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REVERSE GENETIC, PROTEOMIC AND PHYSIOLOGICAL APPROACHES TO UNCOVER THE ROLE OF RICE CHLOROPLASTIC APXs IN DROUGHT AND LIGHT RESPONSES

FORTALEZA
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Orientador: Prof. Dr. Joaquim Albenísio Gomes da Silveira.
Coorientador: Prof. Dr. Milton Costa Lima Neto.

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Dedico esta tese ao meu amado avô Vicente (*In memoriam*). Sentirei saudades eternamente.

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“No one can whistle a symphony. It takes a whole orchestra to play it.”

Halford E. Luccock

RESUMO

A produção de espécies reativas de oxigênio (EROs) é comum ao metabolismo da aeróbico das plantas, porém, quando essa produção atinge níveis excessivos, em situação de estresse abiótico por exemplo, importantes processos como a fotossíntese são fortemente prejudicados. Para combater o excesso de EROs, as plantas apresentam um extenso sistema de defesa antioxidante, com as enzimas peroxidases do ascorbato (APX) exercendo um importante papel em toda a célula. Para compreender a função das isoformas cloroplásticas de APX (chlAPXs) e sua atuação para a manutenção da eficiência fotossintética em plantas de arroz (*Oryza sativa* L.) sob condição de estresse por seca moderada e excesso de luz, este trabalho utilizou uma abordagem integrativa, avaliando aspectos genéticos, proteômicos, bioquímicos e fisiológicos. Para isso, plantas de arroz foram silenciadas (RNAi) individualmente nas isoformas de APX estromal (sAPX – plantas *apx7*) e tilacoidal (tAPX – plantas *apx8*) e seus desempenhos fotossintéticos e de crescimento foram avaliados por meio de perfis proteômicos e fisiológicos em condições normais e de estresse moderado de seca. Plantas *apx7* apresentaram perfil proteômico muito semelhante às plantas NT, embora apresentem um leve aumento na acumulação de H_2O_2 em condições normais de crescimento. Por outro lado, o silenciamento da tAPX levou a intensa alteração proteômica, culminando em uma redução no crescimento e maior acumulação de H_2O_2 quando comparadas às plantas NT e *apx7*. Interessantemente, plantas *apx7* apresentaram um intenso aumento na atividade das isoformas citosólicas de APX (cAPX) e pouca alteração na atividade total de chlAPX. Por outro lado, o silenciamento da tAPX desencadeou uma redução na atividade das cAPX e das sAPX, levando a um comprometimento da atividade total das chlAPX. Provavelmente, tais alterações ocorreram devido ao processo de silenciamento, uma vez que plantas *apx7* e *apx8* apresentaram, aproximadamente, 50% e 90% de redução nos transcritos de *OsApx7* e *OsApx8*, respectivamente. Provavelmente em decorrência dessas e de outras mudanças genéticas, bioquímicas e fisiológicas, plantas *apx7* apresentaram fenótipo morfológico semelhante ao das plantas NT. Porém, plantas *apx7* exibiram melhor performance de crescimento, fotossíntese e de indicadores de estresse em resposta a seca moderada. Aparentemente, a redução parcial dos transcritos de *OsApx7* induz mudanças pós-transcricionais e pós-traducionais, permitindo um arranjo gene-proteína-metabólico mais eficiente para lidar com o estresse de seca. Porém, não foi possível estabelecer relação de causa e efeito entre o proteoma e o fenótipo. Por outro lado, plantas *apx8* demonstraram menor capacidade em

aumentar a abundância de proteínas em resposta à seca, principalmente proteínas envolvidas na fotossíntese, metabolismo redox e de resposta ao estresse, quando comparadas com plantas NT na mesma condição. De fato, plantas *apx8* demonstraram maior dano de membrana, menor conteúdo relativo de água e menores valores de fotossíntese, enquanto que, inversamente, plantas *apx7* mantiveram altos níveis de carboxilação da Rubisco (Vcmax) e parâmetros de trocas gasosas. Além disso, as plantas *apx8* apresentaram um aumento na fotorrespiração em relação às NT, principalmente após o estresse de seca, indicando uma maior dissipação de excesso de energia. Sendo assim, foram investigados quais outros mecanismos possíveis de fotoproteção teriam sido desencadeados pelas plantas *apx8* em situações de moderada e alta pressão de elétrons (luz moderada e alta). Plantas *apx8* apresentam aumento no fluxo cíclico de elétrons e NPQ, principalmente em situações de alta pressão de elétrons, provavelmente como forma de dissipar energia na compensação do desbalanço causado ao ciclo água-água com o silenciamento da tAPX, evitando ou minimizando a fotoinibição do fotossistema I. Os resultados obtidos neste trabalho auxiliam no estudo sobre a função das chlAPXs e regulação da fotossíntese.

Palavras-chave: Peroxidase do ascorbato. Fotossíntese. Mecanismos fotoprotetores.

ABSTRACT

Reactive oxygen species (ROS) production is common to plant aerobic metabolism, but when this production reaches excessive levels, e.i. abiotic stress, important processes such as photosynthesis are strongly impaired. To combat the excess of ROS, plants have an extensive antioxidant defense system, with the ascorbate peroxidase (APX) enzymes playing an important role throughout the cell. In order to understand the role of APX chloroplast isoforms (chlAPXs) and its performance for the maintenance of photosynthetic efficiency in rice plants (*Oryza sativa* L.) under conditions of moderate drought and excess light, this thesis used an integrative approach, evaluating genetic, proteomic, biochemical and physiological aspects. For this, rice plants were silenced (RNAi) individually for the stromal APX (sAPX - *apx7* plants) and thylakoidal (tAPX - *apx8* plants) isoforms of APX and their photosynthetic and growth performances were evaluated under normal conditions and moderate drought stress. *apx7* plants presented a proteomic profile very similar to NT plants, although they present a slight increase in the accumulation of H_2O_2 under normal growth conditions. On the other hand, the tAPX silencing led to intense proteomic alteration as well as growth reduction and greater accumulation of H_2O_2 when compared to NT and *apx7* plants. Interestingly, *apx7* plants showed an intense increase in APX cytosolic isoform activity (cAPX) and little change in total chlAPX activity. On the other hand, tAPX silencing triggered a reduction in the activity of cAPX and sAPX, reducing the total activity of chlAPX. Probably, such changes occurred due to the silencing process, since *apx7* and *apx8* plants presented approximately 50% and 90% reduction in the transcripts of *OsApx7* and *OsApx8*, respectively. Probably as a result of these and other genetic, biochemical and physiological changes, *apx7* plants presented a morphological phenotype similar to NT plants. However, *apx7* plants exhibited better growth performance, photosynthetic efficiency and stress indicators in response to moderate drought. The partial reduction of *OsApx7* transcripts appears to induce post-transcriptional and post-translational changes, allowing for a more efficient gene-protein-metabolic arrangement to cope with the stress of drought. However, it was not possible to establish a cause and effect relationship between the proteome and the phenotype. On the other hand, *apx8* plants showed lower ability to increase protein abundance in response to drought, especially proteins involved in photosynthesis, redox metabolism and stress response when compared to NT plants in the same condition. In fact, *apx8* plants showed higher membrane damage, lower relative water content and lower photosynthesis values, while *apx7*

plants maintained high levels of Rubisco carboxylation (*Vcmax*) and gas exchange parameters. In addition, *apx8* plants showed an increase in photorespiration in relation to NT, especially after drought stress, indicating a greater dissipation of excess energy. Therefore, it was investigated which other possible mechanisms of photoprotection would have been triggered by *apx8* plants in situations of moderate and high electron pressure (moderate and high light). *apx8* plants have an increase in the cyclic electron flow and NPQ, mainly in situations of high pressure of electrons, probably as a way to dissipate energy in the compensation of the unbalance caused to the water-water cycle with the silencing of tAPX, avoiding or minimizing the photoinhibition of the photosystem I. The results obtained in this thesis are important to understand the function of chlAPXs and their role in regulation of photosynthesis.

Keywords: Ascorbate peroxidase. Photosynthesis. Photoprotective mechanisms.

ABBREVIATION LIST

APX	Ascorbate peroxidase	$O^{\bullet-}_2$	Superoxide anion
$^1O_2$	Singlet oxygen	$OH^{\bullet}$	Hydroxil radical
apx8	tAPX silenced rice plants	PET	Photosynthetic electron transport
ASC	Ascorbate	PN	Net photosynthesis
CAT	Catalase	PPFD	Photosynthetic photon flux density
CEF	Cyclic electron flow	PQ	Plastoquinone
chlAPX	Chloroplastic APX	PRX	Peroxiredoxin
Ci	Intercellular CO_2 partial pressure	PSI	Photosystem I
DHA	Dehydroascorbate	PSII	Photosystem II
E	Transpiration	RNAi	Interference RNA
ETRI	PSI electron transport rate	ROS	Reactive oxigen species
ETRII	PSII electron transport rate	Rubisco	Ribulose 1,5 bisphosphate carboxylase/oxygenase
Fv/Fm	Max potential PSII quantum efficiency	sAPX	Stromal APX
GPX	Glutathione peroxidase	SOD	Superoxide dismutase
Gs	Stomatal conductance	tAPX	Thylakoidal APX
GSH	Glutathione	TRX	Thioredoxin
H_2O_2	Hydrogen peroxide	Vcmax	Rubisco carboxylation rate
Jmax	Photosynthetic electron transport	Y(I)	Effective quantum yield of PSI
LEF	Linear electron flux		
MDHA	Monodehydroascorbate	Y(II)	Effective quantum yield of PSII
NPQ	Non-photochemical quenching	Y(NA)	Quantum yield of non-photochemical energy dissipation due to acceptor-side limitation of PSI
NT	Non-transformed plants	Y(ND)	Quantum yield of non-photochemical energy dissipation due to donor-side limitation of PSI

CONTENTS

1 INTRODUCTION

Currently, due to climatic changes and global warming, projections have been shown that the temperature is increasing in the entire world and leading to intense drought areas, especially in low latitudes areas such as Brazil (Rosenzweig et al., 2014; Sakschewski et al., 2014). This scenario, together with the population growth, will require the increase of food demand, which can be a problem without improvement of agricultural technology methods (Swatuk et al., 2015). Rice production has an immense socio-economic impact on human civilization and is the second most important commodity worldwide. In 2016, Brazil produced 11.78 million tonnes of rice, being the 9th larger producer worldwide (FAO, 2016). Also, rice is an important and representative model of cereal food crops and monocotyledons in scientific research (Bennetzen, 2002). Therefore, understanding photosynthetic and photoprotective mechanisms of important agricultural cultures, such as rice, is crucial to increase photosynthetic yield and improve abiotic stress response.

Rice plants are sensitive to several abiotic stresses but especially milder water stress at both vegetative and reproductive stages (Todaka et al., 2015). The effect of drought or high light on rice plants considerably varies with genotypes, different developmental stages, and degree and duration of stress (Kato et al., 2007). In literature, the role of rice chloroplastic APX (chlAPX) during abiotic stress exposure is still insipient. Most of the studies on the effect of the lack or deficiency of chlAPXs have been carried out with *Arabidopsis*; however, studies have shown that plants differ widely in their mechanisms of stress response (Mullineaux and Baker, 2010). Thus, a better understanding of the role played by chlAPXs in monocot crop models such as rice could contribute to a more complete understanding of plant tolerance to abiotic stress.

ChlAPX are classified into thylakoid-bound (tAPX) and stroma-localized forms (sAPX). When the supply of CO_2 to chloroplast is limited or the activities of Calvin-Benson cycle enzymes are inhibited by environmental stress, electrons excised from water at PSII are transferred to oxygen by PSI, resulting in the formation of superoxide radical ($O^{\bullet-}_2$) (Mittler, 2016). The membrane-attached copper/zinc superoxide dismutase (Cu/Zn-SOD) subsequently convert $O^{\bullet-}_2$ into hydrogen peroxide (H_2O_2). tAPX act as the first layer of ROS scavenging and reduces H_2O_2 back into water using ascorbate as an electron donor (Kangasjärvi et al., 2008). Also, the H_2O_2 is removed by sAPX as the second layer in stroma (Miyake, 2010). These reactions participate of the water-water cycle, a mechanism that comprehends the production and scavenging of ROS by enzymes such as Cu/Zn-SOD and chlAPX as well as the regeneration of non-enzymatic molecules in chloroplasts (Asada, 1999).

Maintaining electron flow through the photosynthetic membrane, even under stressful conditions, is therefore vital for preventing damage to plant cells (Cai et al., 2017). In literature, chlAPX, especially tAPX, are considerate to be the source of a bottleneck in the water–water cycle, at least in higher plants, because of their high susceptibility to H_2O_2 (Shigeoka et al., 2002; Foyer and Shigeoka, 2011). This characteristic may affect the capacity of plants to tolerate stress and makes it a good target for improving plant stress tolerance (Maruta et al., 2016). However, a number of recent studies have unexpectedly failed to find a "stress-sensitive phenotype" among chlAPX loss-of-function mutants (Giacomelli et al., 2007; Kangasjärvi et al., 2008; Maruta et al., 2010; Caverzan et al., 2014). Compensation by other antioxidant enzyme(s) represents a plausible explanation for the negligible phenotype of chlAPX deficient mutants.

Apparently, chlAPX knocked down mutants can acclimate and compensate for reduction in this enzymatic activity (Caverzan et al., 2014). Thus, it seems like transient and fluctuating environmental conditions are needed to challenge the mutants and to pinpoint to

the molecular and physiological consequences of the specific defects (Dietz, 2016). Many evidences support the function of ROS as signalling molecules in plant stress responses (Mittler et al., 2011; Shigeoka and Maruta, 2014). During the course of development and environmental fluctuations, ROS are induced and mediates acclimatory changes at diverse levels of regulation or stress perception (Foyer and Noctor, 2005). A previous study demonstrated that the conditional knockdown of tAPX reduces the expression of a large set of ROS-responsive genes under high light stress, suggesting a signalling role of chloroplastic H_2O_2 in stress response (Maruta et al., 2012).

Therefore, some questions still to be solved involving chlAPX importance for antioxidant metabolism in plants. Is chlAPX important to scavenge and to maintain H_2O_2 homeostasis in chloroplasts? How to identify other possible mechanisms? The current thesis is focused on photosynthesis and photoprotection mechanisms and the importance of chlAPX on these mechanisms, especially under abiotic stress exposure.

2 HYPOTHESES

The hypothesis of this thesis is that chloroplastic APXs display a more significant role as signalling redox enzymes through a tight regulation of the H_2O_2 homeostasis instead of the antioxidant protection *per se*, contributing to maintain the photosynthetic efficiency in rice plants submitted to drought and high light exposure.

3 OBJECTIVES

The general objective of this thesis is to elucidate the individual contribution of stromal and thylakoidal APX to chloroplast antioxidant protection and modulation of photosynthetic activity in rice plants submitted to drought and high light.

In order to achieve the general objective of this thesis, some specific goals were proposed:

1. Perform an integrative review in order to understand the current state-of-the-art involving the chloroplast redox systems, the importance of H_2O_2 signalling and the activation of photoprotective mechanisms in plants;
2. Evaluate physiological changes caused by RNAi-knocking down of both chloroplastic APX isoforms in rice plants through proteomic and physiological approaches to characterize the importance of stromal and thylakoidal APX in rice crop model;
3. Investigate which proteins/pathways are regulated in response to stromal and thylakoidal APX silencing in rice plants submitted or not to mild drought stress;
4. Identify the photoprotective mechanisms possibly triggered by rice plants after silencing of thylakoidal APX to overcome this isoform deficiency and avoid photoinhibition in moderate and high light exposure.

(Unpublished review)

4 H_2O_2 SIGNALLING, REDUNDANT SCAVENGING PEROXIDASES AND PHOTOPROTECTIVE MECHANISMS: AN INTEGRATED VIEW OF RICE CHLOROPLAST DEFENSE SYSTEMS

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Introduction

The first atmospheric oxygen molecules (O_2) most likely appeared on Earth about 2.3-3.8 billion years ago, permitting the evolution of an enormous variety of aerobic organisms that use O_2 as electron acceptor (Mittler, 2016). However, aerobic metabolism naturally produces partially reduced or activated forms of atmospheric oxygen, known by reactive oxygen species (ROS). If kept unrestrained, ROS concentrations will increase in cells and cause oxidative damage to membranes (lipid peroxidation), proteins, RNA and DNA molecules, and can even lead to the oxidative destruction of the cell in a process termed oxidative stress (Sies et al., 2017). Higher plants have thus evolved a complex and integrated environment–cell, cell–cell, cell–organelle and organelle-organelle signalling circuits that protect themselves and use ROS as signalling molecules (Mignolet-spruyt et al., 2017).

Acting as signalling molecules, ROS are highly versatile and it has also functions as oxidants of proteins, altering their conformation/function, and of lipids, releasing signal-active compounds (Dietz, 2016). Nevertheless, each cell possesses a redox regulatory network whose state is adjusted by ROS and virtually controls all processes such as gene expression and translation, cell metabolism and protein turnover (Noctor and Foyer, 2016). They are primarily formed in chloroplasts, mitochondria, and peroxisomes, but also at any other cellular compartment that includes proteins or molecules with a sufficiently high redox potential to excite or donate an electron to oxygen (Mittler et al., 2004). In the recent years,

many researches have witnessed significant advancements in understanding the cross talk between organelles in orchestrating the cellular redox state (Foyer and Noctor, 2003; Galvez-Valdivieso and Mullineaux, 2010; Maruta et al., 2012; Dietz et al., 2016a; Exposito-Rodriguez et al., 2017).

Today, there is still a need to understand the mechanisms by which different ROS signals are conditionally transmitted from the plastid to the cytosol, mitochondrion and nucleus and how they feed into the regulatory network of the cell (Noctor et al., 2017). These redox interactions between intracellular compartments are key to stress responses and developmental processes, with non-radical species such as H_2O_2 playing major second messenger functions (Foyer et al., 2017). Among the most sensitive targets for H_2O_2 are thiol peroxidases like glutathione peroxidase and peroxiredoxins. Oxidized peroxiredoxins oxidize redox transmitters such as thioredoxins, which in turn oxidize target proteins (Yoshida and Hisabori, 2016). Besides thiol peroxidases, ascorbate peroxidases are also critical components involved with oxidative stress response in photosynthetic organisms, however they use different electron donors and are linked to distinct redox networks (Noctor et al., 2017).

One of the most important question is why plants contain so many peroxidase systems. Dietz (2016) showed that there are eight hypotheses for this: different types of peroxidases have distinct sub organellar association (1), as well as different substrate preferences (2); there are also metabolic couple of ROS detoxification and the alteration of protein function (3); the protein abundance of peroxidase types is different in response to specific environmental stress (4); their activities are differentially regulated (5); they interact with different proteins (6); each peroxidase is involved in a specific and at least partly unique metabolic and signalling context (7) and they are specifically regulated in dependence on environmental and developmental cues (8). In this review, we will provide an update on findings related to ROS production, H_2O_2 signalling between chloroplast and nuclei, the

redundancy of chloroplast peroxidases types and the consequences of their deficiency in rice plants.

Producing ROS in chloroplasts: reactions and reactivity

Photosynthesis is the key-process to convert light energy to chemical energy. This process begins in chloroplasts thylakoidal membranes, where light harvesting complexes collects energy in specific wavelengths to transport electrons from H_2O to $NADP^+$, producing NADPH. The electron transport chain (ETC) generates a proton gradient (ΔpH) within the lumen and chloroplast stroma which is necessary to produce proton motive force that lead to ATP synthesis (Mano, Endo and Miyake, 2016). These reductant equivalents (ATP and NADPH) are produced on photochemical phase of photosynthesis and utilized by Calvin-Benson cycle reactions to assimilate CO_2 and produce of triose-phosphate. Besides, ATP, NADPH and reduced ferredoxin (Fd_{red}) produced on photochemical phase are also utilized in other metabolical processes such as assimilation of N, S, photorespiration, and others.

Light drives photosynthesis, however, in excess, it can be prejudicial because can produce great amounts of ROS (Apel and Hirt, 2004) (Figure 1). Superoxide anion ($O^{\bullet -}_2$) and its protonated form, the perhydroxyl radical $HO^{\bullet}_2$, are both ROS but also considerate free radicals because the added single electron to O_2 is in the unpaired state (Mittler, 2016). They are produced within photosynthetic electron transport chain (PET) by two mechanisms (i) Reduced plastoquinone donates electrons to O_2 via plastid terminal oxidase (PTOX) and (ii) Over-reduced PSI transfers electrons from ferredoxin to O_2 (Mehler reaction) (Dietz, 2016). Superoxide can react with another very influential signalling free radical species, nitric oxide (NO•), to yield peroxynitrite ($OONO^-$) (Gill and Tuteja, 2010). $OONO^-$ is highly reactive however not toxic in plants, which appear to function without problem even in the presence of elevated levels of this metabolite (Foyer and Shigeoka, 2011). It is likely that $OONO^-$ is

produced in chloroplasts, where it may fulfill signalling functions, since protein S-nitrosylation can modify both protein function and activity (Michelet et al., 2013).

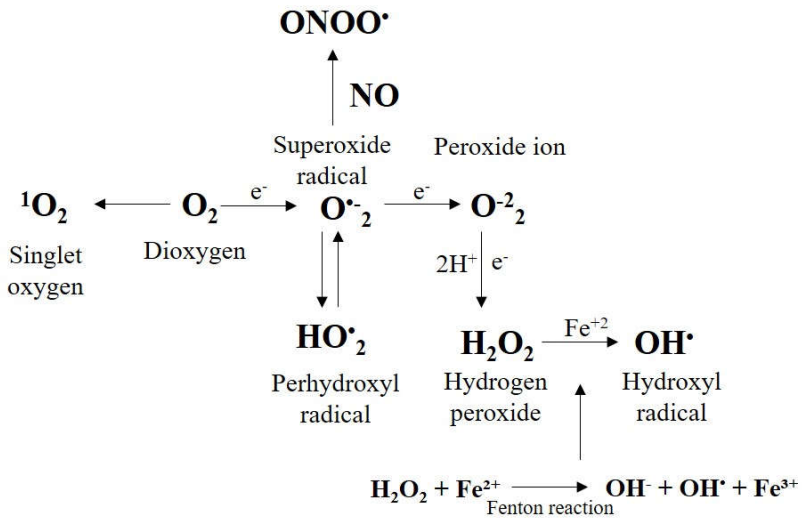

Figure 1. Formation of different ROS and reactive nitrogen species from atmospheric oxygen. Atmospheric oxygen (dioxygen; O_2) is shown to undergo excitation or reduction to form singlet oxygen ($^1O_2$) or superoxide radical ($O^{\bullet-}_2$), respectively. Superoxide is shown to dismutate to form hydrogen peroxide (H_2O_2), and H_2O_2 is shown to interact with Fe^{2+} and to form hydroxyl radicals ($OH^{\bullet}$) via the Fenton reaction. Adapted from Appel and Hirt (2004).

Especially at low pH, dismutation of $O^{\bullet-}_2$ is unavoidable, with one $O^{\bullet-}_2$ giving up its added electron to another $O^{\bullet-}_2$ and then with protonation resulting in the generation of H_2O_2, the uncharged, non-radical ROS (Gill and Tuteja, 2010). H_2O_2 is a weak oxidizing agent but they can react in the presence of transition metals such as copper and iron, e.g. Haber-Weiss/Fenton reaction, to yield the extremely reactive hydroxyl radicals (•OH) (Mignolet-spruyt et al., 2017). OH• has a higher redox potential than $O^{\bullet-}_2$ or H_2O_2, and an increase of OH• production in chloroplasts may lead to oxidative degradation of both ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and glutamine synthase (Fucci et al., 1983; Ishida et al., 1997). Furthermore, at lower temperatures, OH• decomposes the PSI complex in thylakoid membranes (Sonoike, 1996). These oxidative attacks by OH• also cause a reduction in photosynthesis activity. Furthermore, the accumulated H_2O_2 oxidatively degrades ascorbate

peroxidase (APX) in chloroplasts, resulting in the stimulated accumulation of H_2O_2 and enhanced inactivation of photosynthesis (Miyake and Asada, 1992).

As well as H_2O_2, singlet oxygen ($^1O_2$) is a non-radical ROS, however it is formed when an electron from O_2 is elevated to a higher energy orbital, thereby freeing oxygen from its spin-restricted state (Takagi et al., 2016). This removal of spin restriction causes singlet oxygen to rapidly react with organic molecules, potentially causing damage. On plants, singlet oxygen can be formed by the reaction of excited chlorophyll in its triplet state (^{3}Chl) with molecular oxygen in its ground state, which is the triplet state $^3O_2$ (Fischer and Krieger-liszkay, 2013). Normally, efficient photoprotective agents such as carotenoids quench the excited state of chlorophyll, that dissipate excess light energy as heat (Ruban et al., 2012). Other scavengers such as ubiquinol, ascorbate, and glutathione may also quench $^1O_2$ (Laloi and Havaux, 2015). If these limits are exceeded, singlet oxygen kick-starts lipid peroxidation reactions, which may be the main cause of stress-induced photo-oxidation (Farmer and Mueller, 2013). However, it was indicated that singlet oxygen signalling controls the expression of a number of genes and cell fate (Fischer and Krieger-liszkay, 2013).

ROS are very versatile molecules owing to their diverse properties that include different levels of reactivity, sites of production, and potential to cross biological membranes (Mittler, 2016) (Table 1). Each ROS has its own set of distinct chemical properties and reactivity (Møller et al., 2007) as well as their own specific set of targets and signalling routes (Vaahtera et al., 2014). •OH is the most unstable ROS and rapidly reacts with all sorts of cellular components, as reflected by its half-life of only 1 ns (Mittler, 2016). Because of it fast half-life, this molecule does not seem to have a controlled role in signalling but it may well be generated in response to a primary signal (Foreman et al., 2003). However, even the highly reactive $OH^{\bullet}$ plays important roles in plants, notably in cell wall structure and metabolism (Muller et al., 2009).

Table 1. Properties (t1/2, migration distance), reactivity (mode of action), formation (typical production systems), and scavenging (typical scavenging systems) of ROS in plant and animal cells. Abbreviations: APX, ascorbate peroxidase; CAT, catalase; GPX, glutathione peroxidase; PER, peroxidase; PRX, peroxiredoxin; RBOH, respiratory burst oxidase homolog; SOD, superoxide dismutase. Adapted from R. Mittler (2016).

ROS	$t_{1/2}$	*Migration distance*	*Mode of action*	*Production site*	*Scavenging systems*
Hydroxyl radical (•OH)	1 ns	1 nm	Extremely reactive with all biomolecules including DNA, RNA, lipids, and proteins.	Iron and H_2O_2 (Fenton reaction) Peroxisomes	Flavonoids, proline, sugars, ascorbate…
Superoxide ($O^{\bullet-}_2$)	1-4 μs	30 nm	Reacts with Fe-S proteins. Dismutates to H_2O_2.	Apoplast (RBOHs), chloroplasts, mitochondria, peroxisomes, electron transfer chains	SOD, flavonoids, ascorbate…
Singlet oxygen ($^1O_2$)	1-4 μs	30 nm	Oxidizes lipids, proteins (Trp, His, Tyr, Met, and Cys residues), and G residues of DNA.	Membranes, chloroplasts, nuclei	Carotenoids and α-tocopherol
Hydrogen peroxide (H_2O_2)	> 1 ms	> 1 μm	Reacts with proteins by attacking cysteine and methionine residues. Reacts with heme proteins. Reacts with DNA.	Peroxisomes, chloroplasts, mitochondria, cytosol, apoplast	APX, CAT, GPX, PER, PRX, ascorbate, glutathione…

The $O^{\bullet-}_2$ and $^1O_2$ molecules are quite similar in terms of stability, with a half-life of 1-4 μs (Halliwell and Gutteridge, 2015). Nevertheless, they vary greatly in target specificity: whereas $O^{\bullet-}_2$ interacts mainly with protein Fe–S centres, $^1O_2$ may oxidize polyunsaturated fatty acids, guanine and several amino acids. These reactions cause damage inside the cells and induce an oxidative stress. In contrast, H_2O_2 is much more stable (half-life of 1 ms) and can accumulate to relatively high concentrations (μM to mM) (Cheeseman, 2006; Møller et al., 2007). Also, H_2O_2 is freely diffusible across membranes, which enables it to diffuse cell damage (Saxena et al., 2016). The unique property of stability and less reactivity of H_2O_2 when compared to other ROS, makes it a good signalling molecule (Quan et al., 2008).

Photosynthesis and ROS: photoprotective mechanisms in chloroplasts

Photosynthesis consists of a complex series of single redox reactions that, together, regulate the redox state of the entire process, achieved by the balance between light harvesting, physico-chemical energy conversion and metabolic consumption of chemical energy (Maruta et al., 2016). Thus, the redox environment of chloroplasts rapidly changes in dependence on the photosynthetic state, which, in turn, depends on environmental conditions such as light intensity, temperature and CO_2 availability (Dietz and Hell, 2015). For example, excessively accumulation of reducing power in the photosynthesizing chloroplast strongly affects redox homeostasis and normal cell functions, generating many forms of ROS, including H_2O_2 (Kangasjärvi et al., 2008).

