assimilation and photorespiration), the water-water

cycle, and mitochondrial alternative oxidase activity (Hodges et al., 2016; Kono and Terashima, 2016; Takagi et al., 2016a; Alric and Johnson, 2017). Although the photoprotective role of photorespiration and mitochondrial metabolism as electron sinks has been gaining attention in the last years, their importance specifically in avoiding PSI photoinhibition has been neglected.

The water-water cycle is believed to be important for protection from photoinhibition by playing a dual function, as ROS scavenger as well as participating in the dissipation of excess photons and electrons as an alternative electron flux (Asada, 1999; Asada, 2000). However, some studies have questioned the role of the water-water cycle as an excess energy dissipator (Driever and Baker, 2011). As defined by Asada (2000), "the water-water cycle in chloroplasts is the photoreduction of dioxygen to water in photosystem I by the electrons generated in photosystem II from water". A key reaction for this process is the Mehler reaction (Mehler, 1951), which occurs when the photoreduction of O_2 in PSI generates $O_2^{\bullet-}$ (as commented in section 1.2.3) followed by its dismutation to H_2O_2 mainly by SOD. The water-water cycle is a consequence of this reaction and includes the reduction of H_2O_2 to water by the thylakoid APX using ascorbate as an electron donor (Asada, 1999; Foyer and Shigeoka, 2011). A broader version of the water-water cycle, named Foyer-Halliwell-Asada cycle, includes the glutathione-dependent ascorbate regenerating system, also known as the ascorbate-glutathione cycle (Foyer and Halliwell, 1976; Foyer and Shigeoka, 2011). Moreover, because the water-water cycle is directly related to the ROS levels and redox balance within the chloroplasts, it can be an important signal trigger specifically related to the photoinhibition of PSI.

2. AIMS OF THE STUDY

Photosystem I (PSI) inhibition has been shown to significantly suppress photosynthesis and growth, which are essential for plant fitness. Additionally, PSI inhibition has been shown to occur under conditions of high light, fluctuating light and chilling in different species, demonstrating the importance of understanding PSI damage, regulation, and protection also for plant improvement under field conditions. However, PSI photoinhibition and recovery has received little attention, especially compared to PSII photoinhibition and recovery. The central hypothesis of this thesis is that PSI damage and photoinhibition induce strong changes to plant metabolism, mainly to PSI downstream components. Therefore, the main aim of this study was to determine and understand the effects of PSI photoinhibition in plant metabolism by investigating its occurrence in *Arabidopsis thaliana* L. mutant lacking the PGR5 protein, treated with excess light conditions. Specific aims of this thesis were:

1. To develop high light-treated *pgr5* mutant as a model system for induction and study of PSI photoinhibition;

2. To investigate the consequences of PSI inhibition on photosynthetic electron transport, gas exchange, carbon assimilation processes and mitochondrial respiration;

3. To detail the dynamics of PSI inhibition, and to characterise the recovery of PSI function after its photoinhibition;

4. To investigate the impact of PSI photoinhibition on chloroplast retrograde signalling, production, and turnover of reactive oxygen species, and induction of oxidative stress.

3. METHODOLOGY

3.1. Plant material and treatments

Arabidopsis thaliana (L.) Heynh. ecotypes Columbia glabra-1 (*gl1*) and Columbia-0 (Col-0) were used as wild-type controls (WT) for the *pgr5* (Munekage et al., 2002) and *npq4* (Li et al., 2002) mutants, respectively. The *npq4* mutant lacks the PsbS protein and thus NPQ, but the control of cyt *b6f* is retained. Therefore, *npq4* was used as a control for *pgr5* wherein both NPQ and cyt *b6f* control are missing (Tikkanen et al., 2015). Plants were grown for six weeks in a phytotron at 23 °C, relative humidity of 60%, 8 h photoperiod under constant white growth light (GL) of 120 or 125 μmol photons m^{-2} s^{-1}. High-light treatments (HL) involved shifting plants from GL to 1000 μmol photons m^{-2} s^{-1} in temperature-controlled growth chambers set at 23 °C. Time of HL treatment lasted 1 h (Papers I and III) or 4 h (Paper II) for most of the experiments, or as described in the figure legends. For the fluctuating light treatment, the plants were exposed to 50 μmol photons m^{-2} s^{-1} for 5 min and to 500 μmol m^{-2} s^{-1} for 1 min, controlled by an automatic shading system over a photoperiod of 8 h/16 h (light/dark), similarly to previous experiments (Tikkanen et al., 2010; Grieco et al., 2012; Suorsa et al., 2013). Recovery treatments involved returning plants treated with HL or fluctuating light to GL. Other treatments used in this thesis were performed as explained in the figure legends. All the experiments were repeated at least twice and at least three biological replicates were used in every experiment.

3.2. Photochemistry

Photochemistry analyses are detailed in Papers I, II and III. The photochemical parameters of PSI and PSII were simultaneously measured based on chlorophyll *a* fluorescence (Schreiber et al., 1995) and the P700 absorbance (Klughammer and Schreiber, 1998), using a WALZ Dual-PAM-100 system (Papers I and III) or a WALZ Kinetic LED-Array Spectrophotometer (KLAS) (Paper II). Pm and Fm measurements were taken from detached leaves after 30 min of dark acclimation. Light-dependent measurements (Fo, F', Y(NA), Y(ND), and NPQ) were taken after 5 min exposure to each tested actinic light

intensity after the dark acclimation. Changes in redox states of ferredoxin (Fdm) and plastocyanin (PCm) were measured in intact leaves with a KLAS, through the deconvolution of their respective absorbance signals (Klughammer and Schreiber, 2016). Measurements were performed as previously described (Schreiber and Klughammer, 2016; Schreiber, 2017)

3.3. Gas exchange parameters

Evaluation of gas exchange parameters is detailed in Papers I, II and III. Leaves were acclimated in the dark for 15 min and leaf gas exchange parameters were measured in 400 ppm CO_2 (Papers I, II and III) and 2000 ppm CO_2 (Paper I) at 23 °C, using the LI-COR LI-6400XL Portable Infrared Gas Analyzer system (IRGA). Photosynthetic photon flux density (PPFD) values inside IRGA's chamber were set as shown in each figure. Data were taken after IRGA parameters reached a steady-state value following the onset of the respective PPFD (usually around 120 s).

3.4. Mitochondrial respiration

Day respiration was estimated using the data obtained with 0 PPFD from IRGA measurements, as described in section 3.3 and detailed in Paper II. O_2 uptake was measured for 5 min in darkness at 23 °C using an Unisense 'OX-NP' oxygen microsensor, from three detached leaves submerged in 50 mM sodium phosphate buffer (pH 7.2), as detailed in Paper II. Leaves were dark acclimated for at least 15 min prior to each O_2 consumption rate measurement.