Even with chloroplasts being considerate the major source of ROS, over-accumulation of ROS in chloroplasts is one of the most important factors associated to photosynthesis photoinhibition (Murchie et al., 2015). Therefore, chloroplast disposes with different mechanisms that allow the dissipation of the excess of excitation energy to ensure that the output ratio of ATP/NADPH matches the demands of plant metabolism, avoiding excessive production of ROS (Foyer et al., 2012). The dissipation of excess energy by the non-photochemical quenching (NPQ), the cyclic electron flow (CEF) and alternative electron sinks such as the water-water cycle and photorespiration can alleviate the over-reduction of the PET chain, avoiding the production of excessive ROS and photoinhibition (Flexas and Medrano 2002, Horton and Ruban 2005, Asada 2006, Bagard et al. 2008, Takahashi and Murata 2008).

An important process for photoprotection in plants is NPQ, which is induced by a low thylakoid lumen pH and a high ΔpH that are generated by photosynthetic electron transport, especially under excess light conditions. The low pH of thylakoid lumen activates energy state quenching (qE) by protonating the protein PsbS and by activating violaxanthin

deepoxidase, which converts violaxanthin to antheraxanthin and zeaxanthin in the xanthophyll cycle (Li et al., 2000). This process allows the harmless dissipation of excess light energy as heat in PSII (Demmig-Adams and Adams, 2006). Excess energy from linear electron flow (LEF) can be in part redirected to another photoprotective mechanism, the water–water cycle (WWC), which also contribute to transthylakoid ΔpH, as they keep the linear electron transport relatively high (this can be particularly important in abiotic stress exposure).

In WWC (Figure 2), electrons excised from water at PSII are transferred to oxygen by PSI, resulting in the formation of $O^{\bullet-}_2$, which is disproportionated to H_2O_2 and O_2 by membrane-attached copper/zinc superoxide dismutase (Cu/Zn-SOD) (Asada, 2006). tAPX converts the H_2O_2 back into water, using ascorbate as an electron donor (Kangasjärvi et al., 2008). The reactions of these enzymes act as the first layer of ROS scavenging in chloroplasts, followed by their removal by Fe-SOD and sAPX as the second layer in stroma. Ascorbic acid (AsA), used by the chlAPX as a reductant, is converted during this process into ascorbic acid radical (MDH, monodehydroascorbate), and this radical is reduced back to AsA by ferredoxin-, glutathione- and NAD(P)H-dependent pathways (Maruta et al., 2016).

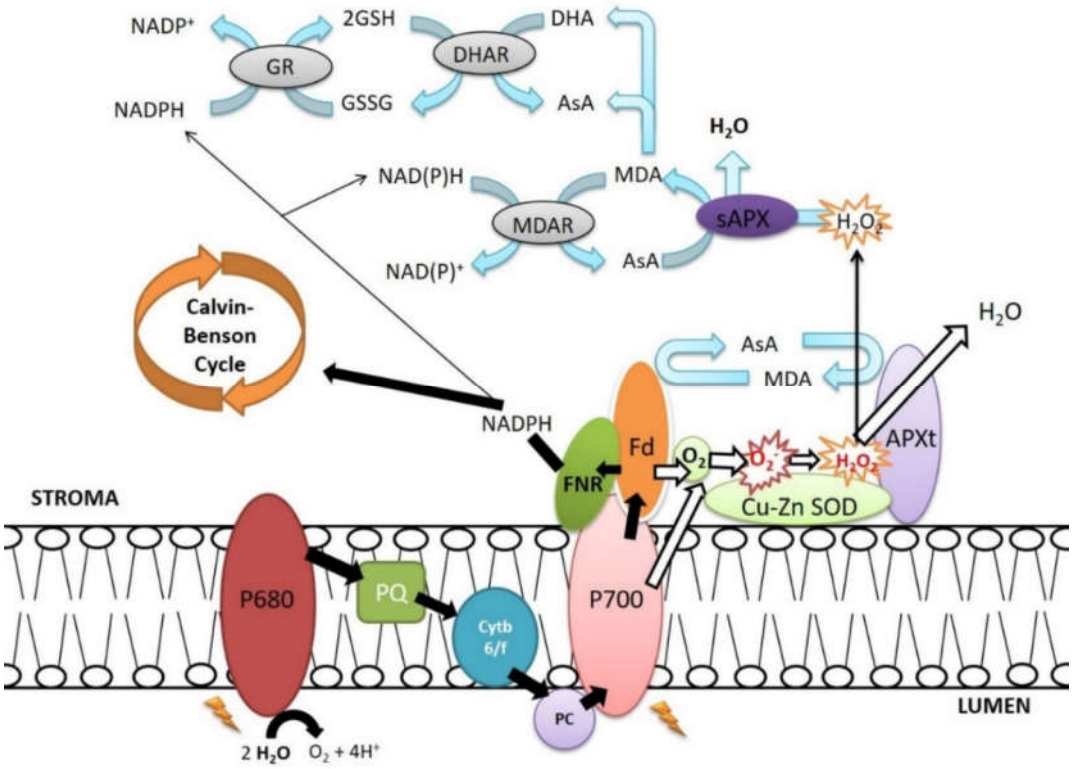

Figure 2. Water-water cycle in chloroplast, showing photoreduction of dioxygen to generate superoxide, disproportionation of superoxide to hydrogen peroxide, reduction of hydrogen peroxide to water and the associated reduction of oxidized ascorbates to ascorbate, and microcompartmentation of the participating enzymes. APX, ascorbate peroxidase; AsA, ascorbate; Cu,Zn-SOD, copper-zinc superoxide dismutase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; Fd, ferredoxin; FR, Fd-NADP+ reductase; GR, glutathione reductase; MDA, monodehydroascorbate radical;MDAR, MDA reductase; sAPX, stromal APX; tAPX, thylakoid-bound APX. Adapted from Asada, 1999.

In the last years, the capacity of the Mehler reaction to act directly in a photo-protective role as an electron sink/safety valve to dissipate excitation energy has been questioned due to its low capacity (Driever and Baker, 2011). These authors have suggested that H_2O_2 is unlikely to leave the chloroplast and act as a mobile signal because of the high antioxidant capacity in that organelle. This view, however, have been refused in the literature. Exposito-Rodriguez et al. (2017) used a genetically encoded fluorescent H_2O_2 sensor, HyPer2, to demonstrate that chloroplast-derived H_2O_2 can be detected in the nucleus. Also, Mubarakshina et al. (2010) showed that the proportion of oxygen photo-reduction compared

to total electron transport capacity increased with increasing light intensity. This indicates that H_2O_2 production is an effective measure of "excess excitation energy" and could therefore be a useful signal to activate acclimatory responses.

Another mechanism that can also modulate the proton circuit of photosynthesis is the cyclic electron flow around photosystem I, which can be up-regulated in stress conditions (Johnson, 2011). In CEF, the electrons photoproduced at PSI of thylakoid membranes return to the PET system through Fd or NADPH, where the electrons are donated to the PQ pool or Cyt b6/f complex (Figure 3) (Breyton et al. 2006, Joliot and Joliot 2006). For CEF to function, Fd, PQ and the mediator catalyzing the electron flow from Fd to the PQ pool are required. Furthermore, the redox ratio of the PQ pool regulates CEF activity (Allen 2003). The maximum activity of CEF is obtained at the half-reduced state of the PQ pool. On the other hand, under extreme conditions of the redox state, which the electron transport chain is perfectly reduced and oxidized, CEF is not active, i.e. the inhibition of CEF by DCMU is due to oxidation of the PQ pool (Hormann et al. 1994). Therefore, both CEF and WWC contribute to the production of additional ATP and the enhancement of non-photochemical quenching (NPQ) in the PSII antenna system, through generation of a proton motive force (Asada 1999).

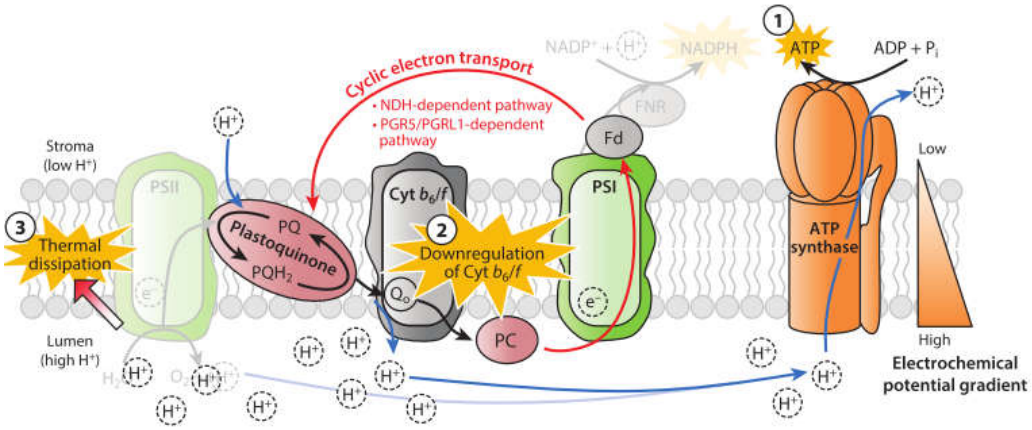

Figure 3. Physiological functions of PSI cyclic electron transport. In angiosperms, PSI cyclic electron transport consists of two partially redundant pathways: the PGR5/PGRL1-dependent pathway and the NDH-dependent pathway. PSI cyclic electron transport is generally considered to be coupled to the generation of a proton gradient across the thylakoid membrane (DpH). The DpH is used primarily to (1) drive ATP synthesis without net production of NADPH in order to increase the ATP/NADPH ratio; in addition, it plays regulatory roles via the acidification of the thylakoid lumen in (2) the downregulation of electron transport through the Cyt b6/f complex (3) and the induction of thermal dissipation (a qE component ofNPQ in PSII). Both (2) and (3) are essential to protect photosystems from irreversible photodamage. Reproduced from Yamori et al. (2016).

There are two cyclic electron flow systems around PSI: the NADH dehydrogenase-like complex-dependent pathway (NDH-mediated CEF, Shikanai et al. 1998, Shikanai 2015) and the proton gradient regulation 5 (PGR5)–PGRL1-mediated pathway (PGR5- mediated CEF, Munekage et al. 2002, Labs et al. 2016). CEF pathways have been proposed to protect PSI from the fluctuating growth light in *A*. *thaliana* (Suorsa et al. 2012) and in rice (Yamori et al. 2016). For shorter times as well, the photosynthetic alternative electron flows and WWC, are essential for protection of PSI from fluctuating light photoinhibition (Kono et al. 2014, Kono and Terashima 2014). These alternative electron flows act as electron sinks and protect plants from photoinhibition, which would otherwise occur.

Photorespiration is another important safety valve to avoid the excess energy in chloroplasts of C3 plants, particularly when plants have low CO_2 assimilation under stressful conditions (Peterhänsel and Maurino 2010, Zivcak et al. 2014). The oxygenation of RuBP by Rubisco produces one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate that would be converted to 3-phosphoglycerate by a series of reactions involving chloroplasts, peroxisomes and mitochondria, releasing CO_2 and NH_3 (Bagard et al. 2008). Photorespiration has the potential to sustain photons in a non-assimilatory pathway, protecting the photosynthetic apparatus against photo-oxidation due to the consumption of reducing equivalents (Niyogi 1999). However, photorespiration produces a great amount of H_2O_2 in the peroxisomes by the glycolate oxidase (GO) (Kangasjärvi et al. 2012). Catalase is also an important enzyme scavenging the excess H_2O_2 in peroxisomes, mainly under high concentrations of H_2O_2 (Suzuki et al. 2012).

Photoinhibition of PSII and PSI by ROS

The photosynthetic machinery is often damaged by high intensity visible light and this phenomenon is called photoinhibition. As already exposed in this review, excess of energy in PET lead to the production of ROS, which promote damaging in PSII complex. $^1O_2$ is thought to stimulate the degradation of an important protein that form the reaction center of PSII: D1 protein. In addition to $^1O_2$, recent studies have revealed that O_2^- is also produced by PSII, which also causes photoinhibition (Bondarava et al., 2010; Zulfugarov et al., 2014). Several studies now suggest that ROS suppress *de novo* D1 protein synthesis (repair) through the oxidative inactivation of the thioredoxin-regulated elongation factor G, an important protein involved with D1 translation (Kojima et al., 2007; Nishiyama et al., 2011). Thus, photoinhibition of PSII becomes apparent when the rate of photodamage exceeds the rate of repair of damaged PSII under high intensity light (Nishiyama et al., 2006).

Photoinhibition has usually been used as a synonym for the inactivation of PSII, however, PSI can also experience photoinhibition caused by ROS production within thylakoid membranes (Terashima et al., 1994; Sejima et al., 2014). In PSI, the risk of ROS production increases when PET chain is in a highly reduced state (Allahverdiyeva et al., 2005; Grieco et al., 2012). In fact, PSI photoinhibition occurs when the PSI electron carriers become reduced, impairing net carbon assimilation, and hence, plant growth (Grieco et al., 2012; Kono et al., 2014). The main cause of photoinhibition of PSI would be the destruction of the iron–sulfur centers by ROS (Tjus et al. 1999). In particular, involvement of •OH, produced from O_2^- via H_2O_2, has been claimed (Tjus et al. 2001). Very recently, it was shown that $^1O_2$ produced in PSI was also involved (Takagi et al. 2016). An important difference between photoinhibition in PSI and PSII is that PSI recovers very slowly, whereas photoinhibited PSII recovers rapidly (Melis, 1999; Sonoike, 2011). Therefore, PSI photoinhibition has more severe consequences than PSII photoinhibition in higher plants.

Hydrogen peroxide role in chloroplast redox signalling

As a reactive oxygen species, H_2O_2 has been given much attention during the past decades. Literature have suggest that H_2O_2 can function as a mobile signal (Karpinski, 1999), but whether it is the sole signal or part of a systemic response remains yet to be determined. Also, H_2O_2 is a very small molecule and it seems unlikely that a receptor exists to recognize it in a classical receptor-binding manner, instead it could be through it weak oxidizing activity that the ‘message’ is transduced (Quan et al., 2008). It induces transcripts encoding proteins with functions in metabolism, protein transport, cellular organization and biogenesis, cell rescue or defense, and transcription (Desikan et al., 2001). The main mechanism by which H_2O_2 may act as signalling molecules seems to be through the oxidation of critical cysteine residues within redox-sensitive proteins, such as the catalytic center of the thiol peroxidases (D’Autréaux and Toledano, 2007).

There are several evidences that H_2O_2 participates in many biological mechanisms, including development of root hair, reinforcement of plant cell wall, xylem differentiation, cell wall structural cross linking and cell wall loosening in stomatal control (Hossain et al., 2015). H_2O_2 has also been shown to act as a key regulator in a broad range of physiological processes, such as senescence (Peng et al. 2005), photorespiration and photosynthesis (Strand et al., 2015; Guo et al., 2016), stomatal movement (Bright et al. 2006), cell cycle (Mittler et al. 2004), growth and development (Foreman et al., 2003) and programmed cell death (Dat et al., 2013). However, it should be noted that the role of H_2O_2 in these processes cannot be separated from that of other ROS and the whole antioxidant response system (Mittler, 2002).

H_2O_2 could cause damage by oxidizing a variety of macromolecular targets, including those Calvin–Benson cycle enzymes that are regulated by the thioredoxin system (Michelet et al., 2013). This is due to the oxidative inactivation by H_2O_2 of Calvin cycle enzymes [fructose 1,6-bisphosphatase (FBPase), NADP-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribulose 5-phosphate kinase (PRK) and sedoheptulose 1,7-bisphosphatase (SBPase)] (Miyake, 2010). These enzymes have thiol groups in their amino acids. The thiol groups are oxidized by H_2O_2, resulting in the formation of disulfide bridges between the groups and leading to the inactive form. In illuminated chloroplasts, disulfide bridges in the inactivated enzymes are reduced to the thiol groups by the Fd–thioredoxin system (FTS), and the enzyme recovers activity (Buchanan, 1991). Therefore, the steady-state activities of these enzymes are determined by the balance between the H_2O_2-depedent oxidation rate and the FTS-dependent reduction rate.

H_2O_2 not only affect the CO_2 assimilation but also the function of photosynthetic electron transport. In fact, LEF, CEF and the Mehler reaction, which appears to be all redox controlled, tightly links the light reactions with the CO_2-fixing pathway (Dietz et al., 2016b). When CO_2 fixation is restricted by the low availability of electron acceptors or the inadequate

activity of redox-regulated enzymes, thioredoxins assume the reduced state, and LEF and CEF are shut down (Buchanan, 2016). So, the toxicity or danger associated with H_2O_2 can lead to signalling cascades thought many important mechanisms but also it concentration needs to be tightly controlled within plant cells to avoid oxidative stress (Foyer et al., 2017). In fact, cellular fate may then be dependent on where in the cell H_2O_2 synthesis is increased (Neill et al., 2002).

H_2O_2 retrograde signalling pathway from chloroplast

H_2O_2 produced inside the chloroplast, have been shown to act as specific signal that trigger responses outside of the organelle. The transmission of redox signals from the chloroplasts to other cellular compartments involves the transport of H_2O_2 through the inner and outer envelopes of the chloroplast and other membranes, however its polar nature might limit its capacity to diffuse through hydrophobic membranes unassisted (Dietz and Hell, 2015). Such movement may be facilitated by 'peroxiporins', speculated water channels, or aquaporins, that may allow for H_2O_2 efflux from the chloroplast, however its presence in the chloroplast envelope remains to be demonstrated (Henzler and Steudle, 2000). However, there is still a need to understand the mechanisms by which the different ROS signals are conditionally transmitted from the plastid to the cytosol and how they feed into the regulatory network of the cell (Dietz et al., 2016a).

Plant retrograde signalling refers to the signal from organelles regulating nucleus gene expression, while the nucleus-to-organelles control is called anterograde signalling (Koussevitzky et al., 2007). Thus, in retrograde signalling, signals originated in the chloroplast are translocated to nucleous and cytosol, regulating gene expression. This mechanism is very important since more than 95% of chloroplast proteins are encoded in the nucleus, and then the demand of those proteins is coordinated by chloroplast status itself (Shi and Theg, 2013). Retrograde signalling has been extensively studied in the context of cell

response to ambient variation, inducing the expression of not only of defense genes, but also resistance genes (Mittler et al., 2004).

It has already been shown that H_2O_2 mobility provides an advantage for it to act as a transducer as well as an initiator of retrograde signalling. In fact, literature has demonstrated that in *Arabidopsis thaliana* up to one-third of the transcriptome is changed upon exposure to H_2O_2 (Gadjev et al., 2006). However, there is still a need to clarify the signalling function of H_2O_2 derived from the chloroplast, since it can be produced by other compartments such as mitochondria, peroxisomes, as well as extracellularly via plasma membrane localized NADPH oxidases. Actually, a H_2O_2 molecule has the limitation that it is a simple molecule unable to store or transmit complex information and will have identical properties, whether it derives from all those cellular compartments (Neill et al., 2002).

It is hypothesized in literature that the movement of H_2O_2 from chloroplasts to nuclei would be facilitated if they were closely or physically associated, involving only a minimal or no transit through the cytosol (Higa et al., 2014). In fact, this physical association has been reported in a wide range of species (Selga et al., 2010; Suetsugu et al., 2016). The nature of the tethering between chloroplasts and nuclei is not known although the endoplasmic reticulum has been noted to form a layer between them (Selga et al., 2010). It has been suggested that stromules (highly mobile chloroplast extensions), formed during the development of the immune hypersensitive response (HR) in Arabidopsis and *Nicotiana benthamiana* could transfer H_2O_2 directly from chloroplasts to nuclei (Caplan et al., 2016) (Figure 4).

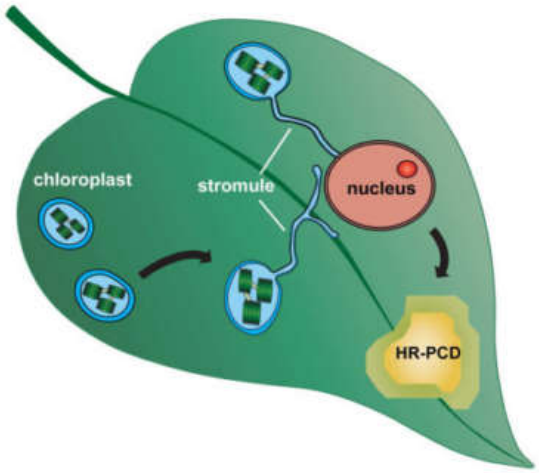

Figure 4. Chloroplast-nucleus communication via stromules during plant immune response. Reproduced from Caplan et al., 2016.

Recently, Exposito-Rodriguez et al. (2017) showed that in photosynthetic *N. benthamiana* epidermal cells, exposure to high light increases H_2O_2 production in chloroplast stroma, cytosol and nuclei. However, they also demonstrated evidences that the H_2O_2 nuclear accumulation is derivate from chloroplast, because: (I) the over-expression of stromal ascorbate peroxidase or treatment with DCMU (photosynthesis inhibitor) attenuates nuclear H_2O_2 accumulation and high light-responsive gene expression and (II) cytosolic ascorbate peroxidase over-expression has little effect on nuclear H_2O_2 accumulation and high light-responsive gene expression. Therefore, they concluded that direct H_2O_2 transfer from chloroplasts to nuclei, avoiding the cytosol, enables photosynthetic control over gene expression.

However, there is a hypothesis proposed by Møller and Sweetlove (2010) that peptides deriving from proteolytic breakdown of oxidatively damaged proteins have the requisite specificity to regulate source-specific genes and in that way it may contribute to retrograde signalling more than H_2O_2 molecules itself. They hypothesized that H_2O_2 might induce a general stress response, but it does not have the required specificity to selectively regulate

nuclear genes required for dealing with localized stress. So, oxidized peptides derived from oxidatively damaged proteins can be specific and selective secondary ROS messengers to the nucleus. This mechanism would work well for membrane-delimited cell compartment such as chloroplasts, mitochondria and peroxisomes, but it could also work for the plasma membrane.

Redundant peroxidase scavenging systems: thiol and ascorbate-dependent peroxidases specific roles in rice chloroplast

Even with chloroplasts being considerate the major source of ROS, including H_2O_2, plants are very resistant to high H_2O_2 concentrations and it is suggested that plant antioxidant response systems are designed more for the control of the cellular redox state than for complete elimination of H_2O_2 (Slesak et al., 2007). In fact, chloroplasts possess a multilayer antioxidative system to assure efficient detoxification of H_2O_2 which includes the thiol-based peroxidases and ascorbate-dependent peroxidase scavenging system (Dietz, 2016).

Although functionally redundant, the two pathways work simultaneously in chloroplasts. The possible simultaneous presence of all these enzymes creates a complex network of peroxidases and raises the question of their specificity and redundancy (Rouhier and Jacquot, 2005). Dominant role of one pathway out of others might be seen because of its enzyme abundance and efficiency. In Table 2 are listed the chloroplast H_2O_2 scavenging enzymes, their affinities (Km value) towards electron donors and the respective electron donors' availability. Thus, the determination factor on to which pathway H_2O_2 flux is the catalytic rate constant (Kcat) and concentration of each enzyme. The kcat for ascorbate peroxidase is about 2 or 3 orders of magnitude larger than PRX and GPX, therefore, ascorbate-glutathione system is much more efficient, assuming local concentrations for the two pathways are the same. In fact, it seems to be an internal coordinating system partitioning the relative abundance of the two systems according to environment.

Table 2. Affinity and constant rate comparison of chloroplast antioxidant enzymes.

Enzyme	Km H_2O_2 (μM)	Km reductant (μM)	Kcat H_2O_2 (s^{-1})
Ascorbate-dependent pathway			
Tobacco stromal APX[a]	22±1	395±27	2510±41
Spinach stromal APX[b]	30	300	290
Thiol peroxidases pathway			
Arabidopsis 2-Cys-PrxA[c]	12±4	17±1	0.27
Pea 2-Cys Prx[d]	27.6	N.A.	0.69
Populus Prx II[e]	21.7±5.8	N.A.	0.57±0.4
Barley 2-Cys Prx[f]	2.1	N.A.	0.23
Arabidopsis GPX1[g]	17.1±0.8	4±0.4	0.83

a: Kitajima et al. (2006); b: Nakano and Asada (1987); c: König et al. (2013); d: Bernier-Villamor et al. (2004); e: Gama et al. (2008); f: Horling et al. (2003); g Iqbal et al. (2006).

Thiol-based peroxidase scavenging system in rice chloroplasts

The thiol redox state constitutes one of the most important process parameters in chloroplasts. Thiol peroxidases are heme-free enzymes that use a cysteinyl thiolate to attack the peroxide substrates using thiol-containing proteins as reductants. Any protein that reacts with peroxides via a cysteinyl thiol tentatively might be called thiol peroxidase, e.g. annexins (Dalal et al., 2014) or glutathione-S-transferases (Dixon et al., 2009). However, two groups of enzymes, the peroxiredoxins (Prx) and GPX, should be considered as thiol peroxidases due to their high affinity to peroxides. Four classes are defined as peroxiredoxins and were already identified by phylogenetic sequence analysis, 1-Cys, 2-Cys, type II, and type Q peroxiredoxins, and the fifth is represented by glutathione peroxidases, which were shown to possess a thioredoxin-dependent activity in plants (Rouhier and Jacquot, 2005).

During catalysis, Prx use a very conserved cysteine (Cys) called the N-terminally located peroxidatic Cys residue to reduce H_2O_2, hydroperoxides and $OONO^-$, even with significant differences in substrate specificity and kinetic properties (Rouhier and Jacquot, 2002). After a nucleophilic attack, this Cys is transformed into a sulfenic acid (SOH), and the reduced product is released (H_2O in case of H_2O_2; correspondent alcohol in case of alkyl hydroperoxides and nitrite in case of $ONOO^-$) (Chae et al., 1994). Among the four classes, only 1-Cys Prx does not possess a second catalytic Cys, so the sulphenic acid is re-reduced by a yet unknown interacting partner before the next catalytic cycle begins (Dietz et al., 2006). However, 2-Cys Prx, PrxQ, and type-II Prx contain a second catalytic Cys residue in a distinct structural context that reacts with the sulphenic acid group of the peroxidatic Cys (König et al., 2013) (Figure 5A).

Comprehensive identification of all Prx members through genome-wide searches provides some evolutionary clues on a potential link between Prx function and photosynthesis. The Figure 5B show a comparison between *Oryza sativa* (monocot) and *Arabidopsis thaliana* (dicot) and reveals conserved and variable features of the Prx gene families. Common to both plants is the presence of at least one gene coding for a 2-Cys Prx-, PrxQ- PrxII E-, PrxII F-, and a cytosolic Prx II-like protein (Dietz et al., 2006). Probably due to recent gene duplication and alternative splicing in the case of Os-*prxQ*, the total number of plastidic Prx is four in *Arabidopsis thaliana* and five in *O. sativa*. In addition, a type-II Prx resides in the mitochondrion, a 1-Cys Prx in the nucleus-cytoplasm, and at least one type-II Prx in the cytosol.

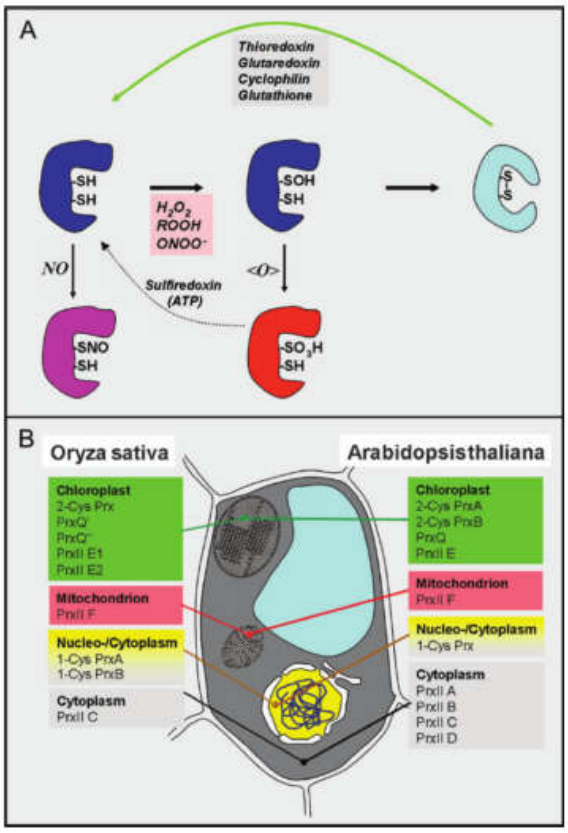

Figure 5. Mechanism of action and subcellular location of Prx isoforms in rice and Arabidopsis. (A) The catalytic Cys is oxidized to sulphenic acid. A disulphide bridge is formed under a conformational switch of the protein. The disulphide-oxidized Prx is regenerated by electron donors such as Trx, Grx, Cyp, and glutathione. Nitrosylation and overoxidation to sulphinic or sulphonic acid derivatives of the catalytic Cys withdraw Prx from the cycle and are considered as regulatory and potentially signalling mechanisms. (B) Subcellular location of Prx isoforms in rice and Arabidopsis. Each species has three different types of Prx in the chloroplast. Reproduced from Dietz et al., (2006).

Typical 2-Cys Prx is most abundant among chloroplastic Prxs. Two pathways have been proposed to reduce typical 2-Cys Prxs: one is based on plastidial Trxs, such as Trx x, which is the most efficient reductant and the other is based on a peculiar type of NADPH thioredoxin reductase (NTR), termed NTRC, with a joint Trx domain at the C-terminus (Serrato et al., 2004; Perez-Ruiz et al., 2006). NTRC conjugates NTR and Trx activities to reduce 2-Cys Prxs with high efficiency (Pérez-Ruiz and Cejudo, 2009). Whereas the Trx-dependent pathway obtains reducing power from ferredoxin (Fd) reduced by the

photosynthetic electron chain and mediated by ferredoxin-dependent thioredoxin reductase (FTR), NTRC uses NADPH as the source of reducing power. This difference is important because NADPH can be produced in darkness from glucose 6-phosphate via the oxidative pentose phosphate pathway, thus supplying reducing power under conditions when the levels of reduced Fd are lower (Spínola et al., 2008).

The peroxidase activity of chloroplastic Prxs, which varies depending on the reducing agent, is generally very low, approximately three–four orders of magnitude lower than that of chloroplastic APX (Dietz et al., 2006). Because of its lower peroxidase activity and higher susceptibility to H_2O_2 inactivation, it has been questioned whether the sole function of 2-Cys Prx protein is to act as a peroxidase. Active 2-Cys Prx is suggested to scavenge H_2O_2 and other hydroperoxides under normal conditions. But once inactivated by H_2O_2 excess, accumulated H_2O_2 would initiate ROS-mediated signalling to induce expression of defense-related genes (Wood et al., 2003). The peroxidase activity of 2-Cys Prx thus functions as ‘‘flood gate’’ of the signalling. Currently, the relative contribution of Prx to the overall water–water cycle activity remains unclear (Liebthal et al., 2017).

The fifth class of peroxiredoxin in plants, GPX, can reduce H_2O_2, organic hydroperoxides and/or lipid peroxides to water or corresponding alcohols with higher efficiency (Herbette et al., 2007). As efficient ROS scavengers, GPX expression and activity were often induced by abiotic stresses such as salinity, heat, and H_2O_2 treatment (Rodriguez Milla et al., 2003; Fischer et al., 2009; Wang et al., 2013; Zhai et al., 2013; Kim et al., 2014). Different from animal GPX, plant GPX preferentially reduce Trx as substract instead of GSH (Toppo et al., 2008). This seems to occur because of differences in the enzyme active center, where plant GPX-like proteins possess a Cys residue instead of a typical seleno-Cys residue in animals (Navrot et al., 2006). However, the use of both molecules as reducing substrates

could contribute to change the redox homeostasis in plant cells, especially under stress conditions (Chang et al., 2009).

In higher plants, GPXs are a family of multiple isozymes and may comprise up to 6 members of isoforms distributed in different subcellular compartments (Navrot et al., 2006). Specifically in rice, GPX (*OsGPX*) gene family consists of 5 members, localized to the mitochondria (GPX1 and GPX3), chloroplasts (GPX4) and cytosol/endoplasmic reticulum (GPX2 and GPX5), but both cytosolic isoforms were also found in chloroplasts (Margis et al., 2008; Passaia et al., 2013). Available microarray data and qPCR analyses of *OsGPX* transcripts revealed that they had different stress-responsive, tissue-specific, and developmental expression profiles (Agrawal et al., 2002; Kang et al., 2003; Passaia et al., 2013). For example, *OsGPX1* and *OsGPX3* were significantly upregulated in both shoots and roots in response to most of the tested stresses, while *OsGPX2* and *OsGPX4* were only induced by drought and oxidative stress but downregulated by salinity, heat, and cold in shoot (Islam et al., 2015).

Ascorbate-dependent peroxidase scavenging system in rice chloroplasts

APXs are heme-peroxidases are members of Class I non-animal peroxidases, which also include cytochrome c peroxidases and bacterial catalase peroxidases (Welinder 1992, Passardi et al. 2007). They are present across a wide spectrum of plastid-containing organisms, with some exceptions (Teixeira et al., 2004). In higher plants, there are multigenic families of APXs, for example, in *Oryza sativa* there are 8 isozymes: two cytosolic (APX1 and 2), two peroxisomal (APX3 and 4), one mitochondrial/chloroplastic (APX5), one mitochondrial (APX6), and two chloroplastic (APX7-stromal and APX8-thylakoid) (Teixeira et al., 2006) (Figure 6). All isoforms differ in their kinetic properties like molecular weight, optimal pH, stability, catalytic rate, and substrate affinity. However, all isoforms are very

sensitive to reduced ascorbate, as well as iron concentrations, and to high concentration of H_2O_2, wherein the activity and stability of the enzymes is negatively affected. (Shigeoka et al., 2002).

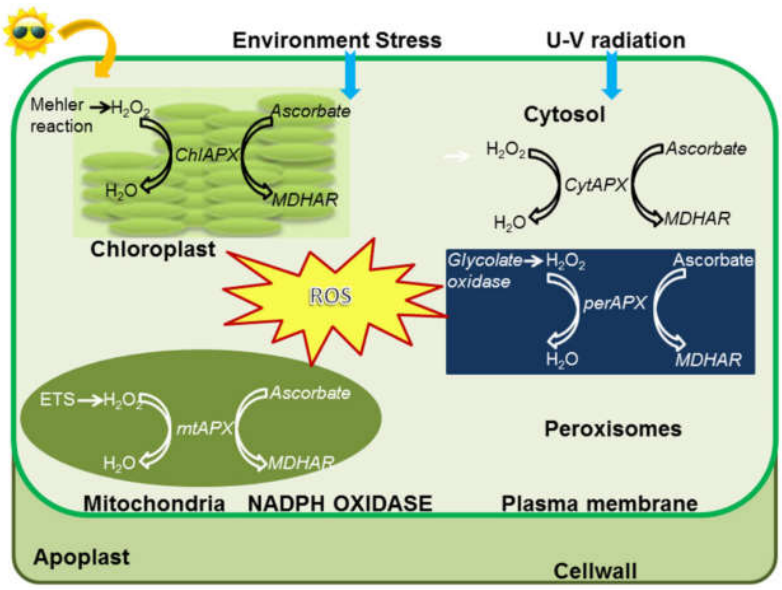

Figure 6. Localization of APX enzymes and detoxification of ROS in subcellular compartments. Reproduced from Pandey et al. (2017).

In the absence of ascorbate, oxidized heme in APX forms an irreversible cross-link to a distal tryptophan residue, leading to a repositioning of the heme (Lad et al., 2002). The cross-linked APX loses its ability to bind to ascorbate and leads to the formation of radical sites inside the protein. Curiously, chlAPXs are more sensitive to H_2O_2 under low ascorbate levels then cAPXs (Miyake and Asada, 1996). The half-inactivation time of chlAPXs is 15 s when the concentration of ascorbate is < 10 mM, while that of the cytosolic enzyme is > 40 min (Kitajima, 2008). This property is very inconsistent with their role in photo-oxidative stress tolerance since *in vitro* studies have already demonstrated that both chlAPX are susceptible to be inactivated by plant oxidative stress, however the presence of ascorbate alleviates the inactivation (Miyake and Asada, 1996; Shikanai et al., 1998; Mano et al., 2001; Hossain and Asada, 1984).

Given the fact that ascorbate is abundant in the chloroplast, some researchers doubt whether the H_2O_2 mediated inactivation of chlAPX actually occurs *in vivo*. There is a hypothesis that the fragile nature of chlAPXs may simply be compensated for by their overaccumulation, possibly by maintaining H_2O_2 in chloroplasts at very low concentrations (Yabuta et al., 2002). This sensitivity to H_2O_2 may be linked to the current concept for the evolution of ROS signalling functions in which plants first acquired ROS-scavenging mechanisms, developed the ability to control intra-cellular levels of ROS and then started to use these molecules for signalling purposes (Mittler et al., 2011).

In fact, chlAPX was showed to be connected with signalling events of photosynthesis protection in rice (Caverzan et al., 2014). Using a double mutant lacking both chlAPXs, they found that these plants were more affected than the non-transformed (NT) plants in the mechanisms of water–water cycle efficiency and photo-oxidative protection in the presence of high light (HL) and methyl viologen treatments (MV). They suggested that sAPX and tAPX are essential to the protection of PSII with effect in the Calvin-Benson cycle reactions under MV-induced acute oxidative stress. However, under HL-induced acute photo-oxidative stress, these proteins are less important to the PSII and overall photosynthesis.

Consequences of peroxidase deficiency in chloroplast

Based on the speculated importance of peroxidases in chloroplast, it is plausible that genetic modifications on those enzymes can be used as a good target for improving plant tolerance (Maruta et al., 2016). However, to rationally improve the plant's response to environmental challenge, it is necessary first a full understanding of the mechanisms involved with the target gene. Many studies have been using genetic engineering technics such as RNAi (knock down) since it has proved to be a helpful research tool, allowing much more rapid characterization of the function of known genes and its importance to abiotic stress response (Castel and Martienssen, 2013). Based on the fragile nature of chlAPXs over thiol

peroxidases, it is plausible that chlAPX activity is a bottleneck in the water–water cycle and a good target for improving plant tolerance (Maruta et al., 2016).

Curiously, several studies employing the use of knocked out/ knocked down plants have reported contrasting results (Danna et al., 2003; Giacomelli et al., 2007; Kangasjärvi et al., 2008; Maruta et al., 2010). In general, no obvious phenotypic difference was observed between these mutants and the wild type in *Arabidopsis* plants. Interestingly, under long-term stress, plants deficient in chlAPXs acquire compensatory mechanisms to contest environmental cues. These findings indicate that chlAPXs contribute, to some extent, to photooxidative stress tolerance, but are not essential in *Arabidopsis*. A similar conclusion was reached in a rice double mutant with the reduced expression of *OsApx7* (sAPX) and *OsApx8* (tAPX) (Caverzan et al., 2014). Thus, no robust evidence currently exists to support the genetic requirement of chlAPXs for antioxidant defense against photooxidative stress tolerance.

Between both chlAPXs, it was observed that the loss of tAPX have more severe impacts on plant photosynthesis than the loss of sAPX, although they share similar enzymatic properties (Maruta et al., 2010). This paradox can be explained because the local concentration of tAPX on thylakoid is sufficient to keep H_2O_2 at safe concentration before it diffuses to stroma (Miyake and Asada, 1992). Actually, the high local concentration of tAPX, high ascorbate level, and high tAPX efficiency keep the H_2O_2 to a safe concentration of 0.3-0.4 nM (Miyake, 2010). Following this deduction, however, there will be no need to express sAPX in plant mesophyll cells wherever tAPX is present. In fact, Kangasjärvi et al. (2008) showed that this affirmative is not right since chlAPXs physiological roles may differ with developmental stage, since sAPX, are of primary importance for photoprotection during the initial greening process.

Awad et al. (2015) showed that in *Arabidopsis* plants under HL conditions, 2-Cys PRXs seems to be more important in maintaining the water-water cycle, preventing excessive $O^{\bullet-}_2$ and H_2O_2 formation as well as photooxidative damage, whereas tAPX is only important in the absence of 2-Cys Prx. They generated an *Arabidopsis* double knock out in the two plastid 2-Cys Prxs (2-Cys Prx A and B) and a triple knock out in both 2-Cys Prxs and tAPX. 2-Cys Prxs double knock out plants showed an impairment of photosynthetic efficiency and photobleached under HL growth conditions, while the single tAPX mutant showed the wild-type phenotype. They also generated elevated levels of $O^{\bullet-}_2$, H_2O_2, and carbonylated proteins but lacked anthocyanin accumulation under HL stress conditions. This phenotype was further facilitated by an additional defect in tAPX (triple mutant), indicating that tAPX partially compensates for the loss of functional 2-Cys PRXs by mutation or inactivation by overoxidation (Figure 7).

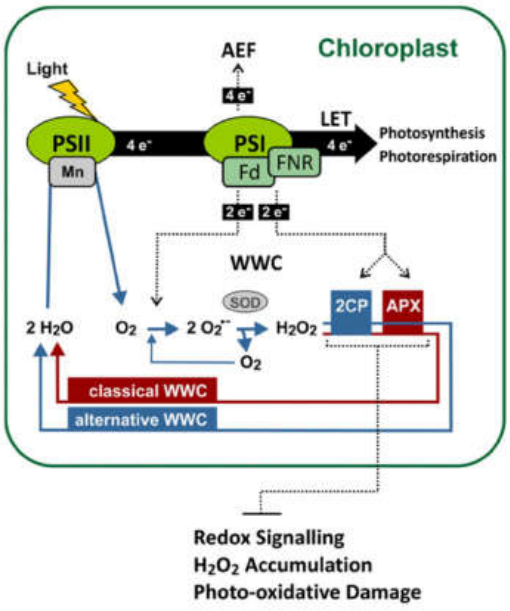

Figure 7. Model of the photoprotective function of 2-Cys Prx and tAPX. The water-water cycle (WWC) and other alternative electron flows (AEFs) are proposed to prevent overreduction of the linear electron transport chain (LET) and protect the photosystems from oxidative damage. Under HL conditions, 2CPs are essential for maintaining a WWC and more

important than plastid APX involved in the classical WWC for protection of the photosystems. 2-Cys PRX and tAPX synergistically inhibit activation of redox-responsive genes and prevent overaccumulation of H_2O_2 as well as photooxidative damage under HL conditions. FNR, Fd NADP oxidoreductase; SOD, superoxide dismutase. Reproduced from Awad et al. (2015).

To date, the biological functions of rice GPX isoenzymes have also been elucidated via transgenic approaches. There are suggestions in literature that plant GPXs may act as ROS sensors, playing critical roles in the redox signal transduction (Rouhier and Jacquot, 2005; Passaia and Margis-Pinheiro, 2015). For example, in *Arabidopsis thaliana*, a GPX loss or gain of function mutants demonstrated the role of these enzymes in H_2O_2 scavenging, signal transduction, photo-oxidative stress tolerance, and nuclear DNA damage protection (Miao et al., 2006; Chang et al., 2009; Gaber et al., 2012). In rice, the knock down of the mitochondrial *OsGPX1* and chloroplastic *OsGPX4* genes affected important processes of development indicating that these enzymes play essential roles in the interaction between redox homeostasis and normal plant development (Passaia et al., 2014). However, the precise physiological functions of different GPXs remain poorly characterized in higher plants.

Remarks and further perspectives

The general idea that "ROS are bad" seems to be too simplistic given the complexity of redox interactions. H_2O_2 is likely involved in many intracellular and systemic signalling routes, conferring plant acclimatory stress tolerance through the modulation of osmotic adjustment, ROS detoxification, carbon fixation and hormonal regulation. In fact, even antioxidants may play an integral part not only in controlling ROS but also in transmitting oxidative signals. However, the mostly unanswered question in plant ROS research has been: How do plant cells sense ROS? Recent findings have shown that effective H_2O_2 signalling may require an increased flux of antioxidants, such as peroxidases, and interaction with other

signalling pathways, such as induction of MAPK. It is unclear, however, whether this systems function in plants, and how are they integrated into the H_2O_2 signalling network of plant cells. It is probable that genetic screens for mutants impaired in H_2O_2 metabolism will reveal some of these mechanisms.

ChlAPXs, particularly tAPX, are still attractive targets for future ROS studies, once their susceptibility to H_2O_2 may have allowed for the flexible use of H_2O_2 as a signalling molecule in plants. In this regard, the following key questions remain to be addressed: is the occurrence of tAPX essential for controlling intracellular levels of ROS? Since chloroplastic H_2O_2 plays a key role in abiotic and biotic stress responses, why transgenic plants lacking chlAPXs showed no apparently impaired phenotype? Since chlAPXs are considerate the bottle-neck of the water-water cycle, why plants rely on multiple peroxidase systems in the chloroplast? Although many decades have passed since the discovery of peroxidases in the chloroplast, there are now more questions than answers. Deciphering ROS signalling may have a significant impact on agriculture and biotechnology in many countries and could lead to the development of important crops, such as rice, with enhanced yield even under suboptimal conditions.

References

AGRAWAL GK, RAKWAL R, JWA NS, AGRAWAL VP. Effects of signaling molecules, protein phosphatase inhibitors and blast pathogen (*Magnaporthe grisea*) on the mRNA level of a rice (*Oryza sativa* L.) phospholipid hydroperoxide glutathione peroxidase (*OsPHGPX*) gene in seedling leaves. **Gene** 283: 227–236, 2002.

APEL K, HIRT H. REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction. **Annu Rev Plant Biol** 55: 373–399, 2004.

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

ASADA, K. Production and scavenging of reactive oxygen species in chloroplasts and their functions. **Plant Physiol** 141: 391–396, 2006.

AWAD J, STOTZ H, FEKETE A, KRISCHKE M, ENGERT C, HAVAUX M, BERGER S, MUELLER MJ. 2-Cys peroxiredoxins and thylakoid ascorbate peroxidase create a water-water cycle that is essential to protect the photosynthetic apparatus under high light stress conditions. **Plant Physiol** 167, 1592-603, 2015.

BENNETZEN J. THE RICE GENOME: Opening the Door to Comparative Plant Biology. **Science**, 296: 60–63, 2002.

BONDARAVA N, GROSS CM, MUBARAKSHINA M, GOLECKI JR, JOHNSON GN, KRIEGER-LISZKAY A. Putative function of cytochrome b559 as a plastoquinol oxidase. **Physiol Plant** 138: 463–473, 2010.

BUCHANAN, BB. Regulation of CO_2 assimilation in oxygenic photosynthesis: The ferredoxin/thioredoxin system. **Arch Biochem Biophys** 288: 1–9, 1991.

BUCHANAN, BB. The carbon (formerly dark) reactions of photosynthesis. **Photosynth Res** 128: 215–217, 2016.

CAI Y-FF, YANG Q-YY, LI S-FF, WANG J-HH, HUANG W. The water-water cycle is a major electron sink in Camellia species when CO_2 assimilation is restricted. **J Photochem Photobiol B Biol** 168: 59–66, 2017.

CAPLAN JL, KUMAR AS, PARK E, PADMANABHAN MS, HOBAN K, MODLA S, CZYMMEK K, DINESH-KUMAR SP. Chloroplast stromules function during innate immunity. **Dev Cell** 34: 48–56, 2016.

CASTEL SE, MARTIENSSEN RA. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. **Nat Rev Genet** 14: 100–112, 2013.

CAVERZAN A, BONIFACIO A, CARVALHO FEL, ANDRADE CMB, PASSAIA G, SCHÜNEMANN M, MARASCHIN F DOS S, MARTINS MO, TEIXEIRA FK, RAUBER R. The knockdown of chloroplastic ascorbate peroxidases reveals its regulatory role in the photosynthesis and protection under photo-oxidative stress in rice. **Plant Sci** 214: 74–87, 2014.

CHAE HZ, UHM TB, RHEE SG. Dimerization of thiol-specific antioxidant and the essential role of cysteine 47. **Proc Natl Acad Sci U S A,** 91: 7022–7026, 1994.

CHANG CCC, SLESAK I, JORDA L, SOTNIKOV A, MELZER M, MISZALSKI Z, MULLINEAUX PM, PARKER JE, KARPINSKA B, KARPINSKI S. Arabidopsis Chloroplastic Glutathione Peroxidases Play a Role in Cross Talk between Photooxidative Stress and Immune Responses. **Plant Physiol** 150: 670–683, 2009.

D'AUTRÉAUX B, TOLEDANO MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. **Nat Rev Mol Cell Biol** 8: 813–824, 2007.

DANNA CH, BARTOLI CG, SACCO F, INGALA LR, SANTA-MARÍA GE, GUIAMET JJ, UGALDE R. Thylakoid-bound ascorbate peroxidase mutant exhibits impaired electron transport and photosynthetic activity. **Plant Physiol** 132: 2116–2125, 2003.

DESIKAN R, A-H-MACKERNESS S, HANCOCK JT, NEILL SJ. Regulation of the Arabidopsis transcriptome by oxidative stress. **Plant Physiol** 127: 159–172, 2001.

DIETZ K-J. Thiol-Based Peroxidases and Ascorbate Peroxidases: Why Plants Rely on Multiple Peroxidase Systems in the Photosynthesizing Chloroplast? **Mol Cells** 39: 20–5, 2016.

DIETZ KJ, HELL R. Thiol switches in redox regulation of chloroplasts: Balancing redox state, metabolism and oxidative stress. **Biol Chem** 396: 483–494, 2015.

DIETZ KJ, JACOB S, OELZE ML, LAXA M, TOGNETTI V, DE MIRANDA SMN, BAIER M, FINKEMEIER I. The function of peroxiredoxins in plant organelle redox metabolism. **J Exp Bot** 57: 1697–1709, 2006.

DIETZ K-J, MITTLER R, NOCTOR G. Recent Progress in Understanding the Role of Reactive Oxygen Species in Plant Cell Signalling. **Plant Physiol** 171: 1535–1539, 2016.

DIETZ K-J, TURKAN I, KRIEGER-LISZKAY A. Redox- and reactive oxygen species-dependent signalling in and from the photosynthesizing chloroplast. **Plant Physiol** 171: 1541–1550, 2016.

DING X, RICHTER T, CHEN M, FUJII H, SEO YS, XIE M, ZHENG X, KANRAR S, STEVENSON RA, DARDICK C. A Rice Kinase-Protein Interaction Map. **Plant Physiol** 149: 1478–1492, 2009.

DRIEVER SM, BAKER NR. The water-water cycle in leaves is not a major alternative electron sink for dissipation of excess excitation energy when CO2 assimilation is restricted. **Plant, Cell Environ** 34: 837–846, 2011.

EXPOSITO-RODRIGUEZ M, LAISSUE PP, YVON-DUROCHER G, SMIRNOFF N, MULLINEAUX PM. Photosynthesis-dependent H_2O_2 transfer from chloroplasts to nuclei provides a high-light signalling mechanism. **Nat Commun** 8: 49, 2017.

FARMER EE, MUELLER MJ. ROS-Mediated Lipid Peroxidation and RES-Activated Signalling. **Annu Rev Plant Biol** 429–450, 2013.

FISCHER BB, DAYER R, SCHWARZENBACH Y, LEMAIRE SD, BEHRA R, LIEDTKE A, EGGEN RIL. Function and regulation of the glutathione peroxidase homologous gene GPXH/GPX5 in Chlamydomonas reinhardtii. **Plant Mol Biol** 71: 569–583, 2009.

FISCHER BB, KRIEGER-LISZKAY A. Production, Detection, and Signalling of Singlet Oxygen. **Antioxid Redox Signal** 18: 2145–2162, 2013.

Food and Agriculture Organization of the United Nations - FAO. Available in < http://www.fao.org/faostat/en/#rankings/commodities_by_country>. Acession in 07.06.2018.

FOREMAN J, DEMIDCHIK V, BOTHWELL JHF, MYLONA P, MIEDEMA H, TORRES MA, LINSTEAD P, COSTA S, BROWNLEE C, JONES JDG. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. **Nature** 442–446, 2003.

FOYER CH, NOCTOR G. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. **Physiol Plantarum**, 355–364, 2003.

FOYER CH, NOCTOR G. Oxidant and antioxidant signalling in plants: A re-evaluation of the concept of oxidative stress in a physiological context. **Plant, Cell Environ** 28: 1056–1071, 2005.

FOYER CH, RUBAN A V, NOCTOR G. Viewing oxidative stress through the lens of oxidative signalling rather than damage. **Biochem J,** 877–883, 2017.

FOYER CH, SHIGEOKA S. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. **Plant Physiol** 155: 93–100, 2011.

FUCCI L, OLIVER CN, COON MJ, STADTMAN ER. Inactivation of key metabolic enzymes by mixed-function oxidation reactions: Possible implication in protein turnover and ageing. **Proc Natl Acad Sci** 80: 1521–1525, 1983.

GABER A, OGATA T, MARUTA T, YOSHIMURA K, TAMOI M, SHIGEOKA S. The involvement of *Arabidopsis* glutathione peroxidase 8 in the suppression of oxidative damage in the nucleus and cytosol. **Plant Cell Physiol** 53: 1596–1606, 2012.

GADJEV I, GADJEV I, VANDERAUWERA S, VANDERAUWERA S, GECHEV TS, GECHEV TS, LALOI C, LALOI C, MINKOV IN, MINKOV IN. Transcriptomic Footprints Disclose Specificity of Reactive Oxygen Species Signalling in *Arabidopsis*. **Society** 141: 436–445, 2006.

GALVEZ-VALDIVIESO G, MULLINEAUX PM. The role of reactive oxygen species in signalling from chloroplasts to the nucleus. **Physiol Plant** 138: 430–439, 2010.

GIACOMELLI L, MASI A, RIPOLL DR, LEE MJ, VAN WIJK KJ. *Arabidopsis thaliana* deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low. **Plant Mol Biol** 65: 627–644, 2007.

GILL SS, TUTEJA N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. **Plant Physiol Biochem** 48: 909–930, 2010.

GUO Z, WANG F, XIANG X, AHAMMED GJ, WANG M, ONAC E, ZHOU J, XIA X, SHI K, YIN X. Systemic induction of photosynthesis via illumination of the shoot apex is mediated by phytochrome B. **Plant Physiol** 172, 1259-1272, 2016.

HENZLER T, STEUDLE E. Transport and metabolic degradation of hydrogen peroxide in Chara corallina: model calculations and measurements with the pressure probe suggest transport of H_2O_2 across water channels. **J Exp Bot** 51: 2053–2066, 2000.

HERBETTE S, ROECKEL-DREVET P, DREVET JR. Seleno-independent glutathione peroxidases: More than simple antioxidant scavengers. **FEBS J** 274: 2163–2180, 2007.

HIGA T, SUETSUGU N, KONG S-G, WADA M. Actin-dependent plastid movement is required for motive force generation in directional nuclear movement in plants. **Proc Natl Acad Sci** 111: 4327–4331, 2014.

HOSSAIN MA, BHATTACHARJEE S, ARMIN S-M, QIAN P, XIN W, LI H-Y, BURRITT DJ, FUJITA M, TRAN L-SP. Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging. **Front Plant Sci** 6: 1–19, 2015.

ISHIDA H, NISHIMORI Y, SUGISAWA M, MAKINO A, MAE T. The Large Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase is Fragmented into 37-kDa and 16-kDa Polypeptides by Active Oxygen in the Lysates of Chloroplasts from Primary Leaves of Wheat. **Plant Cell Physiol** 38: 471–479, 1997.

ISLAM T, MANNA M, KAUL T, PANDEY S, REDDY CS, REDDY MK. Genome-Wide Dissection of Arabidopsis and Rice for the Identification and Expression Analysis of Glutathione Peroxidases Reveals Their Stress-Specific and Overlapping Response Patterns. **Plant Mol Biol Report** 33: 1413–1427, 2015.

KANG S-G, JEONG HK, SUH HS. Characterization of a New Member of the Glutathione Peroxidase Gene Family in Oryza sativa. **Mol Cells** 17: 23–28, 2003.

KANGASJÄRVI S, LEPISTÖ A, HÄNNIKÄINEN K, PIIPPO M, LUOMALA E-MM, ARO E-MM, RINTAMÄKI E, KANGASJARVI S, LEPISTO A, HANNIKAINEN K. Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses. **Biochem J** 412: 275–85, 2008.

KARPINSKI S. Systemic Signalling and Acclimation in Response to Excess Excitation Energy in Arabidopsis. **Science** 284: 654–657, 1999.

KATO Y, KAMOSHITA A, YAMAGISHI J. Evaluating the resistance of six rice cultivars to drought: Restriction of deep rooting and the use of raised beds. **Plant Soil** 300: 149–161, 2007.

KIM Y-J, JANG M-G, NOH H-Y, LEE H-J, SUKWEENADHI J, KIM J-H, KIM S-Y, KWON W-S, YANG D-C. Molecular characterization of two glutathione peroxidase genes of Panax ginseng and their expression analysis against environmental stresses. **Gene** 535: 33–41, 2014.

Kitajima S. Hydrogen peroxide-mediated inactivation of two chloroplastic peroxidases, ascorbate peroxidase and 2-Cys peroxiredoxin. **Photochem Photobiol** 84: 1404–1409, 2008.

KOJIMA K, OSHITA M, NANJO Y, KASAI K, TOZAWA Y, HAYASHI H, NISHIYAMA Y. Oxidation of elongation factor G inhibits the synthesis of the D1 protein of photosystem II. **Mol Microbiol** 65: 936–947, 2007.

KÖNIG J, GALLIARDT H, JÜTTE P, SCHÄPER S, DITTMANN L, DIETZ KJ. The conformational bases for the two functionalities of 2-cysteine peroxiredoxins as peroxidase and chaperone. **J Exp Bot** 64: 3483–3497, 2013.

KONO M, NOGUCHI K, TERASHIMA I. Roles of the cyclic electron flow around PSI (CEF-PSI) and O_2-dependent alternative pathways in regulation of the photosynthetic electron flow in short-term fluctuating light in *Arabidopsis thaliana*. **Plant Cell Physiol** 55: 990–1004, 2014.