3.5. Carbohydrate quantification

Frozen leaves were oven dried at 60 °C for 72 h for the determination of starch, glucose, and fructose contents, as detailed in papers I and II. Starch content was measured using a total starch assay kit (Megazyme K-TSTA assay kit). After ethanolic extraction (80% v/v) at 99 °C for 15 min, glucose and fructose contents were determined using the Sucrose/Fructose/D-Glucose test kit (Megazyme K-SUFRG assay kit). All assays were performed according to the manufacturer's protocol. Leaves from the same plants were fixed with

glutaraldehyde and starch accumulation was analysed through transmission electron microscopy (TEM) imaging.

3.6. Leaf membrane damage

Leaf membrane damage (MD) was estimated through the electrolyte leakage method (Blum and Ebercon, 1981), as shown in Paper III. Detached leaves were placed in tubes containing deionized water and incubated in a shaking water bath at 25 °C for 24 h. After measuring the first electric conductivity (L1), the solution was heated at 95 °C for 1 h and then cooled to 25 °C, after which the second electric conductivity (L2) was measured. Membrane damage was calculated as MD = (L1/L2) × 100.

3.7. 12-Oxo-phytodienoic acid, H_2O_2 and singlet oxygen quantifications

12-Oxo-phytodienoic acid (OPDA) abundance was quantified by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) in frozen leaves after extraction in methanol, as described in Paper I. H_2O_2 content was quantified using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies) according to the manufacturer's protocol, as detailed in Paper III. Singlet oxygen ($^1O_2$) trapping was performed in isolated thylakoids as previously described (Yadav et al., 2010) using a Miniscope (MS5000) electron paramagnetic resonance (EPR)-spectrometer equipped with a variable temperature controller (TC-HO4) and Hamamatsu light source (LC8), as shown in Paper I.

3.8. Histochemical detection of superoxide and hydrogen peroxide

Nitroblue tetrazolium (NBT) and diaminobenzidine (DAB) staining, as detailed in Paper III, were performed in leaves after the light treatments for detection of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), respectively, as previously described (Ogawa et al., 1997; Thordal-Christensen et al., 1997). After staining, leaves were treated with ethanol-chloroform bleaching solutions and results were compared through their photographs.

3.9. Lipid peroxidation imaging and quantification

Lipid peroxidation, as detailed in Papers I and III, was evaluated through autoluminescence imaging and quantification of thiobarbituric acid-reactive substances (TBARS). Autoluminescence analyses were performed in detached leaves and rosettes after 2 h of dark incubation according to the method described in Birtic et al. (2011). The luminescence signal was collected for 20 min using an IVIS Lumina II system (Caliper Life Sciences) containing an electrically-cooled charged-couple device (CCD) camera, which allowed obtaining autoluminescence images for evaluation. TBARS were extracted from frozen leaves in TCA acid and supernatants were evaluated based on the formation of thiobarbituric acid-malondialdehyde complex, as previously described (Heath and Packer, 1968).

3.10. Enzymatic activity assays

Enzymatic activity assays are detailed in Paper III. Total protein was extracted from whole leaves in a potassium phosphate buffer (final concentration of 100 mM; pH 7.0) containing EDTA (final concentration of 1 mM) and used for enzymatic activity assays. Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined based on inhibition of nitro blue tetrazolium chloride (NBT) photoreduction (Giannopolitis and Reis, 1977). Catalase (CAT; EC 1.11.1.6) activity was based on the reduction of H_2O_2 (Beers and Sizer, 1952; Havir and McHale, 1987). Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured based on the oxidation of ascorbate (ASC) (Nakano and Asada, 1981). Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was assayed based on the generation of monodehydroascorbate (MDHA) free radicals by ascorbate oxidase (AO; 1.10.3.3) and following oxidation of NADH (Hossain et al., 1984). Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was assayed based on the oxidation of glutathione (GSH) (Nakano and Asada, 1981).

3.11. Western blotting

Western blotting procedures are detailed in Papers II and III. Thylakoids were isolated from mature leaves as previously described (Järvi et al., 2011). Total thylakoid proteins (Paper II) or total leaf proteins (Paper III) were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes and blotted with polyclonal antibodies against PsaB, PsaC, PsaD and LOX-C.

3.12. RNA isolation and transcriptome analysis

Transcriptomics-related methods are detailed in Paper I and Paper III. Total RNA was isolated from frozen leaves using TRIsure (Bioline) according to the protocol supplied, with an additional final purification in 2.5 M LiCl overnight at -20 °C, and used for RNAseq library construction. Libraries were sequenced in 50 bp single-end reads using Illumina Hiseq 2500 technology (BGI Tech Solutions). Reads were aligned to the reference genome build *Arabidopsis thaliana* TAIR 10 and quantified using the DESeq R package. Gene expression fold changes were calculated using a two-way ANOVA test with Benjamini-Hochberg *p*-value correction. Analyses of enriched Gene Ontology for Biological Process (GO-BP) terms were performed using the enrichment analysis tool of the Gene Ontology Consortium.

4. OVERVIEW OF THE RESULTS

4.1. Characterization of PSI photoinhibition in high light-treated *pgr5* mutants

In order to evaluate PSI photoinhibition, the maximum oxidation capacity of P700 at the PSI reaction centre (Pm) was monitored as an indicator of PSI functionality. The results showed that *pgr5* mutants have levels of oxidisable PSI (Pm) around 25% lower in normal growth light conditions (GL; 125 μmol photons m^{-2} s^{-1}) when compared to wild-type plants (WT) (Figure 1 in Paper I; Figure 1b in Paper II; Figure 1a in Paper III). However, Pm values in *pgr5* mutants decreased to lower than 25% of the WT value within only 1 h of high light (HL; 1000 μmol photons m^{-2} s^{-1}), while Pm in WT was almost unaffected (Figure 1 in Paper I; Figure 1b in Paper II; Figure 1a in Paper III). Pm seemed to reach its minimum value in PSI-photoinhibited plants within only 1 h of HL, and remained this low for at least the next 5 h of the excess light treatment (Figure 1 in Paper I; Figure 1b in Paper II; Figure 1a in Paper III). In accordance, HL-induced weaker PSI donor side limitation (Y(ND)) and stronger PSI acceptor side limitation (Y(NA)) in *pgr5* mutants compared to WT, illustrating unregulated electron transport to PSI in excess of the capacity of stromal electron acceptors from PSI (Figure 2a and 2b in Paper I). The recovery of Pm in HL-treated *pgr5* plants occurred over a period of 4 days, after which time the Pm value of *pgr5* plants was restored to a similar level to that of untreated plants (Figure 1b in Paper II). Similar results were obtained in an experiment using a fluctuating light (FL) treatment, in which FL-treated *pgr5* took more than 5 days to recover to the values observed in the GL treatment (Figure 4). No significant difference was observed between GL- and HL-treated WT plants during the recovery experiment (Figure 1b in Paper II). Furthermore, PSI photoinhibition correlated with the depletion of the PsaB subunit, but not PsaC nor PsaD, of the PSI complex after HL as well as during PSI recovery (Figure 2 in Paper II).