KONO, M., TERASHIMA, I. Elucidation of Photoprotective Mechanisms of PSI Against Fluctuating Light photoinhibition. **Plant Cell Physiol**. 57, 2016.

LAD L, MEWIES M, BASRAN J, SCRUTTON NS, RAVEN EL. Role of histidine 42 in ascorbate peroxidase: Kinetic analysis of the H42A and H42E variants. **Eur J Biochem** 269: 3182–3192, 2002.

LALOI C, HAVAUX M. Key players of singlet oxygen-induced cell death in plants. **Front Plant Sci** 6: 1–9, 2015.

Liebthal M, Maynard D, Dietz K-J. Peroxiredoxins and Redox Signalling in Plants. **Antioxid Redox Signal** 28, 2017.

MARGIS R, DUNAND C, TEIXEIRA FK, MARGIS-PINHEIRO M. Glutathione peroxidase family - An evolutionary overview. **FEBS J** 275: 3959–3970, 2008.

MARUTA T, NOSHI M, TANOUCHI A, TAMOI M, YABUTA Y, YOSHIMURA K, ISHIKAWA T, SHIGEOKA S. H_2O_2-triggered Retrograde Signaling from Chloroplasts to Nucleus Plays Specific Role in Response to Stress. **J Biol Chem** 287: 11717–11729, 2012.

MARUTA T, SAWA Y, SHIGEOKA S, ISHIKAWA T. Diversity and evolution of ascorbate peroxidase functions in chloroplasts: More than just a classical antioxidant enzyme? **Plant Cell Physiol** 57: 1377–1386, 2016.

MARUTA T, TANOUCHI A, TAMOI M, YABUTA Y, YOSHIMURA K, ISHIKAWA T, SHIGEOKA S. *Arabidopsis* chloroplastic ascorbate peroxidase isoenzymes play a dual role in photoprotection and gene regulation under photooxidative stress. **Plant Cell Physiol** 51: 190–200, 2010.

MIAO Y, LV D, WANG P, WANG X-C, CHEN J, MIAO C, SONG C-P. An *Arabidopsis* Glutathione Peroxidase Functions as Both a Redox Transducer and a Scavenger in Abscisic Acid and Drought Stress Responses. **Plant Cell Online** 18: 2749–2766, 2006.

MICHELET L, ZAFFAGNINI M, MORISSE S, SPARLA F, PÉREZ-PÉREZ ME, FRANCIA F, DANON A, MARCHAND CH, FERMANI S, TROST P. Redox regulation of the Calvin-Benson cycle: something old, something new. **Front Plant Sci** 4: 470, 2013.

MIGNOLET-SPRUYT L, XU E, IDÄNHEIMO N, HOEBERICHTS FA, MÜHLENBOCK P. Spreading the news: subcellular and organellar reactive oxygen species production and signalling. **J Exp Bot** 67: 3831–3844, 2017.

MILLER G, SUZUKI N, CIFTCI-YILMAZ S, MITTLER R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. **Plant, Cell Environ** 33: 453–467, 2010.

MITTLER R. Oxidative stress, antioxidants and stress tolerance. **Trends Plant Sci** 7: 405–410, 2002.

MITTLER R. ROS Are Good. **Trends Plant Sci** 22: 11–19, 2016.

MITTLER R, VANDERAUWERA S, GOLLERY M, VAN BREUSEGEM F. Reactive oxygen gene network of plants. **Trends Plant Sci** 9: 490–498, 2004.

MITTLER R, VANDERAUWERA S, SUZUKI N, MILLER G, TOGNETTI VB, VANDEPOELE K, GOLLERY M, SHULAEV V, VAN BREUSEGEM F. ROS signaling: The new wave? **Trends Plant Sci** 16: 300–309, 2011.

MIYAKE C. Alternative electron flows (water-water cycle and cyclic electron flow around PSI) in photosynthesis: Molecular mechanisms and physiological functions. **Plant Cell Physiol** 51: 1951–63, 2010.

MIYAKE C, ASADA K. Thylakoid-Bound Ascorbate Peroxidase in Spinach Chloroplasts and Photoreduction of Its Primary Oxidation Product Monodehydroascorbate Radicals in Thylakoids. **Measurement** 33: 541–553, 1992.

MIYAKE C, ASADA K. Inactivation Mechanism of Ascorbate Peroxidase at Low Concentrations of Ascorbate; Hydrogen Peroxide Decomposes Compound I of Ascorbate Peroxidase. **Plant Cell Physiol** 37: 423–430, 1996.

MØLLER IM, JENSEN PE, HANSSON A. Oxidative Modifications to Cellular Components in Plants. **Annu Rev Plant Biol**. 459–483, 2007.

MØLLER IM, SWEETLOVE LJ. ROS signalling - specificity is required**. Trends Plant Sci** 15: 370–374, 2010.

MOUSTAFA K, ABUQAMAR S, JARRAR M, AL-RAJAB AJ, TRÉMOUILLAUX-GUILLER J. MAPK cascades and major abiotic stresses. **Plant Cell Rep** 33: 1217–1225, 2014.

MUBARAKSHINA MM, IVANOV BN, NAYDOV IA, HILLIER W, BADGER MR, KRIEGER-LISZKAY A. Production and diffusion of chloroplastic H_2O_2 and its implication to signalling. **J Exp Bot** 61: 3577–3587, 2010.

MULLER K, LINKIES A, VREEBURG RAM, FRY SC, KRIEGER-LISZKAY A, LEUBNER-METZGER G. In Vivo Cell Wall Loosening by Hydroxyl Radicals during Cress Seed Germination and Elongation Growth. **Plant Physiol** 150: 1855–1865, 2009.

MULLINEAUX PM, BAKER NR. Oxidative stress: antagonistic signaling for acclimation or cell death? **Plant Physiol** 154: 521–525, 2010.

NAVROT N, COLLIN V, GUALBERTO J, GELHAYE E, HIRASAWA M, REY P, KNAFF DB, ISSAKIDIS E, JACQUOT J-P, ROUHIER N. Plant Glutathione Peroxidases Are Functional Peroxiredoxins Distributed in Several Subcellular Compartments and Regulated during Biotic and Abiotic Stresses**. Plant Physiol** 142: 1364–1379, 2006.

NEILL S, DESIKAN R, HANCOCK J. Hydrogen peroxide signalling. **Curr Opin Plant Biol** 5: 388–395, 2002.

NISHIYAMA Y, ALLAKHVERDIEV SI, MURATA N. Protein synthesis is the primary target of reactive oxygen species in the photoinhibition of photosystem II. **Physiol Plant** 142: 35–46, 2011.

NISHIYAMA, Y, ALLAKHVERDIEV, SI, MURATA, N. A new paradigm for the action of reactive oxygen species in the photoinhibition of photosystem II. **Biochimica et Biophysica Acta - Bioenergetics** 7: 742-749, 2006.

NOCTOR G, FOYER CH. Intracellular Redox Compartmentation and ROS-Related Communication in Regulation and Signalling. **Plant Physiol.** 171: 1581–1592, 2016.

NOCTOR G, REICHHELD J-P, FOYER CH. ROS-related redox regulation and signalling in plants. **Semin Cell Dev Biol**, 80: 3-12, 2017.

PANDEY S, FARTYAL D, AGARWAL A, SHUKLA T, JAMES D, KAUL T, NEGI YK, ARORA S, REDDY MK. Abiotic Stress Tolerance in Plants: Myriad Roles of Ascorbate Peroxidase. **Front Plant Sci** 8: 1–13, 2017.

PASSAIA G, CAVERZAN A, FONINI LS, CARVALHO FEL, SILVEIRA JAG, MARGIS-PINHEIRO M. Chloroplastic and mitochondrial GPX genes play a critical role in rice development. **Biol Plant** 58: 375–378, 2014.

PASSAIA G, MARGIS-PINHEIRO M. Glutathione peroxidases as redox sensor proteins in plant cells**. Plant Sci** 234: 22–26, 2015.

PASSAIA G, SPAGNOLO FONINI L, CAVERZAN A, JARDIM-MESSEDER D, CHRISTOFF AP, GAETA ML, DE ARAUJO MARIATH JE, MARGIS R, MARGIS-PINHEIRO M. The mitochondrial glutathione peroxidase GPX3 is essential for H_2O_2 homeostasis and root and shoot development in rice. **Plant Sci** 208: 93–101, 2013.

PÉREZ-RUIZ JM, CEJUDO FJ. A proposed reaction mechanism for rice NADPH thioredoxin reductase C, an enzyme with protein disulfide reductase activity. **FEBS Lett** 583: 1399–1402, 2009.

PEREZ-RUIZ JM, SPÍNOLA MC, KIRCHSTEIGER K, MORENO J, SAHRAWY M, CEJUDO. Rice NTRC Is a High-Efficiency Redox System for Chloroplast Protection against Oxidative Damage. **Plant Cell Online** 18: 2356–2368, 2006.

POPESCU S, POPESCU G, BACHAN S, ZHANG Z, GERSTEIN M, SNYDER M, DINESH-KUMAR SP. MAPK target networks in Arabidopsis thaliana revealed using functional protein microarrays. **Genes Dev** 23: 80–92, 2009.

QUAN LJ, ZHANG B, SHI WW, LI HY. Hydrogen peroxide in plants: A versatile molecule of the reactive oxygen species network. **J Integr Plant Biol** 50: 2–18, 2008.

RAO KUPR, RICHA TA, KUMAR KU, RAGHURAM BA, SINHA ALOKKR. In Silico Analysis Reveals 75 Members of Mitogen-Activated Protein Kinase Kinase Kinase Gene Family in Rice. **DNA Res.** 139–153, 2010.

RODRIGUEZ MILLA MA, MAURER A, HUETE AR, GUSTAFSON JP. Glutathione peroxidase genes in *Arabidopsis* are ubiquitous and regulated by abiotic stresses through diverse signalling pathways. **Plant J** 36: 602–615, 2003.

ROSENZWEIG C, ELLIOTT J, DERYNG D, RUANE AC, MÜLLER C, ARNETH A, BOOTE KJ, FOLBERTH C, GLOTTER M, KHABAROV N. Assessing agricultural risks of climate change in the 21st century in a global gridded crop model intercomparison. **Proc Natl Acad Sci** 111: 3268–3273, 2014.

ROUHIER N, JACQUOT J. The plant multigenic family of thiol peroxidases. **Free Radic Med** 1413–1421, 2005.

ROUHIER N, JACQUOT JP. Plant peroxiredoxins: Alternative hydroperoxide scavenging enzymes. **Photosynth Res** 74: 259–268, 2002.

RUBAN A V, JOHNSON MP, DUFFY CDP. The photoprotective molecular switch in the photosystem II antenna. **BBA - Bioenerg** 1817: 167–181, 2012.

SÁ, JCM, LAL, R, CERRI, CC, LORENTZ, K, HUNGRIA, M, CARVALHO, PCF. Low-carbon agriculture in South America to mitigate global climate change and advance food security. **Environmental International**, 90: 102-112, 2017.

SAKSCHEWSKI B, VON BLOH W, HUBER V, MÜLLER C, BONDEAU A. Feeding 10 billion people under climate change: How large is the production gap of current agricultural systems? **Ecol Modell** 288: 103–111, 2014.

SAXENA I, SRIKANTH S, CHEN Z. Cross Talk between H_2O_2 and Interacting Signal Molecules under Plant Stress Response. **Front Plant Sci** 7: 1–16, 2016.

SELGA T, SELGA M, GOBINS V, OZOLINA A. Plastid-nuclear complexes: permanent structures in photosynthesizing of vascular plants. **Environ Exp Biol** 8: 85–92, 2010.

SERRATO AJ, PÉREZ-RUIZ JM, SPÍNOLA MC, CEJUDO FJ. A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in Arabidopsis thaliana. **J Biol Chem** 279: 43821–43827, 2004.

SHI LX, THEG SM. The chloroplast protein import system: From algae to trees. **Biochim Biophys Acta - Mol Cell Res** 1833: 314–331, 2013.

SHIGEOKA S, ISHIKAWA T, TAMOI M, MIYAGAWA Y, TAKEDA T, YABUTA Y, YOSHIMURA K. Regulation and function of ascorbate peroxidase isoenzymes. **J Exp Bot** 53: 1305–1319, 2002.

SHIGEOKA S, MARUTA T. Cellular redox regulation, signaling, and stress response in plants. **Biosci Biotechnol Biochem** 78: 1457–1470, 2014.

SIES H, BERNDT C, JONES DP. Oxidative Stress. **Annu Rev Biochem** 715–48, 2017.

SINGH R, JWA NS. The rice MAPKK-MAPK interactome: The biological significance of MAPK components in hormone signal transduction. **Plant Cell Rep** 32: 923–931, 2013.

SINGH R, LEE M-O, LEE J-E, CHOI J, PARK JH, KIM EH, YOO RH, CHO J-I, JEON J-S, RAKWAL R. Rice Mitogen-Activated Protein Kinase Interactome Analysis Using the Yeast Two-Hybrid System. **Plant Physiol** 160: 477–487, 2012.

SLESAK I, LIBIK M, KARPINSKA B, KARPINSKI S, MISZALSKI Z. The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. **Acta Biochim Pol** 54: 39–50, 2007.

SONOIKE K. Photoinhibition of photosystem I: Its physiological significance in the chilling sensitivity of plants. **Plant Cell Physiol** 37: 239–247, 1996.

Spínola MC, Pérez-Ruiz JM, Pulido P, Kirchsteiger K, Guinea M, González M, Cejudo FJ. NTRC new ways of using NADPH in the chloroplast. **Physiol Plant** 133: 516–524, 2008.

STRAND DD, LIVINGSTON AK, SATOH-CRUZ M, FROEHLICH JE, MAURINO VG. Activation of cyclic electron flow by hydrogen peroxide in vivo. **PNAS** 112: 5539–5544, 2015.

SUETSUGU N, HIGA T, GOTOH E, WADA M. Light-Induced Movements of Chloroplasts and Nuclei Are Regulated in Both Cp-Actin-Filament-Dependent and -Independent Manners in *Arabidopsis thaliana*. **PLoS One** 11, 2016.

SUORSA, M., JÄRVI, S., GRIECO, M., NURMI, M., PIETRZYKOWSKA, M., RANTALA, M., KANGASJÄRVI, S., PAAKKARINEN, V., TIKKANEN, M., JANSSON, S., ARO, E.-M. PROTON GRADIENT REGULATION5 is essential for proper acclimation of Arabidopsis photosystem I to naturally and artificially fluctuating light conditions. **Plant Cell** 24, 2934–48, 2012.

SWATUK L, MCMORRIS M, LEUNG C, ZU Y. Seeing "invisible water": Challenging conceptions of water for agriculture, food and human security. **Can J Dev Stud** 36: 24–37, 2015.

TAKAGI D, TAKUMI S, HASHIGUCHI M, SEJIMA T, MIYAKE C. Superoxide and singlet oxygen produced within the thylakoid membranes both cause photosystem I photoinhibition. **Plant Physiol**. vol. 171 no. 3 1626-1634, 2016.

TEIXEIRA FK, MENEZES-BENAVENTE L, GALVÃO VC, MARGIS R, MARGIS-PINHEIRO M. Rice ascorbate peroxidase gene family encodes functionally diverse isoforms localized in different subcellular compartments. **Planta** 224: 300–314, 2006.

TEIXEIRA FK, MENEZES-BENAVENTE L, MARGIS R, MARGIS-PINHEIRO M. Analysis of the molecular evolutionary history of the ascorbate peroxidase gene family: Inferences from the rice genome. **J Mol Evol** 59: 761–770, 2004.

TODAKA D, SHINOZAKI K, YAMAGUCHI-SHINOZAKI K. Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. **Front Plant Sci** 6: 84, 2015.

TOPPO S, VANIN S, BOSELLO V, TOSATTO SCE. Evolutionary and Structural Insights Into the Multifaceted Glutathione Peroxidase (Gpx) Superfamily. **Antioxid Redox Signal** 10: 1501–1514, 2008.

VAAHTERA L, BROSCHÉ M, WRZACZEK M, KANGASJÄRVI J. Specificity in ROS signalling and transcript signatures. **Antioxid Redox Signal** 21: 1422–1441, 2014.

WANG X, HOU C, LIU J, HE W, NAN W, GONG H, BI Y. Hydrogen peroxide is involved in the regulation of rice (*Oryza sativa* L.) tolerance to salt stress. **Acta Physiol Plant** 35: 891–900, 2013.

WOOD ZA, POOLE LB, KARPLUS PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signalling. **Science** 300: 650–653, 2003.

YABUTA Y, MOTOKI T, YOSHIMURA K, TAKEDA T, ISHIKAWA T, SHIGEOKA S. Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. **Plant J** 32: 915–925, 2002.

YAMORI, W., MAKINO, A., SHIKANAI, T. A physiological role of cyclic electron transport around photosystem I in sustaining photosynthesis under fluctuating light in rice. **Sci. Rep.** 6, 1–12, 2016.

YOSHIDA K, HISABORI T. Two distinct redox cascades cooperatively regulate chloroplast functions and sustain plant viability. **PNAS** 3967–3976, 2016.

ZHAI CZ, ZHAO L, YIN LJ, CHEN M, WANG QY, LI LC, XU ZS, MA YZ. Two Wheat Glutathione Peroxidase Genes Whose Products Are Located in Chloroplasts Improve Salt and H_2O_2 Tolerances in *Arabidopsis*. PLoS One, 8: 10-15 2013.

ZHOU J, WANG J, SHI K, XIA XJ, ZHOU YH, YU JQ. Hydrogen peroxide is involved in the cold acclimation-induced chilling tolerance of tomato plants. **Plant Physiol Biochem** 60: 141–149, 2012.

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5 PARTIAL SILENCING OF STROMAL ASCORBATE PEROXIDASE LEAD TO IMPROVEMENT OF PHOTOSYNTHETIC EFFICIENCY IN RICE PLANTS EXPOSED TO MILD DROUGHT STRESS

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Abstract

Ascorbate peroxidase (APX) exerts a significant role in the maintenance of normal levels of reactive oxygen species (ROS) in plants. However, the functional importance of stromal APX for chloroplast oxidative protection and their role in plant stress tolerance remains unknown. In this work, we performed an integrative study of the *OsApx7* gene expression in rice plants. For this, physiological and proteomic analysis of sAPX knockdown silenced plants (*apx7*) exposed to mild drought stress were conducted. Our results demonstrate that *apx7* plants showed higher tolerance to mild drought through the maintenance of higher photosynthetic capacity, when compared to NT. Stromal APX silencing decreased stromal APX activity proportionally to reduction observed in the transcript amount (by 50%) and triggered only a slight decrease in the thylakoidal APX activity. On the other hand, *apx7* plants showed an

intense increase in the cytosolic APX activity probably as a compensatory mechanism. In fact, *apx7* plants showed a slight increase in accumulation of H_2O_2 and TBARS when compared to NT plants. However, NT and *apx7* plants showed a similar proteomic profile under control conditions. After mild drought stress, proteomic analysis showed that both NT and *apx7* plants increased the majority of drought-responsive proteins. However, *apx7* plants kept a higher increase of these proteins when compared to NT, especially of proteins related with photosynthesis and stress. Our data indicate that a partial decrease in *OsApx7* gene expression may trigger genetic and proteomic changes that are favorable to induce a rice phenotype more capable to cope with mild drought, especially in terms of photosynthetic capacity.

Keywords: RNAi silencing; chloroplast; proteomic; drought tolerance.

Introduction

Drought is a major abiotic stressor that limits plant growth and production, especially in drought-susceptible species such as rice (Todaka et al., 2015). During the past few decades, extensive studies on drought resistance have enriched our understanding of this phenomenon (Capell et al., 2004; Sato and Yokoya, 2008; Selvaraj et al., 2017). It is commonly recognized that drought resistance is a complex trait and involves numerous developmental, physiological, biochemical and molecular adjustments (Lenka et al., 2011). Cultivars with better drought tolerance are needed, especially in rainfed environments. Therefore, understanding how plants respond to drought stress can play a key role in stabilizing crop performance through adequate management techniques and plant genetic breeding (Chaves et al., 2009). For this, global analysis of plant function is necessary to obtain a more complete understanding of drought-mediated responses.

Numerous ‘‘omics’’ techniques, namely high-throughput methods for transcriptomic, proteomic, metabolomic analysis and so on, have been used to investigate plant drought

responses (Shu et al., 2012; Wu et al., 2016; Chintakovid et al., 2017). Application of these tools has been proving useful in understanding the molecular mechanisms responsible for the expression of the abiotic stress tolerance traits. Discovery of novel abiotic stress regulatory genes, identification of key pathways of proteins that are altered in response to stress and functional characterization of the proteins involved are imperative to understand stress tolerance mechanisms (Lenka et al., 2011). However, the majority of proteomic studies are quite incomplete because they add few insights to the biochemical mechanisms involved in plant response (Silveira and Carvalho, 2016). Such integrative studies are lacking in plant science, whereas segregated and concise studies lead mostly to controversial results.

Drought stress often induces the overproduction of reactive oxygen species (ROS), such as superoxide anion ($O^{\bullet-}_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) in plants. In fact, ROS are important signaling molecules involved in key physiological pathways, such as cell cycle, programmed cell death (PCD), senescence and stress response (Dat et al., 2000; Hossain et al., 2015; Reczek and Chandel, 2015; Dietz et al., 2016). However, at high concentration, ROS can diffuse through biological membranes reacting with biological compounds such as lipids, proteins and nucleic acids, thus promoting cell damage and, in many cases, cellular death (Foyer and Noctor, 2016). The control of intracellular ROS levels, and consequently, the cellular redox homeostasis, is a very complex process involving many mechanisms and a large network of genes (Hernandez et al., 2010).

The main ROS scavenging mechanism is composed by several enzymes, present in different subcellular compartments. In plants, ascorbate peroxidases (APX, EC, 1.11.1.11) has an important role in the maintenance of ROS levels, catalyzing the reduction of H_2O_2 to H_2O and O_2, using ascorbate as specific electron donor (Asada, 1999). In addition, APX is a component of the ascorbate-glutathione (AsA-GSH) cycle, one of the most important antioxidant systems in plants. In this cycle, glutathione is used as reducing substrate to restore

the ascorbate levels and is ultimately recycled at NAD(P)H expense (Smirnoff, 1996). In rice plants (*Oryza sativa* L.), APX is encoded by a small gene family, in which eight isoforms are targeted to distinct subcellular compartments: two cytosolic (APX1 and 2), two peroxisomal (APX3 and 4), one mitochondrial/chloroplastic (APX5), one mitochondrial (APX6), and two chloroplastic (APX7-stromal and APX8-thylakoid) (Teixeira et al., 2006).

Our group has demonstrated the functional importance of the different APX isoforms in the cellular homeostasis in rice. Previously, we demonstrated that rice plants silenced in both chloroplastic APXs (*OsApx7* and *OsApx8*) exhibited differences in photosynthetic parameters related to efficiency of utilization of light and CO_2 when submitted to stress conditions (Caverzan et al., 2014). This suggests that a partial deficiency of both APX isoforms in chloroplasts modulate the photosystem activity and integrity mainly under abiotic stress conditions (Caverzan et al., 2014). These data suggest that the chlAPXs exert a significant role in stress response, however more studies are needed to evaluate the individual contribution of *OsApx7* and *OsApx8*. In fact, we have demonstrated that the individual silencing of *OsApx8* led to a lower chlAPX activity, which is accompanied by a higher H_2O_2 content in leaves and a decrease stomatal aperture (Jardim-Messeder et al., 2018).

In this work, we performed an integrative study of *OsApx7* gene using molecular, physiological and proteomic approaches. For that, rice plants individually silenced to *OsApx7* (*apx7*) and non-transformed (NT) plants were submitted or not to mild drought stress. These transgenic plants have similar growth compared to NT plants. On the other hand, when subjected to mild drought stress, *apx7* plants show higher capacity to maintain photosynthetic efficiency, resulting in higher tolerance to drought stress, as compared to NT. Proteomic analyses demonstrated that *apx7* plants exposed to drought stress was able to maintain higher levels of increased proteins in many processes, including those involved with light harvesting in photosynthesis, corroborating the improvement in photochemical and gas exchange

parameters when compared to NT plants. Increasing drought stress tolerance may indicate that *OsApx7* gene could be involved in stress signaling pathway of rice plants. The functional role of sAPX in photosynthesis efficiency under drought stress is discussed.

Materials and methods

Construction of the plant vector and plant transformation

A chimeric gene producing mRNA with a hairpin structure (hpRNA) was constructed based on the sequence of the *OsApx7* (LOC Os04g35520) gene. The following primer pairs were used to amplify a 220 bp *RNAiOsApx7* sequence: 5'-CTCCGAGCAATCTGGGTGCAAAAAT-3' and 5'-GGTACCTCGAGGACTCGTGGTCAGGAAAAGC-3'. PCR product was cloned into the Gateway vector pANDA, in which hairpin RNA is driven by the maize ubiquitin promotor and an intron placed upstream of the inverted repeats (Miki and Shimamoto, 2004). Rice plants with knockdown of *OsApx7* were obtained by *Agrobacterium tumefaciens*-mediated transformation of rice embryogenic calli (*Oryza sativa* L. ssp japonica cv. Nipponbare) induced from seeds and cultivated in NB medium at 28°C in the dark (Upadhyaya et al., 2000). In this work, we used RNAi plants at the F3 generation.

Plant growth and water deficit experiment

Experiments were taken place at Federal University of Ceará – Brazil (3°44'43.6"S 38°34'28.3"W). Non-transformed (NT) and RNAi knockdown rice plants were sowed in a 3L vase containing a 1:1 mix of sand and vermiculite. Plants were watered every other day with distilled water, until drainage, and every three days with a half-strength of Hoagland and Arnon's nutrient solution (Hoagland and Arnon, 1950). Plants were grown for 45 days in a greenhouse under natural conditions in a photoperiod of 12h, as described in Supplementary Table 1. For the water deficit treatment, the irrigation of 30 days-old plants was withdrawn for

four consecutive days. Rice plants daily irrigated as described above were used as the control treatment. Daily irrigated plants (to near pot saturation) were used as the control. After control or drought stress treatment, leaf gas exchange and photochemical parameters, as well as phenotypic parameters were obtained and leaf segments were collected with liquid N_2 to further biochemical quantifications.

Phenotypic parameters

All phenotypic parameters were collected before and after four days of control or mild drought exposure treatments in both NT and *apx7* plants. Tillers were hand counted and shoot length was measured from the ligule to leaf tip using a flat ruler. To determine shoot biomass, shoots were harvested, oven dried at 80°C for 72 h, and weighed according to [22].

Relative water content and electrolyte leakage

The leaf relative water content (RWC) was calculated as follows: RWC = [(FW − DW)/(TW − DW)] × 100, where FW is the fresh weight, TW is the turgid weight measured after 6 h of saturation in deionized water at 4 °C in the dark and DW is the dry weight determined after 48 h in an oven at 75 °C (Silveira et al., 2009). Cellular integrity (electrolyte leakage) was measured as described by (Blum and Ebercon, 1981). Leaves samples were placed in tubes containing deionized water. The flasks were incubated in a shaker for 12 h, and the electric conductivity in the medium (L1) was measured. Then, the medium was boiled (95 C) for 60 min and the electric conductivity (L2) was measured again. The relative membrane damage (MD) was estimated by MD = L1/L2 x 100.

Quantitative PCR (qPCR)

Real-time PCR experiments were carried out using cDNA synthesized from total RNA purified with TRIzol (Invitrogen®). The samples were treated with DNAase (Invitrogen®) to

remove the eventual genomic DNA contamination and complementary DNA (cDNA) was obtained using the SuperscriptTMII (Life Technologies®) reverse transcriptase system and a 24-polyTV primer (Invitrogen®). After synthesis, cDNAs were diluted 10–100 times in sterile water for use in PCR reactions. All reactions were repeated four times, and expression data analyses were performed after comparative quantification of the amplified products using the 2−ΔΔCt method (Livak and Schmittgen, 2001). qPCR reactions were performed in an Applied Biosystems StepOne plus Real Time PCR system (Applied Bio- systems®) using SYBR and primer for *Osapx7* gene (F-5'-GGTACCTCGAGGACTCGTGGTCAGGAAA-3' and R-5'-CTCGAGGAGCAATCTGGGTGCAAAAT -3').

Separate assays of APX isoenzymes activities

Activities of cytosolic (cAPX), stromal (sAPX), thylakoidal (tAPX) and peroxisomal (pAPX) in the leaf extract were separately determined as previously reported by (Amako et al., 1994; Miyake and Asada, 1996) utilizing the different sensitivities of these isoenzymes to a low-AsA condition. Rice leaves were ground to a fine powder in liquid N_2 and the homogenate in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ascorbate, 20% (w/v) sorbitol, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 40 mM KCl, 10 mM $CaCl_2$ and 10% Glycerol using a mortar and pestle. Thereafter was added 0.1% *n*-Dodecyl β-D-maltoside to the homogenate and it was kept under stirring for 15 min and followed by centrifugation at 3000 x g for 10 min. The supernatant was centrifuged at 120 000 x g for 20 min to get the soluble fraction (supernatant) and membrane fraction (pellet). The obtained soluble fraction contained activities of sAPX and cAPX.

The soluble fraction (10 µl) was added to 5.0 ml of N_2-bubbling 50 mM potassium phosphate buffer (pH 7.0) containing 10 µM H_2O_2. At 3, 4, 5, and 6 min after the start of the incubation, the incubated mixture (1.98 ml) was sampled and mixed with 10 µl of 100 mM ascorbate to terminate the inactivation. The residual oxidizing activity of ascorbate was then

assayed by adding 10 µl of 10 mM H_2O_2. The oxidation of ascorbate was followed by a decrease in the A_{290n},. cAPX and sAPX activities were calculated from the inactivation curve of each isoenzyme. The 120,000 × g membrane fraction was washed and suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ascorbate. The suspended-membrane fraction contained activities of tAPX and mAPX isoenzyme, which were assayed separately by the same method using each half-inactivation time as measurements of activities of sAPX and cAPX isoenzymes.

Hydrogen peroxide and TBARS content

Hydrogen peroxide content was measured using the Amplex®-red kit (Thermo Fisher Scientific®, USA), based on colorimetric measure of resorufin formation in presence of H_2O_2 (Zhou et al., 1997). Fresh leaf samples were macerated with liquid N_2 in the presence of 100 mM K-phosphate buffer pH 7.5 and centrifuged at 12,000 x *g* (4 ºC) during 30 min. The supernatant was immediately used for H_2O_2 determination. The absorbance at 560 nm was quantified for H_2O_2 measurement. The hydrogen peroxide content was calculated from a standard curve, and the results were expressed as nmol H_2O_2 g^{-1} fresh mater (FM).

Lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) in accordance with (Cakmak and Horst, 1991). The concentration of TBARS was calculated using its absorption coefficient (155 $mM^{-1}cm^{-1}$), and the results were expressed as nmol MDA-TBA g FW^{-1}.

Gas exchange, chlorophyll a fluorescence and P_N-Ci curve

The net CO_2 assimilation rate (P_N), transpiration (E), stomatal conductance (g_S) and intercellular CO_2 partial pressure (Ci) were measured using a portable infra-red gas analyzer system, equipped with an LED source and a leaf chamber (IRGA LI-6400XT, LI-COR, Lincoln, NE, USA). For instantaneous measurements, the PPFD was fixed at 1200 µmol m^{-2}

s^{-1}, temperature equaled to 28 °C, VDP between 1.0 and 1.5 kPa, and external CO_2 was fixed at atmospheric partial pressure (38 Pa). The amount of blue light was set to be 10% of the PPFD to maximize stomatal aperture (Flexas et al., 2007). Also, the P_N was measured in response to changes in the intercellular *Ci* controlled inside the IRGA leaf chamber. A-Ci fitting curves were determined according to models proposed by (Sharkey et al., 2007). The following parameters associated with photosynthetic efficiency were determined: maximum Rubisco carboxylation rate (*Vcmax*), maximum rate of photosynthetic electron transport (*Jmax*), maximum net photosynthesis (P_N *max*) and mesophyll conductance (*gm*). Photorespiratory rate (P_R) was obtained accorded to (Baggard et al., 2008).

In vivo chlorophyll *a* fluorescence was measured using a LI-6400-40 Leaf Chamber Fluorometer (LI-COR, Lincoln, NE, USA) coupled with the IRGA. The actinic light utilized for measuring the gas exchange and chlorophyll *a* fluorescence was 1000 μmol m^{-2} s^{-1} PPFD, which corresponded to the saturation light for CO_2 assimilation in rice (Carvalho et al., 2014). The fluorescence parameters were measured using the saturation pulse method (Klughammer and Schreiber, 1994). in leaves exposed to light or 30 min dark-acclimated conditions. The intensity and duration of the saturation light pulse were 8000 μmol m^{-2} s^{-1} and 0.7 s, respectively. The following parameters were assessed: the maximum quantum yield of PSII [Fv/Fm = (Fm − Fo)/Fm], measured in 30-min dark-adapted leaves, and the effective quantum yield of PSII [ΔF/Fm' = (Fm' − Fs)/Fm'], measured in leaves exposed to actinic light of 1000 μmol m^{-2} s^{-1}.

Protein extraction for proteomic analysis

Leaf samples (500 mg) were ground using a mortar and pestle with liquid nitrogen. Samples were lyophilizate and the samples were added to 10% trichloroacetic acid and 0.07% 2-mercaptoethanol solution in acetone and mixed thoroughly by vortex. The mixture was

sonicated for 10 min and incubated for 1 h at -20°C. After centrifuged at 9,000 x g for 20 min at 4°C, the supernatant was removed and remained pellet was washed twice with 0.07% 2-mercaptoethonal in acetone. The pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and resuspended in a lysis buffer containing 8 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine and homogenized by vigorous vortex for 1 h at 25°C. The suspension was centrifuged at 20,000 x g for 20 min at 25°C and the resulting supernatant was collected as protein extract. Protein concentration was determined using Bradford method (Bradford, 1976) with BSA as standard.

Protein purification, digestion, and desalt for proteomic analysis

Extracted proteins (100 µg) were adjusted to final volume of 100 µL. The samples were enriched with methanol and chloroform to remove detergent from samples, using standard procedures (Komatsu et al., 2013). Briefly, 400 µL of methanol was added to samples and mixed. Chloroform (100 µL) and 300 µL of water were added to samples, mixed, and centrifuged at 20,000 x g for 10 min to achieve phase separation. The upper phase was discarded and 300 µL of methanol was added to lower phase. Samples were centrifuged at 20,000 x g for 10 min and the pellet was dried. Dried samples were reduced with 25 mM dithiothreitol for 30 min at 56°C in dark and alkylated with 30 mM idoacetamide for 30 min at 37°C in dark. Alkylated proteins were digested with trypsin and lysyl endopeptidase (Wako, Osaka, Japan) at 1:100 enzyme/protein ratio at 37°C for 16 h in dark. Peptides were acidified with 20% formic acid (pH < 3) and desalted with a C18-pipette tip (Nikkyo Technos, Tokyo, Japan).

Protein identification using nanoliquid chromatography mass spectrometry

The peptide samples were separated using an Ultimate 3000 nanoLC system (Dionex, Germering, Germany), and the peptide ions were detected using a nanospray LTQ Orbitrap

Discovery MS (Thermo Fisher Scientific, San Joes, CA, USA) with data-dependent acquisition mode with installed Xcalibur software (version 2.1, Thermo Fisher Scientific). The peptide samples were loaded onto a C18 PepMap trap column (300 μm ID x 5mm, Thermo Fisher Scientific) equilibrated with 0.1% formic acid and eluted from the trap column with a linear acetonitrile gradient in 0.1% formic acid at a flow rate of 200 nL/min. The eluted peptides were loaded and separated on a C18 capillary tip column (75 μm ID x 120 mm, Nikkyo Technos) with a spray voltage of 1.5 kV. Full-scan mass spectra were acquired in the Orbitrap MS over 400-1,500 m/z with a resolution of 30,000. The top ten most intense precursor ions were selected for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35%. Dynamic exclusion was employed within 90s to prevent the repetitive selection of peptides (Zhang et al., 2009).

Data acquisition by mass spectrometry analysis

Protein identification was performed using the Mascot search engine (version 2.5.1, Matrix Science, London, U.K.) and a rice protein database (50253 sequences and 15266515 residues) obtained from the Rice Annotation Project Database (RAP-DB, http://rapdb.dna.affrc.go.jp), including protein sequences supported by FL-cDNA and EST data (IRGSP-1.0_protein_20134-24) and protein sequence predicted computationally (IRGSP1.0_predicted protein_2013-3-9). Proteome Discoverer software (version 1.4, Thermo Fisher Scientific) was used to process the acquired raw data files. For the Mascot searches, the carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Peptide mass tolerance was set at 10 ppm, fragment mass tolerance was set at 0.5 Da, the peptide charge was set at +2, +3, and +4. An automatic decoy database search was performed as part of the search. Mascot results were filtered with the Percolator function to improve the accuracy and sensitivity of peptide

identification. The acquired Mascot results were imported to SIEVE software (version 2.1, Thermo Fisher Scientific).

Differential analysis of proteins using mass spectrometry data

For the differential analysis of relative abundances of peptides and proteins between samples the commercial label-free quantification package SIEVE was used. The chromatographic peaks detected by MS were aligned, and the peptide peaks were detected as a frame on all parent ions scanned by MS/MS using 5 min of frame time width and 10 ppm of frame m/z width. Chromatographic peak areas within a frame were compared for each sample, and the ratios between samples in a frame were determined. The frames detected in the MS/MS scan were matched to the imported Mascot results. The peptide ratio between samples was determined from the variance-weighted average of the ratios in frames that matched the peptides in the MS/MS spectrum. The ratios of peptides were further integrated to determine the ratio of the corresponding proteins. In the differential analysis of protein abundance, total ion current was used for normalization. The minimum requirement for identification of a protein was two matched peptides. Significant changes in the abundance of proteins between samples were analyzed ($p < 0.05$).

Functional analysis

Functional analysis of identified proteins was performed using MapMan (http://mapman.gabipd.org/) (Usadel et al., 2005).

Statistical analyses and experimental design

The experiment was arranged in a completely randomized design in a 2x2 factorial: two genotypes (NT and *apx7*) and two treatments (control and drought), with four independent replicates. An individual pot containing 2 plants represented each replicate. Data

were analyzed using ANOVA, and the means were compared using the Tukey's ($p \leq 0.05$). The relative abundance of each protein was expressed as ratio in relation to control NT plants and it was considered statically significant when $p \leq 0.05$.

Results

OsApx7 silencing decreases both OsApx7 gene expression and stromal APX activity in rice plants and induces increasing of cytosolic APX activity

In order to determine the physiological roles of rice stromal APX, RNAi-knockdown plants were obtained by RNAi-silencing approach. Previously, we have characterized two *RNAiApx7* lines and we chose to maintain line-a (cited here as *apx7* plants) as a representative line, since it exhibited the most representative phenotype (Jardim-Messeder et al., submitted data). To verify the effect of *OsApx7* silencing in plant development, the growth of *apx7* plants was analyzed under control growth conditions. Our results demonstrated that, under normal conditions, *apx7* plants did not exhibit any apparent phenotypic changes in number of tillers, shoot length and shoot biomass accumulation in relation to NT plants (Supplementary Table 2).

Both *OsApx7* transcript and sAPX activity was reduced by approximately 45 and 55%, respectively, when compared to NT plants (Supplementary Figure 1). These results indicate that the expression of the *OsApx7* genes has been successfully reduced by RNAi-silencing technic. Knockdown of *OsApx7* gene also triggered an intense increase of cytosolic APX (cAPX) activity by 57,5%, as well as a reduction of peroxisomal APX (pAPX) activity by 66,5% (Figure 1). However, qPCR analyses showed that only *OsApx2* transcript (target to cytosol) was reduced and *OsApx6* transcripts (target to mitochondria) was increased when compared to NT plants (Jardim-Messeder et al., submitted data).

sAPX knockdown improves drought tolerance in rice plants

In order to examine the effect of *OsApx7* silencing in drought stress tolerance, NT and *apx7* plants were exposed to control (well-watered conditions) or mild drought stress (water withdrawal) for 4 days. Our results show that silencing of *OsApx7* lead to improvement of drought tolerance in rice plants (Supplementary Figure 2) evidenced by higher relative water content (RWC) and plant biomass, as well as lower electrolyte leakage (membrane damage) when compared to NT (Supplementary Table 2). Due to the mild condition of drought stress, no changes in the number of tillers or shoot length were induced in both NT and *apx7* plants (Supplementary Table 2).

Drought stress induces the increase of the overall rate of metabolism and eventually accentuates H_2O_2 production. If not scavenged properly, i.e. sAPX reduction in chloroplasts, H_2O_2 can accumulate and/or migrate from chloroplast to another cellular compartment, possibly triggering signaling events. Considering the importance of APX activity to protection of ROS detrimental effects, we evaluate the possible changes in H_2O_2 and TBARS accumulation. In fact, *apx7* control plants showed a slight increase in H_2O_2 accumulation which may cause increase in TBARS content, when compared to NT plants (Figure 2). After mild drought exposure, both plants showed similar levels of H_2O_2 and TBARS content.

apx7 plants showed similar or even improved levels of photosynthetic parameters when compared to NT at both control and mild drought stress condition

In addition, to understand the physiological mechanism of drought response in photosynthetic efficiency of NT and a*px7* plants, we also analyzed leaf gas exchange and photochemical parameters of both plants subjected to control or mild drought stress (Figure 3). *apx7* plants did not showed significative differences in net photosynthesis (P_N), CO_2 partial pressure (*Ci*) and stomatal conductance (*gs*) when compared to NT plants at control conditions, only a small reduction in transpiration (*E*). After drought stress exposure, both plants showed reduction in all gas exchange parameters, however *apx7* maintain higher levels

of P_N and *gs* than NT (Figure 3). *apx7* plants also showed higher effective quantum yield of PSII [Y(II)] under control and mild drought exposure, however both plants showed similar Fv/Fm (an indicator of photoinhibition) (Figure 4).

To verify the photosynthetic capacity of rice plants after *OsApx7* silencing, P_N-*Ci* curves were performed (Supplementary Figure 3) and parameters derived from the curves analyzed at both control and drought stress conditions (Table 1). NT and *apx7* plants showed very similar V_{cmax} (a parameter associated to *in vivo* Rubisco activity), J_{max} (a parameter associated with electron transport to RuBP regeneration), P_{Nmax} (maximum CO_2 assimilation rate) and *gm* (mesophyll conductance) under control conditions. *apx7* plants, however, increased the estimated photorespiration (P_R), when compared to NT. After drought stress, only NT plants showed reduction in all V_{cmax}, J_{max}, P_{Nmax}, *PR* and *gm* parameters when compared to *apx7* plants after drought stress (Table 1).

apx7 *plants showed minor alterations in proteomic profile under control condition*

To explore changes in the proteome, we performed gel-free/label free proteomic analysis at NT and *apx7* plants after control or mild drought stress. After protein identification by MS/MS, we performed pairwise comparisons of NT control plants (as reference) to the other conditions. In total, 1478 significantly changed proteins ($p \leq 0.05$) (Supplementary Table 3) were analyzed by ratio of protein levels > 1.0 (increased) or ≤ 1.0 (decreased). 49 proteins were identified in *apx7* plants exposed to control (4 increased and 45 decreased proteins), 754 proteins in NT plants exposed to drought stress (662 increased and 92 decreased proteins) and 675 in *apx7* plants exposed to drought stress (615 increased and 60 decreased proteins) (Figure 5).

Based on MapMan ontology, all significantly changed proteins could be separated into 19 functional categories: C1 metabolism, cell, DNA, hormone and polyamine metabolism,

carbohydrate metabolism, lipid metabolism, miscellaneous enzyme family, mitochondrial electron transport, unassigned proteins, protein, nucleotide metabolism, protein metabolism, photosynthesis (PS), RNA, redox, secondary metabolism, signalling, stress, tetrapyrrole synthesis and transport (Figure 6). Surprisingly, *apx7* plants at control conditions showed a very similar proteomic profile than NT plants at the same condition, with much lower number of changed proteins than plants exposed to drought stress ($p \leq 0.05$). The majority of drought-induced changes in protein ratio were increasing protein ratio for both plants, but especially in *apx7* (91%) over NT (87.8%) (Figure 6). This could indicate that *OsApx7* silencing might trigger metabolic adjustment that lead to drought stress tolerance in rice plants exposed to mild drought stress.

apx7 plants increased many proteins involved with light harvesting while NT plants increases Calvin-Benson cycle-related proteins more intensely in plants exposed to mild drought stress

Both plants showed major increasing in protein ratio for PS category, however NT presented more increased proteins (96.4%) than *apx7* plants (92.6%). The Calvin-Benson (C3) cycle is the primary pathway of atmospheric CO_2 uptake and fixation into organic molecules. Surprisingly, NT showed more intense increase in Calvin-Benson-related proteins than *apx7* plants, such as: fructose bisphosphate aldolase, phosphoribulokinase, sedoheptulose biphosphatase and transketolase (Table 2, N. 1; 2; 3; 4 and 5). Also, both plants increased protein ratio of glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase (N. 6 and 7), however *apx7* plants showed the more intense increase. Both plants showed similar increasing of Rubisco (N. 8), however NT plants showed more intense increasing in Rubisco activase protein ratio over apx7 (N. 9).

Similar to Calvin-Benson cycle proteins, both plants showed an increase in ratio of proteins involved with photochemical phase of photosynthesis. Interestingly, *apx7* plants

showed more prominent increase than NT in many light-harvesting and photosystems core-related proteins of both PSII and PSI, such as: photosystem II 23 kDa polypeptide, PsbP, PSII stability/assembly factor HCF136, PSII oxygen-evolving complex protein 1, PSII 11 kD protein, LHCII-CP29, PsaF, PSI reaction center subunit V, LHCI protein I, III and V (N. 10 - 20). Besides these proteins, both plants also increased the amount of LHCII - CP24, PsbQ and PSI reaction center subunit IV (N. 21; 22 and 23), however NT showed more intense increase than *apx7* plants. ATP synthase beta and gamma chain (N. 24 and 25) and ferredoxin NADP reductase (N. 26) are also increase more intensely in NT plants.

Proteomic analyses also showed that many proteins involved in photorespiration are increased in both plants, however the increase was higher in NT over *apx7* plants. glycerate kinase, glycine decarboxylase P subunit, glycine dehydrogenase, glycolate oxidase and serine hydroxymethyltransferase (Table 2, N. 27-30). On the other hand, both plants showed an intense increase in proteins involved with carbohydrate metabolism, however the increase was more intense in *apx7* (93%) than NT (90%) plants. Many proteins involved with glycolysis were increase in both NT and *apx7* plants, such as Glyceraldehyde-3-phosphate dehydrogenase, Enolase 1 and 2, Triosephosphate isomerase, Glucose-6-phosphate isomerase, Fructokinase 2, Succinyl-CoA ligase, Sucrose-phosphatase, Sucrose synthase 2 and Citrate synthase (Table 1, N. 31-40).

Drought stress induced increasing in redox and stress-related proteins in both NT and apx7 *plants*

The perception of drought stress followed by succession of signal transduction events to switch on molecular, cellular and whole plant adaptive processes are critical steps for stress tolerance. NT and *apx7* plants showed an intense increase in protein ratio at both redox and stress categories after drought stress exposure. Among changed proteins, cAPX (Table 2, N.

41) showed higher increase in *apx7* plants (13.47) over NT (10.42), corroborating the increase in this isoform activity previously showed. NT plants also exhibited decrease of protein ratio of tAPX and pAPX (N. 42-43) after drought stress, which also is in accordance with each isoform activity. Similarly, the increase in pAPX protein ratio of *apx7* plants after drought stress is also accompanied by increasing in pAPX activity.

Proteomic analysis showed that three proteins involved with AsA-GSH cycle are increased in both plants exposed to mild drought stress: monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase, with the first two more increased in NT plants and the last more increase in *apx7* plants (Table 2, N. 44-46). NT plants also showed an intense increase in protein ratio of Mn and Cu-Zn superoxide dismutase, catalase B and C, peroxiredoxin 2E, 2-cys peroxiredoxin Bas-1 and glutathione peroxidase (Table 2, N. 47-53). On the other hand, NT showed decrease in protein ratio of catalase A and thioredoxin reductase (N. 54 and 55).

After drought stress, many molecular chaperones were identified in both plants. HSPs were increased in both plants, but *apx7* plants showed higher increase in HSP70 and HSP90, when compared to NT plants. On the other hand, another two proteins also involved with chaperone functions, protein disulfide isomerase and calnexin, showed a strong increase in NT over *apx7* plants (Table 2, N. 56-61).

Discussion

Silencing of *OsApx7* transcript reduced sAPX activity by 50% but did not alter sAPX activity. On the other hand, cAPX activity was intensely increased, probably in an attempt to compensate for sAPX deficiency. For the past years, studies have demonstrated a high degree of plasticity in ROS signaling and antioxidant responses in plants suggesting the existence of redundant pathways for ROS protection that might compensate for the

lack/deficiency of classical ROS removal enzymes, such as cytosolic and chloroplastic APXs (Miller et al., 2007; Rosa et al., 2010; Caverzan et al., 2014). However, these complex responses are still debated, and the elucidation of biochemical mechanisms is lacking. In addition, classical antioxidant pathways should be revised, and new metabolic models are needed to explain these complex responses (Mittler et al., 2011).

apx7 plants also showed decrease in peroxisomal APX activity (pAPX) after *OsApx7* silencing. Our group has previously showed that pAPX-silenced rice exhibited an antioxidant response that provided an improved acclimation to oxidative stress that was induced by CAT deficiency and high photorespiration as compared to NT plants (Sousa et al., 2015). Similarly, *apx7* plants also showed higher photorespiration rate, as well as slightly higher H_2O_2 accumulation and TBARS content, however this increase did not lead to growth impairment or increasing of membrane damage. In fact, proteomic analyses showed that *apx7* plants were very similar to NT in well-watered conditions. Our group have recently demonstrated that rice plants silenced for thylakoidal APX (*apx8*) showed an intense mobilization in many proteins especially those involved with carbohydrate, photosynthesis and protein metabolism (Cunha et al., 2018 - Chapter III). One possibility for this difference is that *apx7* plants showed approximately 50% of *OsApx7* transcripts after RNAi-silencing whereas *apx8* plants showed only 10% of *OsApx8* transcripts.

We have previously demonstrated that the double silencing of both chlAPXs (APX7 and APX8) do not result in visible growth changes of rice plants under control growing conditions, but it causes alterations in the level of proteins involved in photosynthesis and in oxidative metabolism. Moreover, under abiotic stress conditions, the double knockdown plants showed some alterations in photosynthesis (Caverzan et al., 2014). On the other hand, the individual silencing of *OsApx8* transcript under severe drought stress condition led to a decrease of chlAPX activity, which results in higher H_2O_2 content and no

change in acute drought stress tolerance of rice plants (Jardim-Messeder et al., 2018). When evaluated these same plants, now under mild drought stress, we showed that *apx8* plants demonstrated more sensitivity than NT, which may indicate that this isoform is important during the onset of drought stress (Cunha et al, 2018 – Chapter III). Although these studies have reported the importance of *OsApx7* and *OsApx8* isoforms in abiotic stress response in rice plants, however the individual role of *OsApx7* remained unknown.

It is valid to observe that both NT and *apx7* plants showed major increased proteins in all protein classes evaluated in response to drought, as demonstrated in Figure 4. This increase is probably due to the mild intensity of stress, since acute drought stress is known to strongly decrease protein abundance, especially those involved with photosynthesis (Ji et al., 2012). In fact, both NT and *apx7* plants showed a very similar proteomic profile after mild drought exposure. However, *apx7* plants showed higher increase in protein ratio than NT plants, which could have triggered tolerance to mild drought, also demonstrated by relative water content, membrane damage and photosynthetic parameters. In fact, we demonstrated here that the drought tolerance mechanism of *apx7* plants is not correlated to decreasing stomatal aperture or transpiration, but to the capacity to maintain a high transpiration and stomatal conductance under stress condition, which lead to improvement in photosynthetic capacity.

The stomata have two key functions: controlling transpiration, which supplies nutrients and regulates leaf temperature, and controlling the entry of CO_2 into the leaf. Stomatal closure in response to water deficit is the primary limitation to photosynthesis (Flexas and Medrano, 2002) and constitutes a key cost in terms of plant growth and temperature regulation under drought conditions. A higher stomatal conductance under stress condition enable a higher photosynthesis, but the water loss by transpiration is also increased. This can be serious in severe and prolonged stress situations, which could compromise not

only the production but also the plant survival. Interestingly, proteomic analysis indicated that *apx7* plants increased more intensely proteins involved with light harvesting during the stress exposure, whereas NT plants increased more intensely Calvin-Benson cycle-related proteins. Since NT plants showed higher stomatal limitation after drought stress, this strategy did not lead to improvement of photosynthesis.

In fact, *apx7* plants showed higher PSII potential quantum yield associated with V_{cmax}, J_{max}, P_{Nmax} and *gm* compared to NT. Photosynthesis is not only restricted by stomatal limitations but also by non-stomatal limitations that impair metabolic reactions such as RuBP synthesis, ATP synthesis, and photosynthetic electron transport (Cossins and Chen, 1997). Nevertheless, glycerate kinase, glycine decarboxylase and serine hydroxymethyltransferase, which are related to photorespiratory metabolism, were increased in both plants under drought stress, especially in NT. In drought-stressed leaves, the reduction of CO_2 assimilation reduces the electron consumption by photosynthesis (Munné-Bosch, Queval and Foyer, 2013). Photorespiration enhancement may, at least partially, use the excess of electrons generated by light capture through photochemical reactions. A direct involvement of the photorespiratory pathway, acting as an electron sink for the protection of the photosynthetic apparatus from electron induced photo-damage, has been reported in drought-stressed C3 plants (Voss et al., 2013).

The increase of photorespiration-related proteins reflected that drought stress induce limitations to photosynthesis, which may in turn result in a positive feedback on photorespiration in both plants. Besides the higher increase in proteins involved with photorespiration, NT plants did not show the higher P_R. Surprisingly, *apx7* plants not only showed increasing in photorespiration rate at control and after drought stress. In fact, there was reported in the literature the existence of a strong interaction between the chloroplast redox status and photorespiration (Voss et al., 2013). Silencing of *OsApx7* gene might result

in up-regulation of photorespiration, as a flexible strategy to optimize photosynthesis while protecting against oxidative stress.

Rubisco activase is a chloroplast protein that activates and maintains Rubisco in an active state by facilitating removal of various sugar phosphates that either block substrate binding or prevent carbamylation (Suganami et al., 2018). In this way, Rubisco activase is also considerate a molecular chaperone, controlling the switching of Rubisco conformation from inactive to active state (Salin, 1988; Spreitzer and Salvucci, 2002). Both NT and *apx7* plants showed an increase in Rubisco activase protein ratio, however NT plants increased more intensely. The increasing of these enzymes might alleviate the damage on Rubisco by drought stress.

Another molecule with molecular chaperone function are the heat shock proteins (HSPs). HSPs functions as molecular chaperones acting as a buffer to limit misfolding and resolve protein aggregates formed during natural conditions or, especially, during abiotic stress exposure (Jacob et al., 2017). It was reported in literature that HSP70/HSP90 machinery plays essential functions in plants to integrate signals from biotic and abiotic stress conditions, modulating physiological responses to abscisic acid (ABA) (Clement et al., 2011). In fact, *apx7* plants were found to increase protein abundance of HSP70/90, after mild drought stress exposure, which may have contributed to a higher tolerance to drought exposure than NT plants. On the other hand, NT plants showed an intense increase in calnexin and protein disulfide isomerase, both molecular chaperones assisting in protein folding in the endoplasmic reticulum (ER) (Wang et al., 2004). This different mobilization of molecular chaperones is also perceived in *apx8* plants under the same condition (Cunha et al., 2018 – Chapter III), indicating that RNAi-silencing induced alteration in the pattern of rice cellular protection.

Chloroplasts are recognized as significant sources of ROS, which are produced as a direct consequence of the highly energetic redox reactions in the photosynthetic electron

transport chain, especially during stress exposure. Various peroxidases link the detoxification of H_2O_2 to the oxidation of specific substrates, of which ascorbate is one of the most important (Dietz, 2016). Ascorbate functions not only as an antioxidant but also as a substrate for APX and violaxanthin de-epoxidase in chloroplasts (Asada, 1999). Therefore, the regeneration of ascorbate is essential for the maintenance of the activity of the antioxidative scavenging system and is achieved by AsA-GSH cycle functioning in the chloroplast stroma. Here, we showed that both plants increase MDAR, DHAR, Cu-Zn SOD and GR protein ratio after mild drought stress, however the increase was higher in NT plants. This mobilization might be expected to provide antioxidant protection for NT against damage by mild drought stress. However, NT plants were not more resistant to drought stress than *apx7* plants.

Interpretation of proteomic data can be challenging. Over the past decade, researchers have witnessed the amazing evolution of computational and experimental technologies in molecular cell biology (Jensen, 2006). At the same time, it has become evident that proteins are much more complex, diverse and dynamic than was originally anticipated. Proteomic technologies have faced a big challenge regarding the intricate control and regulation of protein functions, localizations and interactions in cells and tissues. In this work, proteomic analyses showed many similar pathways increased in both NT and *apx7* plants. Even with the higher amount of identified protein, there is still a question about the surprising tolerance observed in *apx7* plants over NT after drought stress exposure.

We demonstrated here that the silencing of *OsApx7* apparently does not change the H_2O_2 homeostasis in chloroplasts but increase the plant tolerance drought stress. This response indicates that deficiency in the s*APX* activity could be involved in stress signaling via a still unknown mechanism to date, increasing the photosynthetic performance during mild drought stress exposure. The subtle changes showed by proteomic data may have, together, lead to a more tolerant phenotype in *apx7* plants. Therefore, we hypothesized that

RNAi-silencing of *OsApx7* transcript could trigger drought-resistance mechanisms in *apx7* plants which guarantee the maintenance of photosynthetic perfomance under drought stress exposure. More studies are needed to elucidate the specific mechanisms involved with sAPX deficiency and triggering of compensatory processes related to drought tolerance.