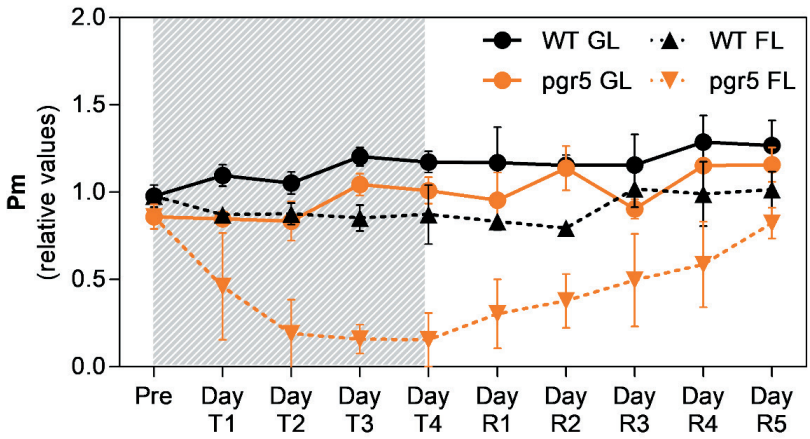

Figure 4. The impact of fluctuating light (FL) treatment on PSI in the *pgr5* mutant. The maximum amount of oxidisable P700 (Pm) in WT and *pgr5* plants treated with growth light (GL, 125 µmol photons m^{-2} s^{-1}) or FL (50 µmol photons m^{-2} s^{-1} for 5 min, 500 µmol m^{-2} s^{-1} for 1 min), controlled by an automatic shading system over a photoperiod of 8 h/16 h (light/dark). Error bars show standard deviation among replicates (n = 4). Significant differences between treatments and between genotypes occurred when error bars do not overlap (Student's t-test, $p < 0.05$). The shaded area represents the time during which the fluctuating light treatment was applied to the FL-treated plants. Measurements of FL-treated plants occurred from day T1 to day T4, whereas measurements with plants in the recovery phase occurred from day R1 to day R5.

4.2. Effects of high light on the photosynthetic electron transport chain of *pgr5* mutants

Photoinhibition of PSI induced malfunction in several components of the photosynthetic electron transport chain (Figure 2 in Paper I; Figure 1 in Paper II; Figure 1 in Paper III). A strong decrease of the maximal reduction state of Fd (Fdm) was observed in *pgr5* mutants, but not in WT, after a 4-h HL treatment (Figure 1c in Paper II). This decrease in Fdm, as well as its recovery, were correlated to the Pm values in HL-treated *pgr5* mutants (Figures 1c and 1e in Paper II), suggesting that PSI photoinhibition led to relative oxidation of the Fd pool, which is the first PSI electron acceptor in linear electron flow. However, no significant changes were observed in the maximum oxidation state of plastocyanin (PCm) after the HL treatment or during the recovery phase (Figure 1d in Paper II), suggesting no correlation between PSI photoinhibition

and the redox state of the plastocyanin pool, the PSI electron donor (Figure 1f in Paper II).

To assess PSII function , the parameters F'/Fm and Fm were used to avoid the confounding effect of PSI photoinhibition on fluorescence, which influences the Fv/Fm parameter because of the effect of PSI status on Fo values (Tikkanen et al., 2017) (Figure S1b in Paper II; Figure 1c and 1d in Paper III). F'/Fm increased in GL-treated *pgr5* that were subjected to HL, demonstrating an increase in the number of closed PSII reaction centres, while this was not observed in the WT (Figure 2c in Paper I). The effects of HL in the F'/Fm parameter measured in *pgr5* mutants may be a consequence of the lack of ΔpH-dependent NPQ in these plants, as observed by measuring NPQ (Figure 2d in Paper I). However, HL-treated *pgr5* mutants showed high values for F'/Fm even when measured under low light, which may be due to limited PSI activity and consequent over-reduction of the electron transport chain (Figure 2c in Paper I). This idea is supported by Fm values measured in WT and *pgr5* mutants after 4 h HL treatment (Figure 1a in Paper II) and from 1-5 h (Figure 1b in Paper III), which in *pgr5* mutants were much lower than those of the GL treatment. Together, these results show that, while PSI photoinhibition occurred only in HL-treated *pgr5* mutants, HL induced PSII photoinhibition in both the WT and the *pgr5* mutant. However, the photoinhibition of PSI (measured through Pm) in *pgr5* mutants was clearly much stronger and more rapid than of PSII (measured through Fm) (Figures 1a and 1b in Paper II; Figures 1a and 1b in Paper III). The recovery of PSII function was also more rapid than that of PSI (Figures 1a and 1b in Paper II).

4.3. Effects of PSI photoinhibition on CO_2 assimilation and gas exchange

High light treatments induced impaired CO_2 assimilation rate (*A*) in *pgr5* mutants compared to WT in all experiments (Figure 3 in Paper I; Figure 3 in Paper II; Figures 2 and 3 in Paper III). Light curves (*A*-PPFD curves) under 400 ppm CO_2 showed that HL-treated *pgr5* mutants have lower *A* compared to WT under all irradiances used in the curve, although the difference between the genotypes was most pronounced under lower irradiances. However, no differences between GL-pre-treated *pgr5* and WT were observed (Figure 3 in Paper I; Figure 3 in Paper III). The effect of HL on CO_2 assimilation was detailed

using a time-course experiment of PSI photoinhibition (Figure 2 in Paper III). This experiment clearly showed the negative impact of HL-induced PSI photoinhibition on *A* in *pgr5* mutants, mainly within the first 30 minutes of the light stress. During illumination with GL directly after 1 h of HL treatment, *A* in *pgr5* mutants was approximately 0, whereas WT exhibited the same *A* rates as before undergoing the HL treatment. In a second HL treatment after 1 h recovery in GL, *A* values were approximately equivalent to the rates observed before the end of the previous HL treatment for both genotypes, which was 35% lower in *pgr5* than in WT. In comparison to the first HL treatment, the rate of increase in *A* during the second HL treatment was slower in both WT and *pgr5*. The rate of *A* decline was similar between WT and *pgr5*, and smaller when compared to the first HL treatment for both genotypes (Figure 2 in Paper III).

To better understand the consequences of PSI damage and recovery on CO_2 assimilation and its relevance under different light intensities, *A* of HL-pretreated *pgr5* mutants and WT were assessed under low (50 µmol photons m^{-2} s^{-1}), growth (125 µmol photons m^{-2} s^{-1}) and high (1000 µmol photons m^{-2} s^{-1}) irradiances (Figure 3 in Paper II). In each case, *pgr5* showed a distinct inhibition of *A* immediately after the HL treatment; however, the magnitude of the decrease depended on the intensity of the light used for the measurement. The impact of PSI photoinhibition on *A* in *pgr5* mutants was greater under lower irradiances. For example, *A* in HL-treated *pgr5* mutants was restored to the pre-treatment level after only 1 day of recovery when measured under high irradiance, while 3 days of recovery was required to restore normal *A* in the same plants when measured under low irradiance (Figure 3 in Paper II).

HL-treated *pgr5* exhibited higher internal CO_2 concentration (C_i), mainly under the lowest irradiances of the *A*-PPFD curve (Figure 3b in Paper III). In accordance, lower *A* in HL-treated *pgr5*, compared to HL-treated WT, was also observed under high CO_2 concentrations (2000 ppm) (Figure 3b in Paper I). These results show that lower assimilation rates in *pgr5* mutants compared to the WT, both after HL, was not associated with CO_2 limitation. In addition, the stomatal conductance (g_s) and the transpiration rates (*E*) of HL-treated *pgr5* and WT were similar, showing that the lower *A* in HL-treated *pgr5*, compared to HL-treated WT, is also not associated with stomatal limitation (Figures 3c and 3d in Paper III). As a consequence of these results, lower maximum

carboxylation efficiency (A/C_i) and water use efficiency (WUE) were observed in HL-treated *pgr5* in comparison to HL-treated WT.

4.4. Effects of PSI photoinhibition on carbohydrate accumulation and mitochondrial respiration

The effects of PSI photoinhibition on carbohydrate accumulation was studied through the evaluation of starch, fructose and glucose contents (Figure 4 in Paper I; Figure 4 in Paper II). The results show that HL induced a strong accumulation of starch in the WT, while only slight accumulation occurred in *pgr5* mutants, both compared to the GL treated controls (Figure 4 in Paper I; Figure 4a in Paper II). During the first day of recovery under GL after the HL treatment, the starch content strongly decreased in *pgr5*, reaching less than half of the content observed in untreated plants, while in the WT the starch content was slightly higher compared to GL-treated WT (Figure 4 in Paper I; Figure 4a in Paper II). The starch content in *pgr5* gradually recovered over a period of 3 days, until it reached GL levels (Figure 4a in Paper II). The HL treatment induced substantial increases in glucose and fructose concentrations in both WT and *pgr5* leaves, but the increase in *pgr5* was approximately half of that in the WT for both sugars (Figure 4b and 4c in Paper II). Glucose content in WT and *pgr5*, which was similar during the whole recovery phase, was slightly lower during the initial 2 days of recovery than in GL-treated controls (Figure 4b in Paper II). No differences between genotypes nor between light treatments were observed for the fructose content during the recovery phase (Figure 4c in Paper II).

As the mitochondrial respiration is directly related to photosynthetic energy production, day and night respiration rates were evaluated in leaves of GL- and HL-treated WT and *pgr5* plants (Figure 5 in Paper II). Day respiration was much higher in HL-treated WT plants than in HL-treated *pgr5*, in relation to their respective GL, but no differences between the genotypes or between the light treatment were observed during the recovery phase (Figure 5a in Paper II). O_2 uptake measurements were performed for 4 h in the dark in order to evaluate the importance of night respiration during the first night after the HL treatment (Figure 5b in Paper II). While the rate of decrease in O_2 uptake was equivalent in both GL-treated genotypes, HL-treated WT had a three-fold slower decrease in O_2 uptake rate compared to HL-treated *pgr5*, which in turn

was similar to that of GL-treated plants (Figure 5b in Paper II). Additionally, night respiration was assessed to investigate possible differences in comparison to the day respiration (Figure 5c and 5d in Paper II). O_2 uptake during night-time respiration showed no significant changes for HL-treated WT throughout the experiment, whereas in the *pgr5* mutants night-time respiration decreased in the second night after the HL treatment and was restored to the level of GL-treated plants by the following night (Figure 5d in Paper II).

4.5. Reactive oxygen species accumulation and oxidative stress in *pgr5* mutants after PSI photoinhibition

The relationship between PSI photoinhibition and oxidative stress was evaluated after GL and 1 h HL treatments of WT and *pgr5* mutant plants through several different approaches described below, which showed no major differences between the genotypes (Figure 7 in Paper I; Figures 4, 5, 6 and 7 in Paper III). The HL treatment induced membrane damage, which is a consistent marker of oxidative stress, in both genotypes; however, no differences were observed between the genotypes in either light treatment (Figure 4a in Paper III). H_2O_2 content showed no differences between genotypes or light treatments (Figure 4b in Paper III), and histochemical analysis showed similar accumulations of H_2O_2 (Figure 4c in Paper III) and superoxide (Figure 4d in Paper III) in both HL-treated WT and the HL-treated *pgr5* mutant. HL induced $^1O_2$ production, but no differences were observed between the WT and the *pgr5* mutant under both light conditions (Figure 7 in Paper I).

Total activities of Foyer-Halliwell-Asada cycle enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) were quantified. The only significant differences were increased MDHAR activity in the HL-treated WT, compared to *pgr5*, and increased DHAR activity in the HL-treated *pgr5* mutant, compared to WT. Additionally, higher CAT activity was detected in the *pgr5* mutant compared to the WT (Figures 5b, 5d and 5e in Paper III). The abundance of transcripts encoding enzymes in the Foyer-Halliwell-Asada cycle was also evaluated in WT and *pgr5* prior to HL treatment, as well as after 15 min and 1 h HL exposure. Most genes were upregulated by HL treatment in both WT and *pgr5* plants but, similarly to the

enzyme activities, there were no strong differences between gene expression of the analysed enzymes in the two genotypes (Figures 6 in Paper III).

Lipid oxidation was also measured as a marker to evaluate the occurrence of oxidative stress. The results clearly show that 1 h HL treatment induces a decrease in the lipid oxidation levels of *pgr5* mutants (Figure 7 in Paper III). For example, the content of thiobarbituric acid reactive substances (TBARS) was similar between the WT and the *pgr5* mutant under GL, but decreased only in the *pgr5* mutant after 1 h HL treatment (Figure 7a in Paper III). Similarly, the increased autoluminescence signal induced by HL occurred in the WT (Figure 7b and 7c in Paper III), while there was no corresponding increase in lipid peroxidation signal in HL-treated *pgr5*. Finally, the abundance of the chloroplast lipoxygenase (LOX-C) was shown to be lower in *pgr5* compared to the WT in both light treatments (Figure 7d in Paper III).