References

AMAKO K, CHEN G-X, ASADA K. Separate Assays Specific for Ascorbate Peroxidase and Guaiacol Peroxidase and for the Chloroplastic and Cytosolic Isozymes of Ascorbate Peroxidase in Plants. **Plant Cell Physiol** 35: 497–504, 1994.

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

M. BAGARD, D. LE THIEC, E. DELACOTE, M.P. HASENFRATZ-SAUDER, J. BANVOY, J. GÉRARD, P. DIZENGREMEL, Y. JOLIVET (2008) Ozone-induced changes in photosynthesis and photorespiration of hybrid poplar in relation to the developmental stage of the leaves, **Physiol. Plant**. 134: 559–574, 2008.

BLUM A, EBERCON A. Cell Membrane Stability as a Measure of Drought and Heat Tolerance in Wheat. **Crop Sci** 21: 43–47, 1981.

BRADFORD MM. A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. **Analy** 254: 248–254, 1976.

CAKMAK I, HORST WJ. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). **Physiol Plant** 83: 463–468, 1991.

CAPELL T, BASSIE L, CHRISTOU P. Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. **Proc Natl Acad Sci U S A** 101: 9909–14, 2004.

CARVALHO FEL, RIBEIRO CW, MARTINS MO, BONIFACIO A, STAATS CC, ANDRADE CMB, CERQUEIRA J V., MARGIS-PINHEIRO M, SILVEIRA JAG. Cytosolic APX knockdown rice plants sustain photosynthesis by regulation of protein expression related to photochemistry, Calvin cycle and photorespiration. **Physiol Plant** 150: 632–645, 2014.

CAVERZAN A, BONIFACIO A, CARVALHO FEL, ANDRADE CMB, PASSAIA G, SCHÜNEMANN M, MARASCHIN F DOS S, MARTINS MO, TEIXEIRA FK, RAUBER R. The knockdown of chloroplastic ascorbate peroxidases reveals its regulatory role in the photosynthesis and protection under photo-oxidative stress in rice. **Plant Sci** 214: 74–87, 2014.

CHAVES MM, FLEXAS J, PINHEIRO C. Photosynthesis under drought and salt stress: Regulation mechanisms from whole plant to cell. **Ann Bot** 103: 551–560, 2009.

CHINTAKOVID N, MAIPOKA M, PHAONAKROP N, MICKELBART M V., ROYTRAKUL S, CHADCHAWAN S. Proteomic analysis of drought-responsive proteins in rice reveals photosynthesis-related adaptations to drought stress. **Acta Physiol Plant.** 2017.

CLEMENT M, LEONHARDT N, DROILLARD M-J, REITER I, MONTILLET J-L, GENTY B, LAURIERE C, NUSSAUME L, NOEL LD. The Cytosolic/Nuclear HSC70 and HSP90 Molecular Chaperones Are Important for Stomatal Closure and Modulate Abscisic Acid-Dependent Physiological Responses in *Arabidopsis*. **Plant Physiol** 156: 1481–1492, 2011.

DAT J, VANDENABEELE S, VRANOVÁ E, VAN MONTAGU M, INZÉ D, VAN BREUSEGEM F. Dual action of the active oxygen species during plant stress responses. **Cell Mol Life Sci** 57: 779–795, 2000.

DIETZ K-J. Thiol-Based Peroxidases and Ascorbate Peroxidases: Why Plants Rely on Multiple Peroxidase Systems in the Photosynthesizing Chloroplast? **Mol Cells** 39: 20–5, 2016.

DIETZ K-J, TURKAN I, KRIEGER-LISZKAY A. Redox- and reactive oxygen species-dependent signalling in and from the photosynthesizing chloroplast. **Plant Physiol** 171: 1541–1550, 2016.

FLEXAS J, MEDRANO H. Drought-inhibition of photosynthesis in C3 plants: Stomatal and non-stomatal limitations revisited. **Ann Bot** 89: 183–189, 2002.

FLEXAS J, ORTUÑO MF, RIBAS-CARBO M, DIAZ-ESPEJO A, FLÓREZ-SARASA ID, MEDRANO H. Mesophyll conductance to CO_2 in Arabidopsis thaliana. **New Phytol** 175: 501–511, 2007.

FOYER CH, NOCTOR G. Stress-triggered redox signalling : what ' s in pROSpect ? **Plant, Cell Environ** 951–964, 2016.

HERNANDEZ M, FERNANDEZ-GARCIA N, DIAZ-VIVANCOS P, OLMOS E. A different role for hydrogen peroxide and the antioxidative system under short and long salt stress in Brassica oleracea roots. **J Exp Bot** 61: 521–535, 2010.

HOAGLAND DR, ARNON DI. The water-culture method for growing plants without soil. 32, 1950.

HOSSAIN MA, BHATTACHARJEE S, ARMIN S-M, QIAN P, XIN W, LI H-Y, BURRITT DJ, FUJITA M, TRAN L-SP. Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging. **Front Plant Sci** 6: 1–19, 2015.

JACOB P, HIRT H, BENDAHMANE A. The heat-shock protein/chaperone network and multiple stress resistance. **Plant Biotechnol J** 15: 405–414, 2017.

JARDIM-MESSEDER D, CAVERZAN A, RAUBER R, CUNHA JR, CARVALHO FEL, GAETA ML, DA FONSECA GC, COSTA JM, FREI M, SILVEIRA JAG, MARGIS-PINHEIRO, M. Thylakoidal APX modulates hydrogen peroxide content and stomatal closure in rice (*Oryza sativa* L.). **Environ Exp Bot** 150: 46–56, 2018.

JENSEN ON. Interpreting the protein language using proteomics. **Nat Rev Mol Cell Biol** 7: 391–403, 2006.

JI K, WANG Y, SUN W, LOU Q, MEI H, SHEN S, CHEN H. Drought-responsive mechanisms in rice genotypes with contrasting drought tolerance during reproductive stage. **J Plant Physiol** 169: 336–344, 2012.

KLUGHAMMER C, SCHREIBER U. An improved method, using saturating light pulses, for the determination of photosystem I quantum yield via P700+-absorbance changes at 830 nm. **Planta.** 1994.

KOMATSU S, NANJOA Y, NISHIMURAB M. Proteomic analysis of the flooding tolerance mechanism in mutant soybean. **J Proteomics** 79: 231–250, 2013.

LENKA SK, KATIYAR A, CHINNUSAMY V, BANSAL KC. Comparative analysis of drought-responsive transcriptome in Indica rice genotypes with contrasting drought tolerance. **Plant Biotechnol J** 9: 315–327, 2011.

MIKI D, SHIMAMOTO K. Simple RNAi vectors for stable and transient suppression of gene function in rice. **Plant Cell Physiol** 45: 490–495, 2004.

MILLER G, SUZUKI N, RIZHSKY L, HEGIE A, KOUSSEVITZKY S, MITTLER R. Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. **Plant Physiol** 144: 1777–1785, 2007.

MITTLER R, VANDERAUWERA S, SUZUKI N, MILLER G, TOGNETTI VB, VANDEPOELE K, GOLLERY M, SHULAEV V, VAN BREUSEGEM F. ROS signaling: The new wave? **Trends Plant Sci** 16: 300–309, 2011.

MIYAKE C, ASADA K. Inactivation Mechanism of Ascorbate Peroxidase at Low Concentrations of. **Plant Cell Physiol** 37: 423–430, 1996.

MUNNÉ-BOSCH, S.; QUEVAL, G.; FOYER CH, MUNNE-BOSCH S, QUEVAL G, FOYER CH. The Impact of Global Change Factors on Redox Signaling Underpinning Stress Tolerance. **Plant Physiol** 161: 5–19, 2013.

RECZEK CR, CHANDEL NS. ROS-dependent signal transduction. **Curr Opin Cell Biol** 33: 8–13, 2015.

ROSA SB, CAVERZAN A, TEIXEIRA FK, LAZZAROTTO F, SILVEIRA JAG, FERREIRA-SILVA SL, ABREU-NETO J, MARGIS R, MARGIS-PINHEIRO M. Cytosolic APx knockdown indicates an ambiguous redox responses in rice. **Phytochemistry** 71: 548–558, 2010.

SATO Y, YOKOYA S. Enhanced tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, sHSP17.7. **Plant Cell Rep** 27: 329–334, 2008.

SELVARAJ MG, ISHIZAKI T, VALENCIA M, OGAWA S, DEDICOVA B, OGATA T, YOSHIWARA K, MARUYAMA K, KUSANO M, SAITO K. Overexpression of an *Arabidopsis thaliana* galactinol synthase gene improves drought tolerance in transgenic rice and increased grain yield in the field. **Plant Biotechnol J** 15: 1465–1477, 2017.

SHARKEY TD, BERNACCHI CJ, FARQUHAR GD, SINGSAAS EL. Fitting photosynthetic carbon dioxide response curves for C3 leaves. **Plant, Cell Environ** 30: 1035–1040, 2007.

SHU L, LOU Q, MA C, DING W, ZHOU J, WU J, FENG F, LU X, LUO L, XU G. Genetic, proteomic and metabolic analysis of the regulation of energy storage in rice seedlings in response to drought. **Proteomics** 11: 4122–4138, 2012.

SILVEIRA JAG, ARAÚJO SAM, LIMA JPMS, VIÉGAS RA. Roots and leaves display contrasting osmotic adjustment mechanisms in response to NaCl-salinity in Atriplex nummularia. **Environ Exp Bot** 66: 1–8, 2009.

SILVEIRA JAG, CARVALHO FEL. Proteomics, photosynthesis and salt resistance in crops: An integrative view. **J Proteomics** 143: 24–35, 2016.

SMIRNOFF N. The Function and Metabolism of Ascorbic Acid in Plants. **Ann Bot** 78: 661–669, 1996,

SOUSA RH V, CARVALHO FEL, RIBEIRO CW, PASSAIA G, CUNHA JR, LIMA-MELO Y, MARGIS-PINHEIRO MM, SILVEIRA JAG. Peroxisomal APX knockdown triggers antioxidant mechanisms favourable for coping with high photorespiratory H_2O_2 induced by CAT deficiency in rice. **Plant, Cell Environ** 38: 499–513, 2015.

SUGANAMI M, SUZUKI Y, SATO T, MAKINO A. Relationship between Rubisco activase and Rubisco contents in transgenic rice plants with overproduced or decreased Rubisco

content. **Soil Sci Plant Nutr** 1–8, 2018.

TEIXEIRA FK, MENEZES-BENAVENTE L, GALVÃO VC, MARGIS R, MARGIS-PINHEIRO M. Rice ascorbate peroxidase gene family encodes functionally diverse isoforms localized in different subcellular compartments. **Planta** 224: 300–314, 2006.

TODAKA D, SHINOZAKI K, YAMAGUCHI-SHINOZAKI K. Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. **Front Plant Sci** 6: 84, 2015.

UPADHYAYA NM, SURIN B, RAMM K, GAUDRON J, SCHÜNMANN PHD, TAYLOR W, WATERHOUSE PM, WANG M-B. Agrobacterium-mediated transformation of Australian rice cultivars Jarrah and Amaroo using modified promoters and selectable markers. **Funct Plant Biol** 27: 201, 2000.

USADEL B, NAGEL A, THIMM O, REDESTIG H, BLAESING OE, STEINHAUSER D, GIBON Y, MORCUENDE R, WEICHT D, MEYER S. Extension of the Visualization Tool MapMan to Allow Statistical Analysis of Arrays , Display of Coresponding Genes , and Comparison with Known Responses 1. **Plant Physiol** 138: 1195–1204, 2005.

VOSS I, SUNIL B, SCHEIBE R, RAGHAVENDRA A. Emerging concept for the role of photorespiration as an important part of abiotic stress response. **Plant Biol (Stuttg)** 15: 713–722, 2013.

WANG W, VINOCUR B, SHOSEYOV O, ALTMAN A. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. **Trends Plant Sci** 9: 244–252, 2004.

WU Y, MIRZAEI M, PASCOVICI D, CHICK JM, ATWELL BJ, HAYNES PA. Quantitative proteomic analysis of two different rice varieties reveals that drought tolerance is correlated with reduced abundance of photosynthetic machinery and increased abundance of ClpD1 protease. **J Proteomics** 143: 73–82, 2016.

ZHANG Y, WEN Z, WASHBURN MP, FLORENS L. Effect of dynamic exclusion duration on spectral count based quantitative proteomics. **Anal Chem** 81: 6317–6326, 2009.

ZHOU M, DIWU Z, PANCHUK-VOLOSHINA N, HAUGLAND RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. **Anal Biochem** 253: 162–168, 1997.

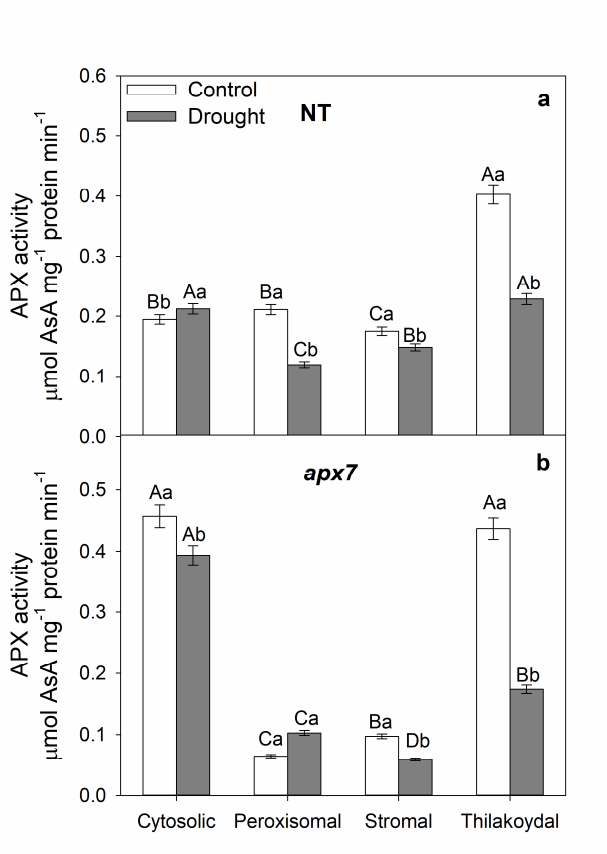


Figure 1. Ascorbate peroxidase activity in cytosolic, stromal, thylakoidal and peroxisomal fractions measured in leaves of rice plants exposed to control (a) or 4 days of drought stress (b). Different capital letters represent significant differences between the treatments within lines (NT and apx7), and different lowercase letters represent significant differences between the lines within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test, ($p \leq 0.05$).

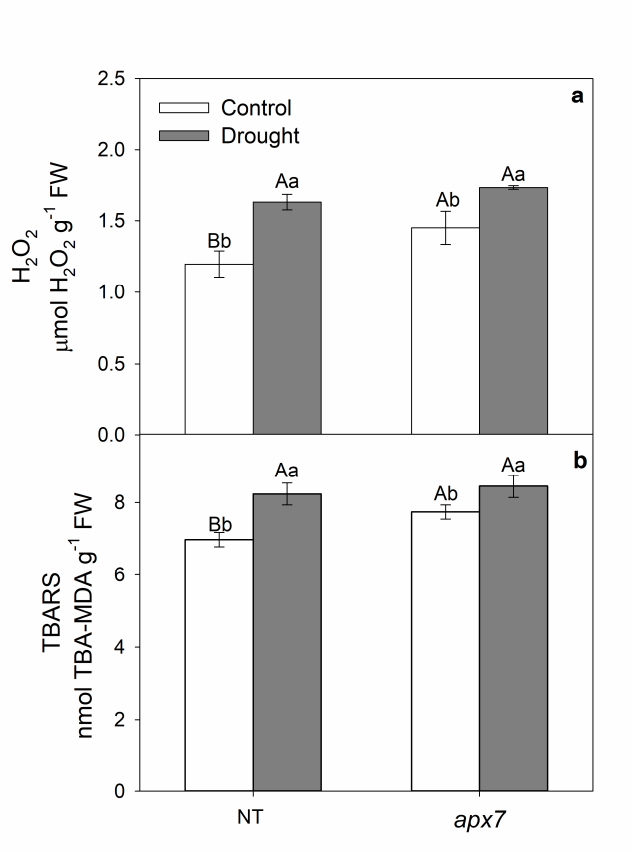


Figure 2. H_2O_2 (a) and thiobarbituric acid-reactive-substances, TBARS (b) contents of NT and *apx7* rice plants exposed to control or drought stress for 4 days. Different capital letters represent significant differences between the treatments within lines (NT and *apx7*), and different lowercase letters represent significant differences between the lines within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test, ($p \leq 0.05$).

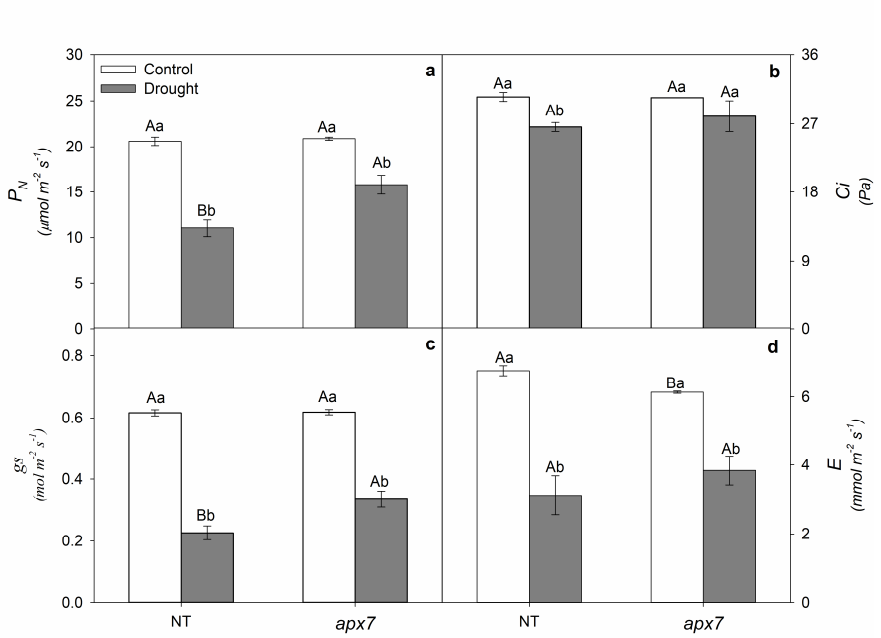


Figure 3. Photosynthesis, P_N (a), intercellular CO_2 partial pressure, Ci (b), stomatal conductance, gs (c) and transpiration, E (d) of NT and apx7 rice plants exposed to control or drought stress for 4 days. Different capital letters represent significant differences between the treatments within lines (NT and apx7), and different lowercase letters represent significant differences between the lines within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test, ($p \leq 0.05$).

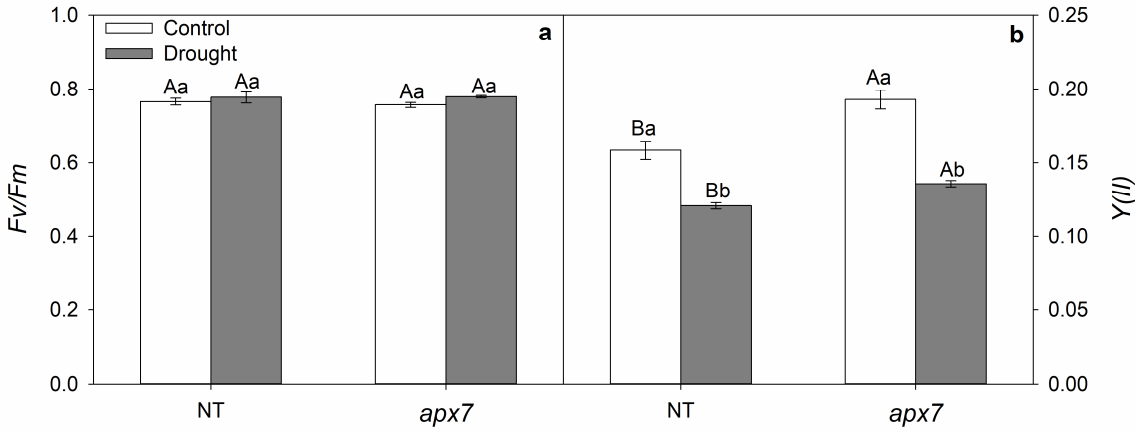


Figure 4. Potential quantum yield (Fv/Fm) (a) and effective quantum yield of PSII [Y(II)] (b) of NT and apx7 rice plants exposed to control or drought stress for 4 days. Different capital letters represent significant differences between the treatments within lines (NT and apx7), and different lowercase letters represent significant differences between the lines within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test, ($p \leq 0.05$).

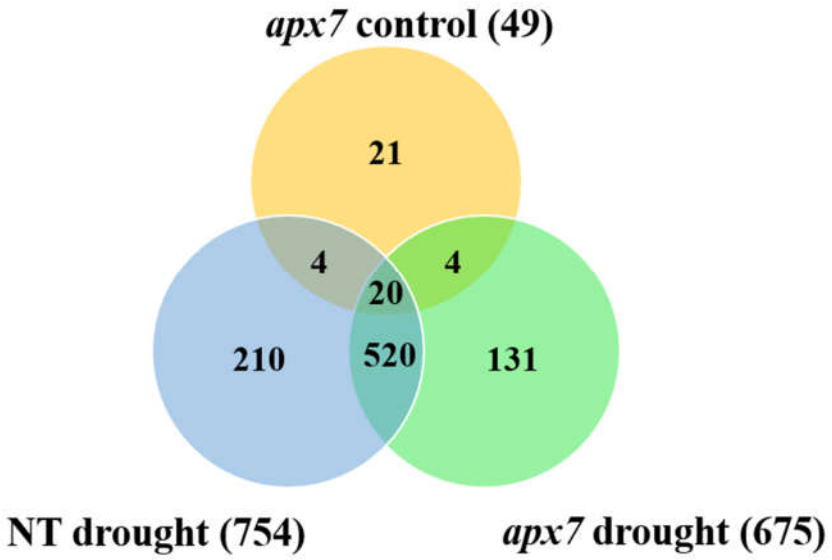


Figure 5.Venn diagram depicting proteins of NT and *apx7* rice leaves after control or 4 days of drought stress. The Venn diagram was constructed in the Pangloss Venn diagram generator (http://www.pangloss.com/seidel/Protocols/venn.cgi).

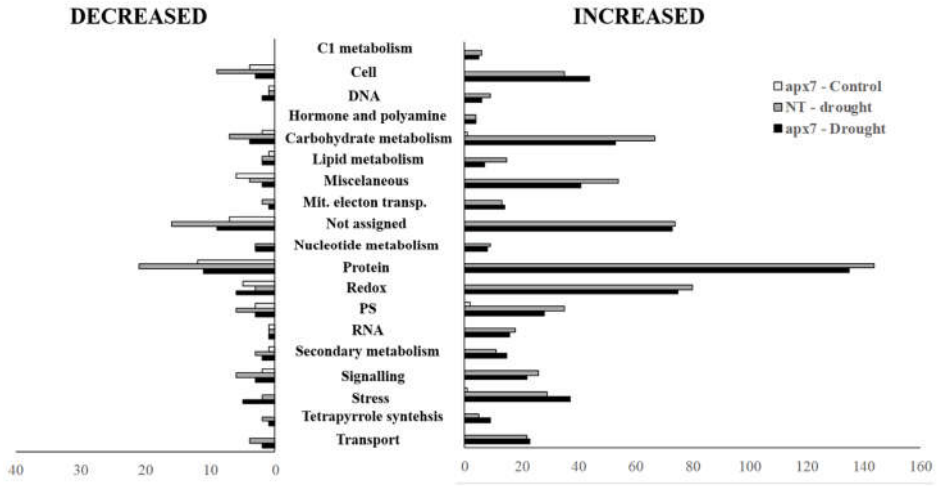


Figure 6. Proteome changes of rice NT and *apx7* leaves after control or 4 days of drought stress. Proteins were extracted from rice leaves using the TCA method. After digestion with trypsin and lysyl endopeptidase, peptides were compared using LC−MS/MS-based proteomic analysis. Protein abundance ratios were estimated using SIEVE software. One-way ANOVA tests were performed with GraphPad software ($p \leq 0.05$). Significantly changed proteins were analyzed by ratio of protein levels > 1.0 (increased) or ≤ 1.0 (decreased). Functional categories of significantly changed proteins were performed using MapMan ontology.

Table 1. V_{cmax} (in vivo Rubisco carboxylation rate), J_{max} (maximum electron transport rate), P_N (the maximum CO_2 assimilation rate), P_R (photorespiration) and gm (mesophyll conductance) of NT and *apx7* rice plants exposed to control or drought stress for 4 days. Different capital letters represent significant differences between the treatments within lines (NT and *apx7*), and different lowercase letters represent significant differences between the lines within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test, ($p \leq 0.05$).

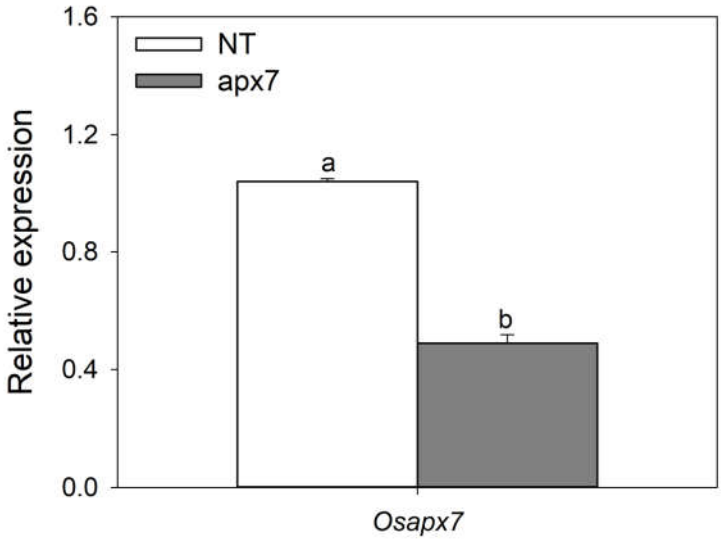
		V_{cmax}		J_{max}		P_{Nmax}		P_R		g_m	
NT	**Control**	156.1	Aa	111.8	Aa	24.9	Aa	20.2	Bb	10.2	Aa
	Drought	122.3	Bb	90.1	Bb	17.7	Bb	26.9	Ba	7.5	Bb
apx7	**Control**	165.8	Aa	116.4	Aa	25.8	Aa	23.5	Ab	10.4	Aa
	Drought	150.0	Ab	113.9	Aa	25.2	Aa	30.9	Aa	8.3	Ab

Table 2. Summary of differentially accumulated proteins of NT and *apx7* rice plants exposed to control or drought stress for 4 days. The relative abundance of each protein was expressed as ratio in relation to control NT plants and it was considered statically significant when $p \leq 0.05$. *ND: not detected.*

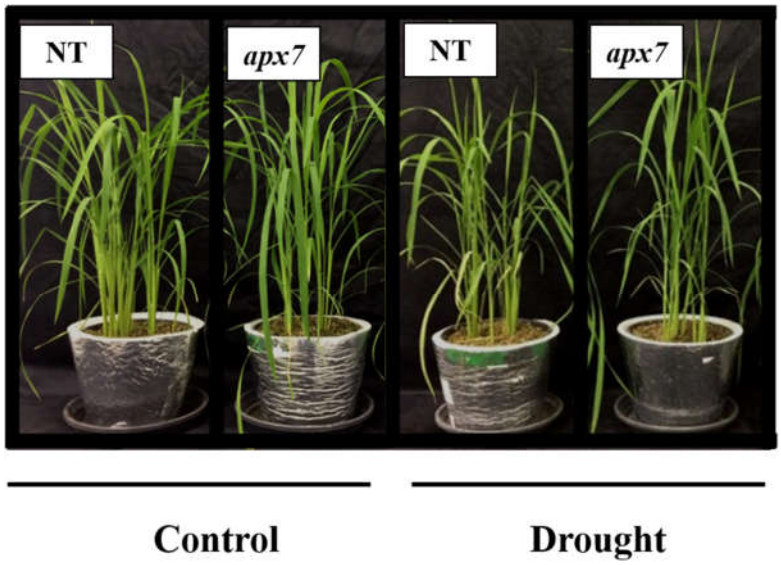
Function	Acession*	N.	Protein name	*p-value*	NT-D x NT-C	*p-value*	APX7-D x NT-C	*p-value*	APX7-C x NT-C
PS	Os06g0608700	1	Fructose bisphosphate aldolase	0.00	20.70	0.00	3.36	0	ND
	Os02g0698000	2	Phosphoribulokinase	0.00	54.03	0.00	27.14	0	ND
	Os04g0234600	3	Sedoheptulose-1,7-bisphosphatase.	0.00	8.53	0.03	2.29	0	ND
	Os04g0266900	4	Transketolase	0.05	110.23	0.04	5.31	0	ND
	Os06g0133800	5	Transketolase	0.00	11.32	0.00	6.98	0	ND
	Os04g0459500	6	Glyceraldehyde-3-phosphate dehydrogenase	0.00	43.29	0.00	52.78	0	ND
	Os05g0496200	7	Phosphoglycerate kinase	0.00	7.71	0.00	14.01	0	ND
	Os01g0899425	8	Ribulose bisphosphate carboxylase/oxygenase large subunit	0.00	15.23	0.00	15.96	0	ND
	Os11g0707000	9	Ribulose bisphosphate carboxylase activase	0.00	87.87	0.00	29.56	0	ND
	Os07g0141400	10	Photosystem II 23 kDa polypeptide	0.00	8.31	0.00	9.30	0	ND
	Os08g0512500	11	PsbP	0.02	4.00	0.04	38.22	0	ND
	Os06g0729650	12	Photosystem II stability/assembly factor HCF136	0.00	1.24	0.00	1.88	0	ND
	Os01g0501800	13	Photosystem II oxygen-evolving complex protein 1	0.00	105.35	0.00	183.26	0	ND
	Os03g0333400	14	Photosystem II 11 kD protein	0.01	2.00	0.03	3.64	0	ND
	Os07g0558400	15	LHCII - Chlorophyll a/b_binding protein CP29 precursor.	0.00	97.42	0.00	10.31	0	ND
	Os03g0778100	16	PsaF	0.05	15.87	0.01	70.16	0	ND
	Os09g0481200	17	Photosystem I reaction center subunit V	0.02	4.66	0.00	4.71	0	ND
	Os06g0320500	18	LHCI - Chlorophyll a/b binding protein 1	0.00	5.66	0.00	655.37	0	ND
	Os02g0197600	18	LHCI - Chlorophyll a/b binding protein type III	0.01	26.40	0.00	37.14	0	ND
	Os02g0764500	20	LHCI - Chlorophyll a/b binding protein 5	0.01	2.27	0.00	2.48	0	ND
	Os04g0457000	21	LHCII - Chlorophyll a/b binding protein CP24	0.00	342.96	0.00	34.88	0	ND
	Os07g0105600	22	PsbQ	0.00	10.97	0.00	1.93	0.0	0.8
	Os07g0435300	23	Photosystem I reaction center subunit IV	0.00	505.73	0.03	45.08	0	ND
	Os10g0355800	24	ATP synthase CF1 beta subunit	0.00	16.35	0.00	15.81	0	ND
	Os07g0513000	25	ATP synthase gamma chain	0.00	9.48	0.00	3.16	0	ND
	Os02g0103800	26	Ferredoxin NADP reductase	0.00	32.53	0.00	24.60	0	ND
	Os01g0682500	27	Glycerate kinase	0.00	3.53	0.00	4.30	0	ND
	Os06g0611900	28	Glycine decarboxylase P subunit	0.00	7.34	0.00	40.83	0	ND

	Os07g0152900	29	Glycolate oxidase	0.00	9.97	0.00	8.79	0	ND
	Os03g0738400	30	Serine hydroxymethyltransferase	0.00	33.22	0.00	14.81	0	ND
Carbohydrate metabolism	Os04g0486600	31	Glyceraldehyde-3-phosphate dehydrogenase	0.00	20.75	0.00	19.33	0	ND
	Os06g0136600	32	Enolase 1	0.00	8.46	0.00	17.05	0	ND
	Os03g0248600	33	Enolase 2	0.00	13.31	0.00	19.60	0	ND
	Os01g0147900	34	Triosephosphate isomerase	0.00	4.53	0.00	6.19	0	ND
	Os03g0776000	35	Glucose-6-phosphate isomerase	0.00	1.83	0.00	2.08	0	ND
	Os06g0232200	36	Fructokinase 2	0.00	6.29	0.00	9.20	0	ND
	Os02g0621700	37	Succinyl-CoA ligase	0.00	12.82	0.00	21.72	0	ND
	Os02g0143100	38	Sucrose phosphatase	0.01	6.24	0.04	19.25	0	ND
	Os06g0194900	39	Sucrose synthase 2	0.00	3.78	0.04	1.54	0	ND
	Os02g0194100	40	Citrate synthase	0.00	5.27	0.00	2.18	0	ND
Redox	Os07g0694700	41	Cytosolic ascorbate peroxidase.	0.00	10.92	0.00	13.47	0	ND
	Os04g0434800	42	Thylakoid bound ascorbate peroxidase	0.05	0.89	0	ND	0	ND
	Os08g0549100	43	Peroxisome type ascorbate peroxidase	0.00	0.84	0.00	1.32	0	ND
	Os09g0567300	44	Monodehydroascorbate reductase	0.00	5.01	0.02	2.92	0	ND
	Os06g0232600	45	Dehydroascorbate reductase	0.00	12.70	0.00	2.10	0.02	0.7
	Os02g0813500	46	Glutathione reductase	0.00	3.08	0.01	10.58	0	ND
	Os05g0323900	47	Mn Superoxide dismutase	0.00	9.92	0.00	2.09	0.05	1.0
	Os08g0561700	48	Cu-Zn Superoxide dismutase	0.00	1001.10	0.00	259.11	0	ND
	Os06g0727200	49	Catalase isozyme B	0.00	20.09	0.00	1.04	0	ND
	Os03g0131200	50	Catalase isoenzyme C	0.00	9.62	0.00	1.04	0	ND
	Os02g0192700	51	Peroxiredoxin-2E-2	0.00	14.95	0.00	6.01	0	ND
	Os02g0537700	52	2-cys peroxiredoxin BAS1	0.00	28.63	0.00	18.04	0	ND
	Os06g0185900	53	Glutathione peroxidase	0.00	1.63	0.00	0.63	0	ND
	Os02g0115700	54	Catalase isozyme A	0.00	0.75	0.00	1.67	0	ND
	Os07g0657900	55	Thioredoxin reductase	0.04	0.65	0	ND	0	ND
Stress	Os12g0244100	56	Heat shock 70 protein	0.00	1.62	0.02	3.26	0	ND
	Os05g0303000	57	Heat shock 70 protein	0.00	3.48	0.00	9.07	0	ND
	Os08g0487800	58	Heat shock protein 90-5	0.00	6.49	0.00	9.06	0	ND
	Os12g0514500	59	Heat shock protein 90-6	0.01	1.76	0.04	2.07	0	ND
	Os11g0199200	60	Protein disulfide isomerase	0.01	12.65	0.00	3.43	0	ND
	Os04g0402100	61	Calnexin	0.01	10.08	0.03	1.97	0	ND

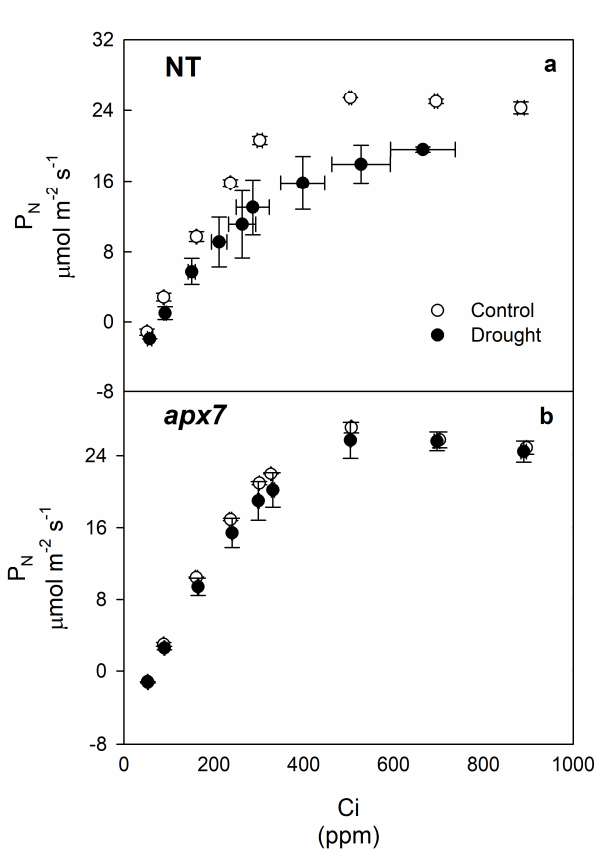
*Acession: protein identification according to the The Rice Annotation Project Database (RAP-DB).



Supplementary Figure 1. Transcript levels of *OsApx7* in plants grown in control conditions. Different capital letters represent significant differences among the NT and *apx7* plants. The relative expression of APX locus was normalized by the average value obtained from the NT and *apx8* plants. The relative expression of APX locus was normalized by the average value obtained from the NT control plants.



Supplementary Figure 2. Morphological aspects of shoots from NT and *apx7* rice plants exposed to control or drought stress for 4 days. Plants are the most representative from four biological replicates.



Supplementary Figure 3. Photosynthesis response to increasing internal CO_2 partial pressure (P_N/Ci curve) in NT and *apx7* rice plants exposed to control or drought stress for 4 days. Data are the means of four replications ± SD.

Supplementary Table 1. Minimum and maximum changes in air temperature, humidity, vapor pressure deficit (VPD) and light intensity of greenhouse conditions measured in a typical day. Data are means of four days.

	Air temperature (ºC)	**Humidity (%)**	**VPD (kPa)**	**Light intensity (µmol m^{-2} s^{-1})**
Minimum	24.3 ± 0.5	42.7 ± 3.2	0.7 ± 0.1	0
Maximum	33.7 ± 0.6	80.2 ± 5.2	1.5 ± 0.2	500.3 ± 5.8

Supplementary Table 2. Number of tillers, shoot length, shoot biomass, membrane damage and relative water content (RWC) of NT and *apx7* rice plants exposed to control or drought stress for 4 days. Different capital letters represent significant differences between the treatments within lines (NT and *apx7*), and different lowercase letters represent significant differences between the lines within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test ($p \leq 0.05$).

		Number of tillers[1]	Shoot lenght[2]	Shoot biomass[3]	Membrane damage[4]	RWC[4]
NT	**Control**	3.5 Aa	86.8 Aa	30.2 Aa	26.5 Ab	75.3 Aa
	Drought	3.0 Aa	84.8 Aa	24.3 Bb	36.5 Ba	54.4 Bb
apx7	**Control**	3.0 Aa	85.0 Aa	29.7 Aa	27.8 Aa	75.2 Aa
	Drought	3.0 Aa	84.2 Aa	28.7 Aa	28.8 Aa	73.9 Aa

[1] plant^{-1}; [2] cm; [3] g plant^{-1}; [4] %

Supplementary Table 3. Differentially accumulated proteins in leaves of NT and *apx7* rice plants exposed to control or drought stress for 4 days. Proteins were identified by label-free proteomics and compared to NT control treatment. (Excel file)

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6 PROTEOMIC AND PHYSIOLOGICAL APPROACHES REVEAL NEW INSIGHTS FOR UNCOVER THE ROLE OF RICE THYLAKOIDAL APX IN RESPONSE TO DROUGHT STRESS

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Abstract

Chloroplast APX isoforms display controversial roles as H_2O_2 scavengers and signaling players in response to abiotic stress and conclusive results are lacking. We tested the hypothesis that thylakoidal APX displays an important role for drought tolerance, especially by regulating abundance of essential protein species. For this, *OsApx8* RNAi-silenced rice (*apx8*) and non-transformed plants (NT) were exposed to mild water deficit. The drought-sensitivity in *apx8* plants was revealed by decreases in shoot growth, relative water content and photosynthesis, which was accompanied by increased membrane damage, all compared to NT plants. This higher sensitivity of *apx8* plants to mild drought stress was also related to a lower accumulation of important protein species involved in several metabolic processes,

especially photosynthesis, photorespiration and redox metabolism. Despite *apx8* plants have displayed an effective induction of compensatory antioxidant mechanisms in well-watered conditions, it was not enough to maintain H_2O_2 homeostasis and avoid oxidative and physiological disturbances under mild drought conditions. Thus, thylakoidal APX is involved in several phenotypic modifications at proteomic profile level, possibly via a H_2O_2-induced signaling mechanism. Consequently, this APX isoform is crucial for rice plants effectively cope with a mild drought condition.

Keywords: ascorbate peroxidases; photosynthesis; proteome; redox metabolism; water deficit; *Oryza sativa*.

Introduction

Ascorbate peroxidases (APXs) are important antioxidant enzymes catalyzing the reduction of H_2O_2 into water using ascorbate as an electron donor [1]. In rice, two isoforms are addressed to chloroplasts, the APX7 (stromal) and APX8 (thylakoidal) [2]. In addition, rice APX6 isoform is supposed to be addressed for both mitochondria and chloroplasts [2]. Despite its potential roles in plant defense metabolism, chlAPX are known to be highly inhibited by H_2O_2 in absence of ascorbate, which is possibly linked to an irreversible cross-linking of the protein heme group to the distal Trp41 and radical formation in Cys31 and Cys125 [3]. These mechanisms could be related to the controversy concerning the biological importance of the chlAPX in plants, especially under stressful conditions [4].

Maintaining the electron flow through the thylakoid membranes, especially under stressful conditions, is vital for preventing oxidative damage to chloroplasts [5]. The water-water cycle (WWC) comprehends the production and scavenging of ROS by the copper/zinc superoxide dismutases (Cu/Zn-SOD) and chlAPX, besides the regeneration of non-enzymatic molecules in chloroplasts, such as DHA and MDHAR [6]. In these reactions, the membrane-attached Cu/Zn-SOD convert the O_2^- produced in PSI to H_2O_2. Subsequently, thylakoidal

APX is supposed to act as the first line of ROS scavenging, reducing H_2O_2 back into water using ascorbate as an electron donor. Also, H_2O_2 could be removed by stromal APX (sAPX) as a second defense layer in stroma, avoiding H_2O_2 accumulation [7]. Thus, chlAPXs, especially thylakoidal APX, have been considered to be a bottleneck in the water–water cycle, at least in higher plants, because of their high susceptibility to H_2O_2 [2]. This characteristic may affect the capacity of plants to tolerate stress, which should make this a good gene-target for improving plant stress tolerance [3].

Several studies have failed to find a "stress-sensitive phenotype" among chlAPX loss-of-function mutants [8–11]. Works involving *Arabidopsis thaliana* transformed plants deficient in sAPX and/or tAPX isoforms have suggested for a minor importance of these enzymes in the plant growth and development [8–10]. Indeed, these plants do not display any visible phenotypic alterations under non-stressful growth conditions. In opposition, under photooxidative stress, tAPX knocked out mutants exhibited increased accumulation of H_2O_2 and oxidized proteins, rather than sAPX knocked out plants [10]. In rice, similar results have been reported for plants silenced by RNAi for cytosolic, peroxisomal and chloroplastic APX isoforms [11–13]. These plants exhibited normal growth as compared to non-transformed (NT) plants and a great tolerance to several abiotic stress conditions [13,14]. Remarkably, double chlAPX knockdown rice plants exhibited no differences in terms of photosynthetic performance under severe high light stress [11].

Recently, much have been discussed about a more complex function of these enzymes, acting not only as classic peroxidase proteins, but also as regulators of retrograde H_2O_2 signaling in order to fine-tuning abiotic and biotic stress responses [15]. Therefore, two important questions are still open: 1) Which is the real physiological importance of chloroplastic APX isoforms under abiotic stress conditions? 2) Which are the protein species/pathways regulated by thylakoidal APX activity or downstream H_2O_2 levels involved

in tolerance/sensitivity against abiotic stress conditions? Particularly, in response to water deficit stress, the studies involving the role of chloroplast APX are scarce. On other hand, the majority of the rice cultivars are adapted to paddy soils and, as an adaptive consequence, commonly they are drought-sensitive [16]. Unfortunately, in the majority of the carried out works, the plants are exposed to acute water deficit, a condition that is commonly associated to drastic stress symptoms [17]. Recently, we demonstrated that rice plants deficient in thylakoidal APX exposed to an acute drought stress triggered increased H_2O_2 accumulation which was associated with stomatal closing, evidencing the role of that enzyme in these processes [18].

In this current study, thylakoidal APX knockdown rice plants (*apx8*) were exposed to mild water deficit to evaluate the role of thylakoidal APX isoform in drought tolerance. To achieve an integrative view, we employed reverse genetic, proteomics and physiological approaches. Our data clearly evidence that silenced rice plants displayed massive changes in protein species expression profile, especially related to redox metabolism and photosynthesis. Remarkably, proteomic analysis suggests that *apx8* knockdown plants are not able to trigger accumulation of essential protein species in suitable levels to effectively cope with drought like the non-transformed plants. These responses suggest that thylakoidal APX protein is essential to phenotypic alterations involving several physiological processes, mainly photosynthetic efficiency and oxidative protection under mild water deficit. The physiological importance of thylakoidal APX isoform in these processes is discussed.

Materials and methods

Construction of the plant vector and plant transformation

A chimeric gene producing mRNA with a hairpin structure (hpRNA) was constructed based on the sequence of the *OsApx8* (LOC_Os02g34810) gene. The following primer pairs

were used to amplify a 227 bp *RNAiOsApx8* sequence: 5'-CTCGAGGCTGCGAAATACTCCTACGG-3' and 5'-GGTACCTCGAGAGGAGGTCATCAGACCATCG-3'. PCR product was cloned into the Gateway vector pANDA, in which hairpin RNA is driven by the maize ubiquitin promotor and an intron placed upstream of the inverted repeats [19]. Rice plants with knockdown of *OsApx8* (*apx8* plants) were obtained by *Agrobacterium tumefaciens*-mediated transformation of rice embryogenic calli (*Oryza sativa* L. ssp japonica cv. Nipponbare) induced from seeds and cultivated in NB medium at 28°C in the dark [20]. In this work, we used RNAi plants at the F3 generation.

Plant growth and drought stress treatment

Experiments were taken place at Federal University of Ceará – Brazil (3°44'43.6"S 38°34'28.3"W). Non-transformed (NT) and RNAi knockdown rice plants were sowed in a 3L vase containing a 1:1 mix of sand and vermiculite. Plants were watered every other day with distilled water, until drainage, and every three days with a half-strength of Hoagland and Arnon's nutrient solution [21]. Plants were grown for 45 days in a greenhouse under natural conditions in a photoperiod of 12h, as described in Supplementary Table 1. For the water deficit treatment, the irrigation of plants was withdrawn for four consecutive days. Rice plants daily irrigated as described above were used as the control treatment.

Phenotypic parameters

All phenotypic parameters were determined before and after four days of well-watered control or mild water deficit (drought) exposure in both NT and *apx8* plants. Tillers were hand counted and shoot length was measured from the ligule to leaf tip using a flat ruler. To determine shoot biomass, shoots were harvested, oven dried at 80°C for 72 h, and weighed according to [22].

Relative water content and electrolyte leakage

The leaf relative water content (RWC) was calculated as follows: RWC = [(FW − DW)/(TW − DW)] × 100, where FW is the fresh weight, TW is the turgid weight measured after 6 h of saturation in deionized water at 4 °C in the dark and DW is the dry weight determined after 48 h in an oven at 75 °C [22]. Cellular integrity (electrolyte leakage) was measured as described previously [23]. Leaf samples were placed in tubes containing deionized water. The flasks were incubated in a shaker for 12 h, and the electric conductivity in the medium (L1) was measured. Then, the medium was boiled (95 °C) for 60 min and the electric conductivity (L2) was measured again. The relative membrane damage (MD) was estimated by MD = L1/L2 x 100.

Quantitative PCR (qPCR)

Real-time PCR experiments were carried out using cDNA synthesized from total RNA purified with TRIzol (Invitrogen®). The samples were treated with DNAase (Invitrogen®) to remove the eventual genomic DNA contamination and complementary DNA (cDNA) was obtained using the SuperscriptTMII (Life Technologies®) reverse transcriptase system and a 24-polyTV primer (Invitrogen®). After synthesis, cDNAs were diluted 10–100 times in sterile water for use in PCR reactions. All reactions were repeated four times, and expression data analyses were performed after comparative quantification of the amplified products using the 2−ΔΔCt method [24,25]. qPCR reactions were performed in an Applied Biosystems StepOne plus Real Time PCR system (Applied Bio- systems®) using SYBR and primer for *OsApx8* gene (F-5'-GGTACCTCGAGAGGAGGTCATCAGACCA-3' and R-5'-CTCGAGGCTGCGAAATACTCCTACCG-3'). The following genes were used as normalizers: *OsFDH3* (F-5'-TTCCAATGCATTCAAAGCTG-3' and R-5'-

CAAAATCAGCTGGTGCTTCTC-3'), *OsUBI* (F-5'- ACCACTTGCACCGCCACTACT-3' and R-5'- ACGCCTAAGCCTGCTGGTT-3') and *OsACT2* (F-5'- GGACGTACAACTGGTATCGTGTT-3' and R-5'- GTTCAGCAGTGGTAGTGAAGGAG-3').

Assays for APX isoenzyme activities

Activities of cytosolic (cAPX), stromal (sAPX), thylakoidal (tAPX) and peroxisomal (pAPX) in the leaf extract were separately determined as previously reported by [26,27] utilizing the different sensitivities of these isoenzymes to a low-ASC concentration conditions. Rice leaves were ground to fine powder in liquid N_2 and then macerated in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ascorbate, 20% (w/v) sorbitol, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 40 mM KCl, 10 mM $CaCl_2$ and 10% Glycerol using a mortar and pestle. Thereafter was added 0.1% *n*-Dodecyl β-D-maltoside to the homogenate and it was kept under stirring for 15 min and followed by centrifugation at 3000 x g for 10 min. The supernatant was centrifuged at 120 000 × g for 20 min to get the soluble fraction (supernatant) and membrane fraction (pellet). The obtained soluble fraction contained activities of sAPX and cAPX.

The soluble fraction (10 µl) was added to 5.0 ml of N_2-bubbling 50 mM potassium phosphate buffer (pH 7.0) containing 10 µM H_2O_2. At 3, 4, 5, and 6 min after the start of the incubation, the incubated mixture (1.98 ml) was sampled and mixed with 10 µl of 100 mM ascorbate to terminate the inactivation. The residual oxidizing activity of ascorbate was then assayed by adding 10 µl of 10 mM H_2O_2. The oxidation of ascorbate was followed by a decrease in the A_{290n}. cAPX and sAPX activities were calculated from the inactivation curve of each isoenzyme. The 120,000 × g membrane fraction was washed and suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ascorbate. The suspended-membrane

fraction contained activities of tAPX and mAPX isoenzymes, which were assayed separately by the same method using each half-inactivation time as measurements of activities of sAPX and cAPX isoenzymes.

Hydrogen peroxide and TBARS content

Hydrogen peroxide content was measured using the Amplex®-red kit (Thermo Fisher Scientific®, USA), based on colorimetric measure of resorufin formation in presence of H_2O_2 [28]. Fresh leaf samples were macerated with liquid N_2 in the presence of 100 mM K-phosphate buffer pH 7.5 and centrifuged at 12,000 × g (4 ºC) during 30 min. The supernatant was immediately used for H_2O_2 determination. The absorbance at 560 nm was quantified for H_2O_2 measurement. The hydrogen peroxide content was calculated from a standard curve, and the results were expressed as ηmol H_2O_2 g^{-1} fresh mater (FM). Lipid peroxidation was measured based on the formation of thiobarbituric acid- reactive substances (TBARS) in accordance with [29]. The concentration of TBARS was calculated using its absorption coefficient (155 $mM^{-1}cm^{-1}$), and the results were expressed as ηmol MDA-TBA g FW^{-1}.

Gas exchange, chlorophyll a fluorescence and P_N-Ci curve

The net CO_2 assimilation rate (P_N), transpiration (E), stomatal conductance (g_S) and intercellular CO_2 partial pressure (Ci) were measured using a portable infra-red gas analyzer system, equipped with an LED source and a leaf chamber (IRGA LI-6400XT, LI-COR, Lincoln, NE, USA). For instantaneous measurements, the PPFD was fixed at 1200 μmol m^{-2} s^{-1}, temperature equaled to 28 °C, VDP between 1.0 and 1.5 kPa, and external CO_2 was fixed at atmospheric partial pressure (38 Pa). The amount of blue light was set to be 10% of the PPFD to maximize stomatal aperture [30]. Also, the P_N was measured in response to changes in the intercellular Ci controlled inside the IRGA leaf chamber. A-Ci fitting curves were

determined according to models proposed by [31]. The following parameters associated with photosynthetic efficiency were determined: maximum Rubisco carboxylation rate (*Vcmax*), maximum rate of photosynthetic electron transport (*Jmax*), maximum net photosynthesis (P_N *max*) and mesophyll conductance (*gm*). Photorespiratory rate (P_R) was obtained accorded to [32].

In vivo chlorophyll *a* fluorescence was measured using a LI-6400-40 Leaf Chamber Fluorometer (LI-COR, Lincoln, NE, USA) coupled with the IRGA. The actinic light utilized for measuring the gas exchange and chlorophyll *a* fluorescence was 1000 μmol m^{-2} s^{-1} PPFD, which corresponded to the saturation light for CO_2 assimilation in rice [12]. The fluorescence parameters were measured using the saturation pulse method [33] in leaves exposed to light or 30 min dark-acclimated conditions. The intensity and duration of the saturation light pulse were 8000 μmol m^{-2} s^{-1} and 0.7 s, respectively. The following parameters were assessed: the maximum quantum yield of PSII [Fv/Fm = (Fm − Fo)/Fm], measured in 30-min dark-adapted leaves, and the effective quantum yield of PSII [ΔF/Fm' = (Fm' − Fs)/Fm'], measured in leaves exposed to actinic light of 1000 μmol m^{-2} s^{-1}.

Protein extraction for proteomic analysis

Leaf samples (500 mg) were ground using a mortar and pestle with liquid N_2. Samples were lyophilized and the samples were added to 10% trichloroacetic acid and 0.07% 2-mercaptoethanol solution in acetone (v/v) and mixed thoroughly by vortex. The mixture was sonicated for 10 min and incubated for 1 h at -20°C. Afterwards, centrifuged at 9,000 × g for 20 min at 4°C, the supernatant was removed, and the remaining pellet was washed twice with 0.07% 2-mercaptoethanol in acetone (v/v). The pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and resuspended in a lysis buffer containing 8 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine and

homogenized by vigorous vortex for 1 h at 25°C. The suspension was centrifuged at 20,000 x g for 20 min at 25°C and the resulting supernatant, protein extract, was collected. Protein concentration was determined using Bradford method [34] with BSA as a standard.

Protein purification, digestion, and desalt for proteomic analysis

Extracted proteins (100 μg) were adjusted to final volume of 100 μL. The samples were enriched with methanol and chloroform to remove detergent from samples, using standard procedures [35]. Briefly, 400 μL of methanol was added to samples and mixed. Chloroform (100 μL) and 300 μL of water were added to samples, mixed, and centrifuged at 20,000 x g for 10 min to achieve phase separation. The upper phase was discarded and 300 μL of methanol was added to lower phase. Samples were centrifuged at 20,000 x g for 10 min and the pellet was dried. Dried samples were reduced with 25 mM dithiothreitol for 30 min at 56°C in dark and alkylated with 30 mM idoacetamide for 30 min at 37°C in dark. Alkylated proteins were digested with trypsin and lysyl endopeptidase (Wako, Osaka, Japan) at 1:100 enzyme/protein ratio at 37°C for 16 h in dark. Peptides were acidified with 20% formic acid (pH < 3) and desalted with a C18-pipette tip (Nikkyo Technos, Tokyo, Japan).

Protein species identification using nanoliquid chromatography mass spectrometry

The peptide samples were separated using an Ultimate 3000 nanoLC system (Dionex, Germering, Germany), and the peptide ions were detected using a nanospray LTQ Orbitrap Discovery MS (Thermo Fisher Scientific, San Jose, CA, USA) with data-dependent acquisition mode with installed Xcalibur software (version 2.1, Thermo Fisher Scientific). The peptide samples were loaded onto a C18 PepMap trap column (300 μm ID x 5mm, Thermo Fisher Scientific) equilibrated with 0.1% formic acid and eluted from the trap column with a linear acetonitrile gradient in 0.1% formic acid at a flow rate of 200 ηL/min. The eluted

peptides were loaded and separated on a C18 capillary tip column (75 μm ID x 120 mm, Nikkyo Technos) with a spray voltage of 1.5 kV. Full-scan mass spectra were acquired in the Orbitrap MS over 400-1,500 m/z with a resolution of 30,000. The top ten most intense precursor ions were selected for collision-induced fragmentation in the linear ion trap at normalized collision energy of 35%. Dynamic exclusion was employed within 90s to prevent the repetitive selection of peptides [36].

Data acquisition by mass spectrometry analysis

Protein species identification was performed using the Mascot search engine (version 2.5.1, Matrix Science, London, U.K.) and a rice protein database (50253 sequences and 15266515 residues) obtained from the Rice Annotation Project Database (RAP-DB, http://rapdb.dna.affrc.go.jp), including protein sequences supported by FL-cDNA and EST data (IRGSP-1.0_protein_20134-24) and protein sequence predicted computationally (IRGSP1.0_predicted protein_2013-3-9). Proteome Discoverer software (version 1.4, Thermo Fisher Scientific) was used to process the acquired raw data files. For the Mascot searches, the carbamethylation of cysteine was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Peptide mass tolerance was set at 10 ppm, fragment mass tolerance was set at 0.5 Da, the peptide charge was set at +2, +3, and +4. An automatic decoy database search was performed a part of the search. Mascot results were filtered with the Percolator function to improve the accuracy and sensitivity of peptide identification. The acquired Mascot results were imported to SIEVE software (version 2.1, Thermo Fisher Scientific).