4.6. Chloroplast retrograde signalling in PSI-photoinhibited *pgr5* mutants

The effects of PSI photoinhibition on chloroplast retrograde signalling is closely related to results on ROS and lipid oxidation described above. The transcriptome profiles of *pgr5* mutants were shown to be severely altered during light stress and recovery. The low occurrence of oxidative stress in HL-treated *pgr5* plants were supported by an analysis of enriched Gene Ontology for Biological Process (GO-BP) terms in lists of genes differentially expressed in the mutants (Figure 5; Table 1 in Paper I). The results show that several GO-BP terms related to signalling and/or oxidative stress are downregulated in *pgr5* compared with WT under GL and even more after 1 h HL (Figure 5A; Table 1 in Paper I). Some of the 31 enriched GO-BP terms of downregulated genes in *pgr5* compared with WT under GL are “response to hydrogen peroxide” (GO:0042542), “response to reactive oxygen species” (GO:0000302), and “response to oxidative stress“ (GO:0006979), in addition to several other GO-BP terms related to stressful conditions and signalling (Figure 5A; Table 1 in Paper I). 62 GO-BP terms were enriched in downregulated genes in *pgr5* after 1 h HL treatment, compared with HL-treated WT. These include several terms related to lipid peroxidation and jasmonic acid metabolism. For example, 6 GO-BP terms directly related to jasmonic acid (JA) metabolism are present in the top 10 most enriched GO-BP terms of the list (Figure 5B; Table 1 in Paper I).

Upregulated genes in GL-treated *pgr5* compared with WT contained no enriched GO-BP terms, and only five GO-BP terms were classified as statistically enriched genes upregulated in *pgr5* after HL treatment ("intracellular sequestrating of iron ion", "sequestrating of iron ion", “hormone metabolic process", "regulation of hormone levels", and "regulation of biological quality") using the criteria selected for this study (Figure 5C; Table 1 in Paper I).

In accordance with the analysis of enriched GO-BP terms, several specific genes related with oxylipin biosynthesis and signalling (e.g. lipoxygenases and JA signalling regulation factors), and abiotic stress response (e.g. heat shock protein chaperones and the cytosolic APX2) were strongly downregulated in *pgr5* mutants in comparison to the WT after 1 h HL (Table 2 in Paper I). Interestingly, even more genes in the list were further downregulated during the recovery treatment (1 h under GL after the 1 h of HL) in comparison to the 1 h HL treatment (Table 2 in Paper I). Additionally, a clustered heatmap of HL-responsive genes showed that approximately 400 genes induced by 12-oxophytodienoic acid (OPDA), which is an oxylipin hormone and chloroplast precursor for JA, were downregulated in *pgr5* compared to WT in the 1-h HL treatment and in the recovery (1 h of GL after 1 h HL) (Figure 5 in Paper I). In accordance, the relative quantification of OPDA showed that *pgr5* mutants indeed have a lower abundance than the WT before and after HL, as well as after 1 h recovery in GL (Figure 6 in Paper I). Transcriptomics analysis also revealed that H_2O_2-responsive genes were upregulated in both genotypes after 1 h HL treatment, but were under-expressed in *pgr5* compared to the WT. This is in accordance with results showing that PSI damage limits the occurrence of oxidative stress, suggesting compromised chloroplast retrograde signalling.

Figure 5. Enriched Gene Ontology for Biological Process (GO-BP) terms in lists of genes differentially expressed in *pgr5* mutants. Genes in *pgr5* mutants treated with growth light (GL; 125 µmol m^{-2} s^{-1}; A) and high light (HL; 1000 µmol m^{-2} s^{-1}; B and C) for 1 h with expression values lower than 0.5 (downregulated; A and B) and higher than 2 (upregulated; C) compared to WT under the respective light treatments were submitted to the enrichment analysis tool of the Gene Ontology Consortium (http://geneontology.org) using Fisher's exact test with FDR correction (≤0.05). Only GO terms with fold enrichment values higher than 2.0 are shown.

5. DISCUSSION

The proper balance between light reactions and electron consumption is important to maintain fluent photosynthetic activity during environmental conditions that are constantly changing. When photosynthetic electron transport exceeds the capacity of electron acceptors, saturation of electron carriers in the photosynthetic electron transport chain can lead to the photoinhibition of photosystem II (PSII) and photosystem I (PSI). Both conditions are limiting for plant fitness and crop yield (Barber and Andersson, 1992; Adams et al., 2013; Kromdijk et al., 2016; Kaiser et al., 2018; Slattery et al., 2018), but much less is known about PSI photoinhibition in comparison to PSII photoinhibition. Although PSI has been considered to be more stable than PSII for most of the species and environmental conditions (Barth et al., 2001; Huang et al., 2010), PSI can be very sensitive to photodamage under certain conditions such as fluctuating light and chilling stress under moderate light, which are typical conditions in nature (Sonoike, 1996; Scheller and Haldrup, 2005; Sonoike, 2011). In this thesis, high light-treatments of Arabidopsis *pgr5* mutants were used to investigate the dynamics of PSI photoinhibition, and its consequences on photosynthetic electron transport, primary metabolism, ROS production and chloroplast retrograde signalling of plants during stress and recovery.

5.1. The role of PROTON GRADIENT REGULATION 5

Thanks to studies using the *pgr5* mutant, the importance of PGR5 in PSI protection has been slowly revealed over recent years. The role of PGR5 in limiting the overreduction of the acceptor side of PSI, thus preventing PSI photoinhibition, has been known for more than 17 years (Munekage et al., 2002). This function has been credited to the existence of a PGR5-mediated cyclic electron flow (CEF) around PSI (Munekage et al., 2002; Munekage et al., 2004), which may be compared to the NADH dehydrogenase-like (NDH)-mediated CEF pathway (Burrows et al., 1998; Shikanai et al., 1998). Despite this, there has been no direct demonstration that PGR5 is involved in electron transport to plastoquinone. Although the exact mechanism of PGR5 photoprotection of PSI is not known and the molecular function of this protein

has not been fully resolved to date, there is a consensus about the role of PGR5 in the establishment of the proton gradient (ΔpH) across the thylakoid membrane through lumen acidification. As a consequence, PGR5 has an essential role in preventing overreduction of the photosynthetic electron transport chain, and thus avoiding photoinhibition, by regulating the activation of NPQ and downregulation of electron flow through the cytochrome *b6f* complex (Tikhonov, 2014; Tikkanen and Aro, 2014). The exact function of PGR5 has been intensively investigated in many recent studies of rice and Arabidopsis *pgr5* mutants (Suorsa et al., 2012; Tiwari et al., 2016; Yamori et al., 2016; Kawashima et al., 2017; Wada et al., 2018; Wang et al., 2018; Yamamoto and Shikanai, 2019). The current study did not aim at determining the mechanism of action of PGR5. Instead, the work in this thesis aimed to exploit the effect of PGR5 in photoprotection of PSI, using Arabidopsis *pgr5* mutants as an experimental tool to better understand the consequences of PSI photoinhibition. Nonetheless, the results presented here clearly show that PGR5 has an essential function in controlling the electron pressure at the donor side of PSI and in avoiding PSI photoinhibition.

5.2. PSI is rapidly photoinhibited and recovers slowly in high light-treated *pgr5* mutants

PSI photoinhibition has been previously reported under high irradiance and fluctuating light conditions in Arabidopsis and rice *pgr5* mutants (Munekage et al., 2002; Suorsa et al., 2012; Kono et al., 2014; Kono and Terashima, 2016; Tiwari et al., 2016; Yamori et al., 2016). In the current work, PSI photoinhibition was shown to occur rapidly under conditions of a severe imbalance between photosynthetic electron transport and acceptor capacity, as is the case for HL-treated *pgr5* mutant (Paper III). Indeed, several results in this thesis show that an exposure of *pgr5* mutants to HL (1000 μmol photons m^{-2} s^{-1}) for 15 min is enough to induce strong PSI photoinhibition followed by severe impairments on plant metabolism (Papers I and III). Rapid inhibition of PSI in *pgr5* presumably occurred due to a rapid increase in the production of ROS that subsequently inactivated the PSI FeS clusters (Sonoike, 2011). This result demonstrates the susceptibility of PSI to photoinhibition, in spite of the fully

operational ROS detoxification network including SOD and ascorbate cycle enzymes in *pgr5* (Paper III).

Previous studies with plants of different species treated with chilling stress under moderate light showed that PSI damage takes much more time to fully recover its activity when compared to PSII (Li et al., 2004; Zhang and Scheller, 2004; Zhang et al., 2011). For this reason, PSI photoinhibition is believed to have more severe consequences than PSII photoinhibition in higher plants (Takagi et al., 2016b; Huang et al., 2017). The results in the current study highlight the importance of PGR5-dependent regulation of the ΔpH across the thylakoid membrane to avoid PSI photoinhibition under natural environmental conditions by showing that PSI recovers very slowly in Arabidopsis *pgr5* mutants treated with high light or fluctuating light (Figure 4; Paper I; Paper II). Gradual recovery of Pm in PSI-photoinhibited plants (HL-treated *pgr5* plants) was accompanied by gradual recovery of CO_2 assimilation measured under low light, which was restored to the pre-treatment level after 3 days of recovery (Paper II). This demonstrates that, although the PSI pool of HL-treated *pgr5* mutants experienced severe photoinhibition, CO_2 assimilation was still possible, which allowed plants to recover (Paper II). PSI functionality despite severe photoinhibition was probably partly enabled by LHCII phosphorylation, which increases the quantity of excitation directed towards PSI (Wientjes et al., 2013; Grieco et al., 2015), improving the efficiency of PSI (Tiwari et al., 2016). These observations may also suggest recruitment of a hypothetical reserve of PSI in order to support electron transport under conditions of damaged PSI that was evident in HL-treated *pgr5* mutants (Paper II). Indeed, a stable intermediate in PSI assembly named PSI*, that contains only a specific subset of the PSI core subunits, (Ozawa et al., 2010; Wittenberg et al., 2017; Marco et al., 2018) is a candidate to restore PSI function by renewing the damaged PSI pool. However, further experiments are necessary to test this hypothesis.

5.3. PSI photoinhibition and recovery affects photosynthetic electron transport and limits electron flow to PSI acceptor side

The results obtained here show that PSI photoinhibition was accompanied by changes in other components of the photosynthetic electron transport chain. For example, HL clearly induced photoinhibition not only of PSI

but also of PSII in *pgr5* as measured by maximum chlorophyll *a* fluorescence (Fm). However, these results were expected because the effects of HL on PSII photoinhibition has been known for a long time (reviewed in Aro et al., 1993; Gururani et al., 2015). Indeed, not only *pgr5* mutants but also the WT experienced some level of PSII damage in all HL-treatments. The photoinhibition of PSII was however much less severe than PSI photoinhibition in HL-treated *pgr5*, as shown by the relative difference between PSII and PSI parameters after HL. This is due to over-reduction of the intersystem when PSI is inactivated.

Several results in this thesis provide strong evidence that PSI photoinhibition limits electron flow to its acceptors. For example, ferredoxin (Fd) capacity was strongly decreased in HL-treated *pgr5* plants and followed the same recovery pattern as for PSI capacity, as shown by a strong positive correlation between the maximal reduction state of Fd (Fdm) and Pm. However, no changes were observed for the plastocyanin (PC) capacity during PSI photoinhibition or during its recovery (Paper II). Interestingly, the oxidation of Fd pool was not associated with any changes in thylakoid Fd abundance (Paper II, Figure S2), rather suggesting that inhibited PSI was unable to reduce its primary electron acceptor Fd. The low capacity for reduction of the Fd pool, and the normal capacity of oxidation of the PC pool, which directly donates electrons to PSI, both under conditions of PSI photoinhibition (Paper II), are key evidences that PSI photoinhibition limits electron flow to PSI acceptors. These observations were supported by the findings of low limitation of electron transfer to the donor (lumenal) side of PSI (Y(ND)) and high limitation of electron transfer from the acceptor (stromal) side of PSI (Y(NA)) in *pgr5* mutants under HL. This means that, under conditions of PSI photoinhibition, electrons are delivered to PSI but do not efficiently flow to downstream pathways. Indeed, the metabolic events downstream of PSI presented as results in this study were clearly downregulated in the PSI-photoinhibited plants used in this thesis. For example, CO_2 assimilation was clearly negatively affected by PSI inhibition in HL-treated *pgr5* leaves in the current study (Papers I, II and III). Consequently, other downstream pathways dependent on CO_2 assimilation were also downregulated in HL-treated *pgr5* mutants. This is the case, for example, for sugar and starch accumulation, and mitochondrial respiration. Low PSI-dependent ROS production were also observed in *pgr5* mutants under

HL (Paper I and III), indicating that the O_2 reduction rate was also downregulated as a consequence of PSI inhibition, similarly to the other PSI downstream pathways. Furthermore, low lipid oxidation and attenuated chloroplast signalling mediated by oxylipins in HL-treated *pgr5* may also be effects of limited PSI electron transport. These events are summarised as a hypothetical scheme showing the limitation in electron flow to the PSI acceptor side and the dependent metabolism involved (Figure 6), which are discussed in the following sections.

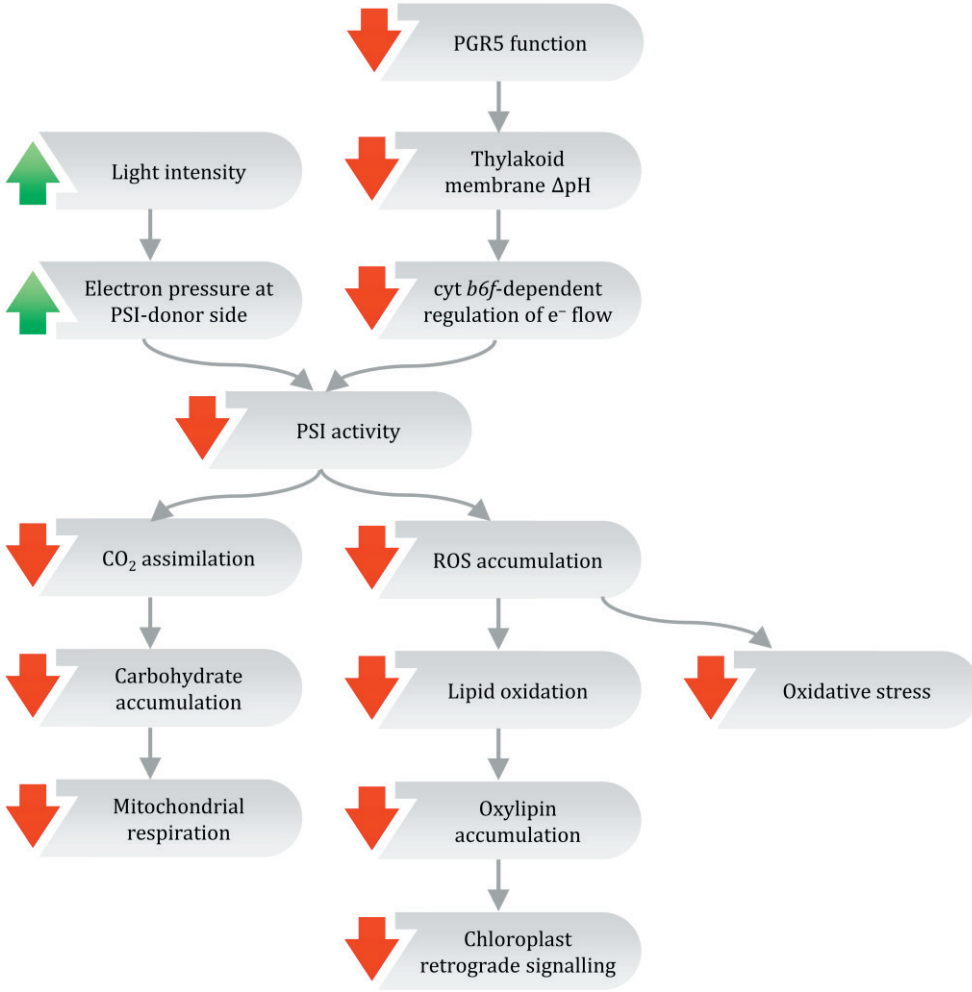

Figure 6. Causes and consequences of PSI photoinhibition on plant metabolism observed in this thesis.

5.4. PSI photoinhibition induces a strong metabolic penalty

The current studies highlight the sustained negative impact of PSI photoinhibition on plant metabolism, including metabolic processes directly related to crop production like CO_2 assimilation, carbohydrates accumulation and mitochondrial respiration. The current results show that HL-treated *pgr5* mutants have low CO_2 assimilation rates, as previously reported (Munekage et al., 2008; Nishikawa et al., 2012). The primary reason for the low CO_2 assimilation in HL-treated *pgr5* mutants was probably the effect of severe PSI photoinhibition on limiting the stromal content of NADPH to supply the CBB cycle. A secondary reason may have been the low reduction levels of the stromal thioredoxin network mediated by the ferredoxin-thioredoxin reductase (FTR), resulting in an impaired redox activation of the CBB cycle enzymes under non-saturating light conditions (Haldrup et al., 2003; Nikkanen et al., 2016; Souza et al., 2018).

PSI photoinhibition also induced altered carbohydrate metabolism. The data show diminished starch accumulation during HL treatment of *pgr5* mutants, as well as during recovery under GL conditions (Papers I and II). Starch synthesis can serve as a transient sink to allocate excess reducing power, like under HL conditions (Paul and Foyer, 2001), suggesting a lack of excess reductants after PSI photoinhibition that is consistent with diminished PSI activity. Although *pgr5* mutants were able to synthesize D-glucose and D-fructose, the concentration increases for these sugars were half of those observed for WT leaves. Changes in leaf starch concentration could also be correlated with lower accumulations of D-glucose and D-fructose in *pgr5* during HL treatments, as starch synthesis has been linked to soluble sugar concentrations (Paul and Foyer, 2001). The fact that the sugar concentrations quickly decreased after the HL treatment mainly in *pgr5* mutants may be related to the plant's demand for energy to recover from HL stress. This would be in agreement with results observed during the recovery phase, in which HL-treated plants, mainly the *pgr5* mutants, slowly recover their starch concentration to the GL levels (Paper II). In addition, the lower starch concentration was an expected result in HL-treated *pgr5* mutants because CO_2 assimilation decreased as a consequence of PSI photoinhibition.

The data presented here demonstrate that PSI damage in HL-treated *pgr5* mutants also limits mitochondrial respiration during both day and night (Paper III), in accordance with other recently published data (Florez-Sarasa et al., 2016). Although the regulatory link between mitochondria and photosynthesis has been demonstrated through different pathways and mechanisms, many fundamental questions regarding this cross-talk are unanswered. For example, little is known about the consequences of PSI photoinhibition on plant respiration and the role of mitochondria, an important source of energy in the cell, on PSI recovery. It is well accepted that reducing equivalents generated in the chloroplasts can be transported to other locations in the cell, including mitochondria, via shuttle machineries such as the malate/oxaloacetate shuttle (Heineke et al., 1991; Raghavendra and Padmasree, 2003; Scheibe, 2004; Vishwakarma et al., 2015; Alric and Johnson, 2017). Specifically, carbohydrates produced from photosynthesis can generate respiratory substrates for the mitochondria like malate and pyruvate through cytosolic glycolysis (O'Leary and Plaxton, 2016; O'Leary et al., 2017), making mitochondria important electron sinks during conditions of high electron pressure in the chloroplast transport electron chain. Recently, night-time leaf respiration rate has been shown to correlate with stored carbon substrates, including starch, in Arabidopsis (O'Leary et al. 2017). These observations are in agreement with the lower mitochondrial respiration caused by lower carbohydrate synthesis in PSI-photoinhibited *pgr5* mutants, which in turn was a consequence of low CO_2 assimilation. Indeed, the plant mitochondrial respiration is mostly dependent on carbohydrates (Plaxton and Podestá, 2006). Thus, the low mitochondrial activity in HL-treated *pgr5* mutants may be a consequence of low malate/oxaloacetate shuttle activity and low carbohydrate availability, both being consequences of low PSI activity.

5.5. PSI photoinhibition prevents oxidative stress

Photosynthetic electron transport generally occurs in an oxygen-rich environment, and the transfer of electrons or energy to oxygen is a frequent occurrence. Thus, the photosynthetic electron transport chain is associated with the generation of ROS which, although important in plant signalling, can cause oxidative stress when accumulated in cells (Czarnocka and Karpiński,

2018; Foyer, 2018; Mullineaux et al., 2018). The results presented here show no greater occurrence of oxidative stress in PSI-photoinhibited plants, compared with control plants, with the exception of PSI photoinhibition itself that is thought to occur through oxidative inactivation of FeS clusters. Additionally, the data clearly show lower lipid oxidation in HL-treated *pgr5* compared to HL-treated WT, which is attributed to lower oxidative stress (Mueller, 2004; Mosblech et al., 2009; Wasternack and Hause, 2013) (Paper III) and under-expression of genes associated with H_2O_2 signalling (Paper I). The absence of any abnormally high accumulation of ROS or oxidative stress in HL-treated *pgr5* could be the result of an efficient scavenging and antioxidant system. However, no substantial increase in ROS scavenging capacity was observed in the PSI-photoinhibited plants (Paper III). Instead, the results shown here suggest that the rapid occurrence of PSI photoinhibition stops the transfer of electrons to O_2, thus preventing excess production of ROS. In accordance, a recent study showed that the production rate and the accumulation of ROS is probably not related to PSI photoinhibition (Takagi et al., 2016b). Furthermore, the same study suggests that the ROS production site, rather than the quantity of ROS, is critical for PSI photoinhibition (Takagi et al., 2016b), which is in accordance with the results presented here. Therefore, PSI photoinhibition seems to prevent oxidative stress by downregulating ROS production because the inactivated PSI pool is probably unable to donate electrons to molecular oxygen. This hypothesis is in line with the other results of this thesis which show that photoinhibition of PSI blocks the electron flow to its electron acceptors, impairing their downstream events.

ROS and their oxidation products generated in chloroplasts can also serve as important signalling mechanisms for plant reprogramming, which is required to face changes in the environment (Geigenberger and Fernie, 2014; Gollan et al., 2015; Dietz et al., 2016). The results presented here clearly show that oxylipin signalling, which is a chloroplast retrograde signalling pathway dependent on lipid peroxidation (Pintó-Marijuan and Munné-Bosch, 2014; Satoh et al., 2014; Gollan et al., 2015; Savchenko et al., 2017), was severely affected in the *pgr5* mutant, being more evident under HL, when this pathway is activated in WT plants (Figure 3; Paper I). The oxylipin metabolic pathway includes the 12-oxophytodienoic acid (OPDA), which is produced in the chloroplast from polyunsaturated fatty acids, after enzymatic peroxidation by

lipoxygenase (LOX) (Howe, 2018). Both, OPDA and chloroplastic LOX, were shown to be downregulated in *pgr5* mutants under GL and HL (Papers I and III), in line with the disrupted oxylipin-dependent chloroplast signalling observed in the mutant. The lower levels of lipid peroxidation observed in HL-treated *pgr5* (Paper III) are also in line with its downregulated oxylipin-dependent chloroplast signalling since lipid peroxidation is an early step in enzymatic oxylipin synthesis and provides the material for oxylipin production (Mueller, 2004; Mosblech et al., 2009; Wasternack and Hause, 2013). These findings, in addition to the consistent results about the photoinhibition of PSI in the HL-treated *pgr5* mutants, suggest that PSI activity is important for chloroplast retrograde signalling through both the oxylipin-dependent and H_2O_2-dependent pathways.

6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This thesis investigated the detrimental impact of photosynthetic imbalance on PSI and revealed important details about the depletion and restoration of photosynthesis and primary metabolism after severe PSI photoinhibition. The data presented here show new insights into the occurrence of PSI photoinhibition and its negative consequences on plant metabolism and chloroplast retrograde signalling. Highlight findings of this thesis were:

1. High light treatment of the *pgr5* mutants is a valuable model for the study of PSI photoinhibition and recovery, as well as the study of related phenomena including the reduction state of photosynthetic electron carriers;

2. PSI photoinhibition is rapidly induced under conditions of reduction-pressure imbalance between PSI donor and acceptor sides, which severely inhibits CO_2 fixation, carbohydrate accumulation and mitochondrial respiration;

3. Plants are able to rapidly recover their CO_2 fixation despite PSI inhibition, by improving PSI efficiency through LHCII phosphorylation and activation of "reserve" PSI;

4. Chloroplast regulation of nuclear gene expression is dependent on PSI activity under high light stress through enzymatic oxylipin synthesis and H_2O_2 production;

5. Inactivation of PSI can be a protective mechanism against oxidative stress in the chloroplast stroma and in the wider cell by preventing ROS over-production.

Although the use of *pgr5* mutant combined with high light treatments has been shown in this thesis and in literature as a very good model for studying PSI photoinhibition, future work on this topic involving other model systems

could strengthen the conclusions obtained here. For example, the use of other mutants with compromised PSI activity and/or protection, or other methods for inducing PSI photoinhibition (Sejima et al., 2014; Tikkanen and Grebe, 2018), are promising perspective for deepening the knowledge on PSI photoinhibition. In addition, ongoing work to determine the exact function of the PGR5 protein opens a vast field for exploration and should receive more research attention.

This study strengthens the importance of regulation of balance between the photosynthetic light reactions and CO_2 fixation, which is vital for normal photosynthesis, carbon metabolism and chloroplast signalling, thus contributing to plant fitness. Some attempts for plant improvement focusing on upregulation of photosynthetic electron transfer have neglected the importance of developing strong electron sinks, including the maintenance of CO_2 assimilation and carbohydrate metabolism. Findings in this thesis show that strong electron sinks and protection of the stromal components of photosynthesis are ultimately important. In addition, these events are essential for the maintenance and protection of the electron transport chain at the thylakoid membrane. Therefore, this thesis highlights the importance of considering the prospect of damage and recovery of PSI, and the consequent impact on plant metabolism, as well as the importance of balancing photosynthetic electron transfer in thylakoids with stromal sink strength, during development of bioengineering strategies designed to improve yield in crop plants.

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