Differential analysis of proteins using mass spectrometry data

For the differential analysis of relative abundances of peptides and protein species between samples the commercial label-free quantification package SIEVE was used. The chromatographic peaks detected by MS were aligned, and the peptide peaks were detected as a frame on all parent ions scanned by MS/MS using 5 min of frame time width and 10 ppm of frame m/z width. Chromatographic peak areas within a frame were compared for each sample, and the ratios between samples in a frame were determined. The frames detected in the MS/MS scan were matched to the imported Mascot results. The peptide ratio between samples was determined from the variance-weighted average of the ratios in frames that matched the peptides in the MS/MS spectrum. The ratios of peptides were further integrated to determine the ratio of the corresponding protein species. In the differential analysis of protein species abundance, total ion current was used for normalization. The minimum requirement for identification of a protein species was two matched peptides. Significant differences between abundance of each protein species were analyzed by Student's T-test ($p \leq 0.05$). The average test was performed between the ratios of the values obtained from each treatment and NT-control plants.

Principal component analysis

In order to synthesize the large number of variables evaluated in the current proteomic study, a principal component analysis (PCA) was performed employing normalized ratios of protein species from the different treatments (APX8C, NTD and APX8D) all compared with NTC as reference (see Supplementary table 3). The PCA was performed employing the OriginPro 2017 software (Origin Lab Corporation, Northampton, USA), according to the software user guide.

Functional analysis

Functional analysis of identified protein species was performed using MapMan (http://mapman.gabipd.org/) [37].

Statistical analyses and experimental design

The experiment was arranged in a completely randomized design in a 2x2 factorial: two genotypes (NT and *apx8*) and two treatments (control and drought), with four independent replicates. An individual pot containing 2 plants represented each replicate. Physiological data were analyzed using ANOVA, and the means were compared using the Tukey's test ($p \leq 0.05$). Proteomics data were tested by Student's T-test ($p \leq 0.05$).

Results and discussion

OsApx8 silencing decreases strongly OsApx8 gene expression, APX8 protein abundance and APX8 activity under well-watered condition

In order to determine the physiological role of rice thylakoidal APX under well-watered and mild drought conditions, RNAi-knockdown plants were obtained (Supplementary Figure 1). Silenced rice plants displayed a slight decrease in shoot biomass, number of tillers and shoot length compared to NT plants in well-watered conditions (Supplementary Table 2). Our group has previously reported that double silencing of chloroplast isoforms (*OsApx7* and *OsApx8*) did not display phenotype differences in rice plants grown under optimal conditions, despite the expression of both genes have been greatly reduced [11]. In this study, RNAi-knockdown caused a strong decrease of *OsApx8* transcript amount (by 90%) in *apx8* plants (Supplementary Figure 2). Proteomic analysis corroborated these results evidencing that APX8 protein amount in silenced plants represented only 6% of NT (Table 1). This decrease was associated to a significant reduction in APX activity in the thylakoidal fraction that

reached approximately 60% when compared to NT plants (Figure 1). Unexpectedly, *OsApx8* silencing also negatively affected the activities of cytosolic (cAPX), peroxisomal (pAPX) and stromal (sAPX) isoforms, reducing by approximately 88, 15 and 23% respectively, compared to NT plants, (Figure 1). The reduction in the activities of these APX isoforms could have contributed for the slight increase noticed in the H_2O_2 content of silenced plants (Figure 2A).

Silenced plants accumulate H_2O_2 in both control and mild drought stress conditions and exhibit higher TBARS content and membrane damage

In order to investigate the effects of *OsApx8* silencing and water deficit on redox homeostasis, we evaluated the level of oxidative stress markers (H_2O_2 content, TBARS content – an indicative of lipid peroxidation and membrane damage – by electrolyte leakage measurement), in both silenced and NT plants. In parallel to reduction in the activities of thylakoidal APX and other APX isoforms, the water deficit induced increases in the contents of H_2O_2 and TBARS in *apx8* plants that were related to increase in membrane damage, compared with NT plants (Figure 2 and Supplementary Table 2). These results indicate that silenced rice plants suffered higher redox disturbances, evidencing the importance of thylakoidal APX as a H_2O_2 scavenger in chloroplasts [10,38]. We have previously showed that rice plants knocked down for both chlAPXs (*apx7/8*) also exhibit higher levels of H_2O_2, membrane damage and TBARS content, than NT plants, in both control conditions and after oxidative stress induced by methyl viologen [11]. Since H_2O_2 steady-state concentration is increased in *apx8* plants, it is plausible to argue that this ROS could act as a signal involved in the oxidative response network in conditions of thylakoidal APX deficiency, as has been previously demonstrated for cytosolic APXs-knockdown rice [13] and in several other plant species [8,9,10,39].

Much have been discussed in the literature about the function of H_2O_2 as a signaling molecule in plants and it has been speculated that this ROS could act through the oxidation of cysteine residues in redox-sensitive proteins, such as the catalytic center of the thiol peroxidases [40]. Altogether, the obtained data indicate that *apx8* rice plants showed markedly changes in their redox homeostasis in normal growth conditions and these changes could have led to distinct sensitive responses when they are exposed to mild drought stress compared to NT plants.

Proteomic analysis evidences that silenced plants display a great number of decreased protein species and after drought exposure the intensity and amount of increased protein species are restricted compared to NT plants

In this study, 1065 protein species had its abundance significantly changed ($p \leq 0.05$) in APX8-control, NT-D and APX-D, compared to NT-control plants (Supplementary Table 3). Among these, 298 protein species were identified in *apx8* plants exposed to control (90 increased and 208 decreased), 754 protein species in NT plants exposed to drought stress (662 increased and 92 decreased) and 809 in *apx8* plants exposed to drought stress (511 increased and 296 decreased) (Figure 3). We also performed a principal component analysis (PCA) in order to reduce the large number of variables to a smaller number of groups that can be more readily visualized and interpreted. The PCA was performed employing normalized protein species ratios, with NT- control (NT-C) as a reference (value 1.0) and the principal components 1 and 2 were able to explain 71.73% of the variance. The loading plot based on PCA results evidenced that the changes in protein species ratios presented by drought stress NT plants (NT-D) were distinct from *apx8* control (APX8-C) and *apx8* drought stressed plants (APX8-D) - (Figure 4). These responses indicate that drought treatment was able to induce stronger changes in the proteomic profile in NT plants as compared to *apx8* silenced

ones. A possible explanation for these interesting results consists in the fact that changes induced by APX8 silencing in rice plants could be able to produce a pre-acclimation condition to abiotic stress [18]. Accordingly, the initial changes in *apx8* plants should result in a reduced plasticity of proteomic profile when a second stress situation was imposed, which was already observed in other studies involving *apx* gene family silencing in rice [11–13].

Based on MapMan ontology, all significantly changed protein species were separated into 19 functional categories: C1 metabolism, cell, DNA, hormone and polyamine metabolism, carbohydrate metabolism, lipid metabolism, miscellaneous enzyme family, mitochondrial electron transport, unassigned protein species, protein metabolism, nucleotide metabolism, photosynthesis (PS), RNA, redox, secondary metabolism, signaling, stress, tetrapyrrole synthesis and transport (Figure 5). Under control conditions, *apx8* plants only exhibited 29.5% of increased protein species in total, and maintained "carbohydrate metabolism", "signaling", "stress" and "transport" categories strongly decreased. After drought stress, most of protein species were increased in both genotypes, especially in NT (87.8%) in comparison to *apx8* plants (63.3%), suggesting a substantial number of drought-responsive protein species in rice plants (Figure 5, Table 1).

In both genotypes, the major increased protein species categories were the "carbohydrate" and "protein metabolism" but the number of these increased protein species was much higher in NT plants. Both categories are considered key-processes involved in stress response, once they support the cellular energy costs required to trigger effective plant defense responses and provide specific molecular mediators in many plant cell processes [41]. In contrast, *apx8* plants showed higher number of decreased protein species than NT plants, in all categories, and a strong increase in the quantity of protein species classified in "stress" category, after the drought treatment. These results suggest that *apx8* plants could have triggered a different pattern of protein regulation in response to drought stress after silencing,

in comparison to NT plants, as is corroborated by PCA (Figure 4). That differential metabolic reprogramming in silenced plants could be a consequence of a previous increased H_2O_2 steady state level and/or other redox alterations [42]. Thus, these distinct responses highlight the great physiological importance of thylakoidal APX, especially in the maintaining of the redox balance, possibly affecting several metabolic pathways by H_2O_2 signaling.

In vivo gas exchange measurements indicate that silenced plants are more sensitive to drought compared to NT but they display similar photochemical activity

In order to investigate differences in the photosynthetic capacity, gas exchanges and photochemical measurements were performed in well-watered and drought conditions. In non-stressful conditions, both silenced and NT plants displayed similar photosynthetic perfomance. However, under mild water deficit the *apx8* plant presented higher diffusive limitations indicated by higher reduction in stomatal conductance (g_S), which was related to decreases in net CO_2 assimilation (P_N), intercellular CO_2 concentration (Ci) and transpiration (E) – Figure 6. These changes were associated with higher decreases of *in vivo* maximum Rubisco carboxylation (Vcmax) and maximum electron transport (Jmax), obtained from P_N-Ci fitted curves (Supplementary Figure 3). In addition, drought induced strong increase in photorespiration rates (PR) and this raise was more prominent in silenced plants (Table 2). Conversely, the photochemical indicators, maximum (Fv/Fm) and actual quantum efficiency of PSII (YII), did not change by effect of silencing or drought in the two genotypes (Figure 7).

In this study plants suffered a mild water stress (withdraw for 4 days), as revealed by the values of membrane damage and relative water content in leaves (Supplementary Table S2). In these conditions, CO_2 assimilation is initially decreased by stomatal limitation and further by biochemical restriction in the Calvin-Benson cycle reactions, especially by decrease in Rubisco activity [43]. Previously, we have demonstrated that *apx8* plants display

high stomatal restriction associated to H_2O_2 accumulation in response to acute water deficit [18]. In this study, data suggest that higher reduction in photosynthetic CO_2 assimilation in silenced plants is due to restriction in both stomatal and Rubisco activity, assuming that *Vcmax* is a good indicator for activity of this enzyme, as has been widely reported [44]. In parallel, the restriction for carboxylation and CO_2 diffusion in silenced plants could have favored the Rubisco oxygenation activity, stimulating photorespiration [45] as was observed under drought condition. On the other hand, some reports have evidenced that CO_2 assimilation reactions are limiting steps for photosynthesis compared to photochemical phase [12] and this feature could explain why the photochemistry did not change by effect of mild drought in silenced plants.

Proteomic analysis reveals that drought induces increase in photosynthetic and photorespiration related-protein species

To verify if the *in vivo* photosynthetic responses displayed by silenced and NT plants could be explained by changes at protein species profile level, proteomic analyses were performed. The results indicate that 60% of protein species involved in PS category are increased in *apx8* plants under well-watered condition compared to NT (Figure 5). After drought stress, proteomic analyses showed that both NT and *apx8* plants presented great increase in PS-related protein species, but the former presented higher increased amount of protein species (96% and 64%, respectively). The drought-induced changes on the protein species levels belonging to Calvin-Benson, were not closely associated with *in vivo* CO_2 assimilation measurements, in both silenced and NT plants. Indeed, despite Rubisco large subunit (N. 18), transketolase (N. 19), phosphoribulokinase (N. 20), aldolase (N. 22), fructose-bisphosphate aldolase (N. 23) and Rubisco activase (N. 25) have been increased in both genotypes (Table 1), CO_2 assimilation rates decreased greatly especially in drought-

stressed silenced plants. Interestingly, only chloroplastic triose phosphate isomerase (N. 21) was decreased in both genotypes, whereas sedoheptulase-1,7-bisphosphatase (N. 24) decreased only in *apx8* plants.

The low correlation observed between *in vivo* CO_2 measurements and changes in the amount of Calvin-Benson cycle protein species is possible since the majority of these enzymes are regulated post-translationally, especially by redox reactions via thioredoxins [46,47]. Moreover, Rubisco might be synthetized in amounts over the minimum required for its optimum activity and it may be accumulated in leaf tissues under stress condition as a storage protein [12,48]. The fact that sedoheptulase-1,7-bisphosphatase has been decreased in silenced plants, in parallel to decreases in CO_2 assimilation and RuDP regeneration rates (estimated by *Jmax*), reinforces the importance of this enzyme for the Calvin-Benson cycle activity [49].

The proteomics also revealed that drought differentially affected the amount of photochemical protein species in silenced plants compared to NT plants despite the *in vivo* activity has remained unchanged. Indeed, some chlorophyll *a*/*b* binding proteins (N.1, 3 and 4), PsbS (N. 5), PsbP (N.6), subunit IV of PSI reaction center (N. 7), cytochrome b6-f complex iron-sulfur subunit (N. 8), FNR (N. 9), plastocyanin (N. 10) and ATP synthase (N. 11 and 12) were increased by drought in both genotypes but they were more prominently enhanced in NT plants (Table 1). The low correlation between *in vivo* photochemical measurements and protein species abundance was previously reported in rice plants deficient in cytosolic APX exposed to high light [12]. These relationships are very complex since photochemical activity involves integrated actions of several protein species in a modular way and an isolated protein amount might not represent the activity in a physiological context.

The over-accumulation of some protective protein species might have favored the PSII activity. The elongation factor Tu (N. 44), an important protein involved in the translation of

chloroplastic genome encoded protein species, was greatly increased in both drought-stressed genotypes but its abundance was prominently higher in silenced plants. This response occurred in parallel to some important heat shock proteins, HSP10 (N. 27), HSP70 (N. 28 and 29) and HSP90 (N. 30 and 31), which were strongly increased in drought-stressed *apx8* plants. The over-accumulation of these protein species could have contributed to synthesis and stability of PSII proteins [50,51] and, consequently favoring photoprotection and activity of PSII in absence or deficiency of thylakoidal APX.

The NADH dehydrogenase (NDH) complex (N. 26) amount was increased by drought effect in both genotypes but it was more notably increased in silenced plants. This protein displays a key role in cyclic electron flow (CEF) of PSI contributing for energy dissipation, ATP synthesis and photoprotection [52]. Thus, the response displayed by *apx8* plants could represent part of a compensatory mechanism for thylakoidal APX deficiency since this enzyme plays a crucial role in the water-water cycle (WWC), involved in energy dissipation and photoprotection [3]. An increased CEF activity could contribute to keep unchanged PSII quantum efficiency in drought-stressed silenced plants even in presence of significant impairment in CO_2 assimilation.

Similar to the noticed for CO_2 assimilation and photochemical activity measured *in vivo*, the trend of photorespiration estimated by gas exchange was not well correlated with abundance of some photorespiratory-protein species in drought-stressed plants. Indeed, silenced plants grown in well-watered conditions exhibited higher values of photorespiration rate than NT plants and this was positively associated with higher amounts of some important photorespiratory protein species: glycerate kinase (N. 13), glycine dehydrogenase – P protein (N. 15), glycolate oxidase (N. 16) and serine hydroxymethyltransferase (N. 17). Nevertheless, in drought condition, when the photorespiration rate intensely increased in *apx8* plants compared to NT plants, only glycine dehydrogenase was increased. Photorespiration is an

important process for dissipation of excess energy and ATP in C3 plants under drought stress [53] and, thus, this mechanism might have contributed for photoprotection in drought-stressed *apx8* plants.

The PCA results suggest that apparently silenced plants displayed restrict phenotypic plasticity to cope with mild drought and, as a consequence, they are less able to perform effective responses, compared to NT plants. An important example for explains this condition is the limitation in antioxidant and photosynthetic capacity exhibited by rice plants deficient in thylakoidal APX. The phenotypic plasticity of these plants is apparently not enough to perform an effective protection against adverse effects induced by a mild stress, as that performed by NT plants.

OsApx8 silencing decreased protein species involved in ASC-GSH cycle leading to metabolic rearrangement of different peroxidases for partial APX8 compensation

This proteomic analysis was performed to evaluate if silencing of *OsApx* is able to trigger a compensatory mechanism involving other antioxidant protein species, especially under drought conditions. In the well-watered conditions, the silenced plants increased the amount of the following protein species: peroxiredoxin 2C (N. 68), peroxiredoxin 2E II (N. 69), 2-cys Prx (N. 70), catalases A, B and C (N. 71, 72 and 73) and thioredoxin M5 (N. 79). Under drought conditions, the redox protein species displayed two trends: a group was increased in both genotypes but NT exhibited higher amount. In this group are cytosolic APX2 isoform (N. 63), glutathione reductase (N. 66), glutamate cysteine ligase (N. 67), peroxiredoxin 2E II (N. 69), 2-cys Prx (N. 70), Mn-SOD (N. 74), monodehydroascorbate reductase (N. 75), Prx Q (N. 76), thioredoxin F (N. 77) and Cu/Zn-SOD (N. 78). The other group includes protein species that were increased in NT but decreased in the silenced plants:

dehydroascorbate reductase (N. 65), CAT B (N. 72), CAT C (N. 73) and thioredoxin M5 (N. 79).

The literature has already proposed possible involvement of other peroxidases, such as glutathione peroxidase (GPX) and peroxiredoxins (Prx), involved in H_2O_2 homeostasis and signaling [13,39]. For instance, redundant pathways for ROS protection may exist in an attempt to compensate for the lack of important antioxidant enzymes, such as chloroplastic APXs. In fact, mechanisms such as ASC-GSH cycle have important roles in defending oxidative damage and balancing the redox status of ascorbate and glutathione under control and abiotic stresses [54]. It is well known that an efficient antioxidant system in chloroplasts and cytosol is vital for photosynthetic protection [10,55]. Thus, balancing of ROS production and detoxification in chloroplasts is crucial during stressful and non-stressful conditions. Here, NT plants showed much more increased protein species related to antioxidant response after drought stress (85.4%) than *apx8* after single silencing and drought stress (35% and 64%, respectively). This result demonstrates that reducing *OsApx8* expression triggered major changes in plant redox-related mechanisms in rice plants and this probably led to a more sensitive phenotype of *apx8* plants after mild drought stress.

Although rice silenced plants have increased the amount of some important peroxidases under well-watered conditions, this mechanism is apparently not enough for a full redox compensation to cope with a mild water deficit. Indeed, in this circumstance, H_2O_2 is accumulated and lipid peroxidation is increased in parallel to membrane damage in leaves of both genotypes but these changes are more intense in silenced plants. The proteomic responses indicate that apparently this changed redox homeostasis is not able to trigger signaling and antioxidant protection mechanisms as that triggered by non-transformed plants, reinforcing that thylakoidal APX isoform might be part of a signaling mechanism involved in several physiological processes. In this vein, it could participate of the regulation of H_2O_2

homeostasis mediating the abundance steady state of several protein species. Figure 8 presents a scheme highlighting the main differences in redox, photosynthesis and photorespiration related to changes in protein species abundance triggered by *apx8* and NT plants in response to a mild drought.

Conclusion

Rice thylakoidal APX displays a central role in the regulation of abundance steady state of several important protein species, especially in response to a mild water deficit. In this physiological circumstance, some crucial processes are adjusted in a minor scale in APX8 deficient plants, compared to non-transformed plants. This feature contributes to compromising the functioning of key metabolic pathways especially those involved with photosynthesis, photorespiration and redox protection. The obtained data reinforce that thylakoidal APX could display an overall signaling role in plant cells, besides its canonical function as a chloroplastic peroxidase. These roles displayed by thylakoidal APX are essential to plants coping with mild water deficit, a common challenge for plants in field conditions.

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References

[1] SHIGEOKA, T. ISHIKAWA, M. TAMOI, Y. MIYAGAWA, T. TAKEDA, Y. YABUTA, K. YOSHIMURA, Regulation and function of ascorbate peroxidase isoenzymes., **J. Exp. Bot.** 53. 1305–1319, 2002.

[2] TEIXEIRA, L. MENEZES-BENAVENTE, V.C. GALVÃO, R. MARGIS, M. MARGIS-PINHEIRO, Rice ascorbate peroxidase gene family encodes functionally diverse isoforms localized in different subcellular compartments, **Planta**. 224, 300–314, 2006.

[3] MARUTA, Y. SAWA, S. SHIGEOKA, T. ISHIKAWA, Diversity and evolution of ascorbate peroxidase functions in chloroplasts: More than just a classical antioxidant enzyme? **Plant Cell Physiol**. 57, 1377–1386, 2016.

[4] KITAJIMA, Hydrogen peroxide-mediated inactivation of two chloroplastic peroxidases, ascorbate peroxidase and 2-Cys peroxiredoxin, **Photochem. Photobiol**. 84, 1404–1409, 2008.

[5] CAI, Y. F.F., Q.-Y.Y. YANG, S.-F.F. LI, J.-H.H. WANG, W. HUANG, The water-water cycle is a major electron sink in Camellia species when CO_2 assimilation is restricted, **J. Photochem. Photobiol. B Biol.** 168, 59–66, 2017.

[6] ASADA, The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons, **Annu. Rev. Plant Physiol. Plant Mol. Biol.** 50, 601–639, 1999.

[7] MIYAKE, Alternative electron flows (water-water cycle and cyclic electron flow around PSI) in photosynthesis: Molecular mechanisms and physiological functions, **Plant Cell Physiol**. 51, 1951–1963, 2000.

[8] GIACOMELLI, A. MASI, D.R. RIPOLL, M.J. LEE, K.J. VAN WIJK, *Arabidopsis thaliana* deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low, **Plant Mol. Biol**. 65, 627–644, 2007.

[9] KANGASJÄRVI, A. LEPISTÖ, K. HÄNNIKÄINEN, M. PIIPPO, E.-M.M. LUOMALA, E.-M.M. ARO, E. RINTAMÄKI, Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses, **Biochem. J.** 412 275–85, 2008.

[10] MARUTA, A. TANOUCHI, M. TAMOI, Y. YABUTA, K. YOSHIMURA, T. ISHIKAWA, S. SHIGEOKA, Arabidopsis chloroplastic ascorbate peroxidase isoenzymes play a dual role in photoprotection and gene regulation under photooxidative stress, **Plant Cell Physiol**. 51, 190–200, 2010.

[11] CAVERZAN, A. BONIFACIO, F.E.L. CARVALHO, C.M.B. ANDRADE, G. PASSAIA, M. SCHÜNEMANN, F.D.S.F. DOS S. MARASCHIN, M.O. MARTINS, F.K. TEIXEIRA, R. RAUBER, R. MARGIS, J.A.G. SILVEIRA, M. MARGIS-PINHEIRO, The knockdown of chloroplastic ascorbate peroxidases reveals its regulatory role in the photosynthesis and protection under photo-oxidative stress in rice, **Plant Sci**. 214, 74–87, 2014.

[12] CARVALHO, F.E.L., C.W. RIBEIRO, M.O. MARTINS, A. BONIFACIO, C.C.

STAATS, C.M.B. ANDRADE, J.V. CERQUEIRA, M. MARGIS-PINHEIRO, J.A.G. SILVEIRA, Cytosolic APX knockdown rice plants sustain photosynthesis by regulation of protein expression related to photochemistry, Calvin cycle and photorespiration, **Physiol. Plant.** 150, 632–645, 2014.

[13] BONIFACIO, M.O. MARTINS, C.W. RIBEIRO, A. V. FONTENELE, F.E.L. CARVALHO, M. MARGIS-PINHEIRO, J.A.G. Silveira, Role of peroxidases in the compensation of cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress, **Plant, Cell Environ**. 34 (2011).

[14] SOUSA, F.E.L. CARVALHO, C.W. RIBEIRO, G. PASSAIA, J.R. CUNHA, Y. LIMA-MELO, M. MARGIS-PINHEIRO, J.A.G. SILVEIRA, Peroxisomal APX knockdown triggers antioxidant mechanisms favourable for coping with high photorespiratory H_2O_2 induced by CAT deficiency in rice, **Plant Cell Environ**. 38, 499–513, 2015.

[15] DANNA, C.G. BARTOLI, F. SACCO, L.R. INGALA, G.E. SANTA-MARÍA, J.J. GUIAMET, R.A. UGALDE, Thylakoid-bound ascorbate peroxidase mutant exhibits impaired electron transport and photosynthetic activity, **Plant Physiol**. 132, 2116–2125, 2003.

[16] D. TODAKA, K. SHINOZAKI, K. YAMAGUCHI-SHINOZAKI, Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants., **Front. Plant Sci.** 6, 84, 2015.

[17] PINHEIRO, M.M. CHAVES, Photosynthesis and drought: Can we make metabolic connections from available data?, **J. Exp. Bot.** 62, 869–882, 2011.

[18] JARDIM-MESSEDER, A. CAVERZAN, R. RAUBER, J.R. CUNHA, F.E.L. CARVALHO, M.L. GAETA, G.C. DA FONSECA, J.M. COSTA, M. FREI, J.A.G. SILVEIRA, R. MARGIS, N.J.M. SAIBO, M. MARGIS-PINHEIRO, Thylakoidal APX modulates hydrogen peroxide content and stomatal closure in rice (*Oryza sativa* L.), **Environ. Exp. Bot.** 150, 46–56, 2018.

[19] MIKI, K. SHIMAMOTO, Simple RNAi vectors for stable and transient suppression of gene function in rice, **Plant Cell Physiol**. 45, 490–495, 2004.

[20] UPADHYAYA, B. SURIN, K. RAMM, J. GAUDRON, P.H.D. SCHÜNMANN, W. TAYLOR, P.M. WATERHOUSE, M.-B. WANG, Agrobacterium-mediated transformation of Australian rice cultivars Jarrah and Amaroo using modified promoters and selectable markers, **Funct. Plant Biol**. 27, 201, 2000.

[21] HOAGLAND, D.I. ARNON, The water-culture method for growing plants without soil, **Calif. Agric. Exp. Stn. Circ.** 347, 1–32, 1950.

[22] SILVEIRA, S.A.M. ARAÚJO, J.P.M.S. LIMA, R.A. VIÉGAS, Roots and leaves display contrasting osmotic adjustment mechanisms in response to NaCl-salinity in *Atriplex nummularia*, **Environ. Exp. Bot**. 66, 1–8, 2009.

[23] BLUM, A. EBERCON, Cell membrane stability as a measure of drought and heat tolerance in wheat., **Crop Sci.** 21, 43–47, 1981.

[24] K.J. LIVAK, T.D. SCHMITTGEN, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods. 25, 402–408, 2001.

[25] SCHMITTGEN, K.J. LIVAK, Analyzing real-time PCR data by the comparative CT method, **Nat. Protoc**. 3, 1101–1108, 2008.

[26] AMAKO, G.-X. CHEN, K. ASADA, Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants, **Plant Cell Physiol**. 35, 497–504, 1994.

[27] MIYAKE, K. ASADA, Inactivation mechanism of ascorbate peroxidase at low concentrations of ascorbate; hydrogen peroxide decomposes compound I of ascorbate peroxidase, **Plant Cell Physiol**. 37, 423–430, 1996.

[28] ZHOU, Z. DIWU, N. PANCHUK-VOLOSHINA, R.P. HAUGLAND, A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases., **Anal. Biochem.** 253, 162–168, 1997.

[29] CAKMAK, W.J. HORST, Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*), **Physiol. Plant**. 83, 463–468, 1991.

[30] FLEXAS, M.F. ORTUÑO, M. RIBAS-CARBO, A. DIAZ-ESPEJO, I.D. FLÓREZ-SARASA, H. MEDRANO, Mesophyll conductance to CO_2 in *Arabidopsis thaliana*, **New Phytol.** 175, 501–511, 2007.

[31] SHARKEY, C.J. BERNACCHI, G.D. FARQUHAR, E.L. SINGSAAS, Fitting photosynthetic carbon dioxide response curves for C3 leaves, **Plant, Cell Environ**. 30, 1035–1040, 2007.

[32] BAGARD, D. LE THIEC, E. DELACOTE, M.-P.P. HASENFRATZ-SAUDER, J. BANVOY, J. GÉRARD, P. DIZENGREMEL, Y. JOLIVET, Ozone-induced changes in photosynthesis and photorespiration of hybrid poplar in relation to the developmental stage of the leaves., **Physiol. Plant**. 134, 559–574, 2008.

[33] BAKER, Chlorophyll fluorescence: A probe of photosynthesis *in vivo*, **Annu. Rev. Plant Biol.** 59, 89–113, 2008.

[34] BRADFORD, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, **Anal. Biochem**. 72 , 248–254, 1976.

[35] KOMATSU, Y. NANJOA, M. NISHIMURAB, Proteomic analysis of the flooding tolerance mechanism in mutant soybean, **J. Proteomics**. 79, 231–250, 2013.

[36] ZHANG, Z. WEN, M.P. WASHBURN, L. FLORENS, Effect of dynamic exclusion duration on spectral count based quantitative proteomics, **Anal. Chem**. 81, 6317–6326, 2009.

[37] USADEL, A. NAGEL, O. THIMM, H. REDESTIG, O.E. BLAESING, N. PALACIOS-ROJAS, J. SELBIG, J. HANNEMANN, M.C. PIQUES, D. STEINHAUSER, W.-R. SCHEIBLE, Y. GIBON, R. MORCUENDE, D. WEICHT, S. MEYER, M. STITT, Extension of the visualization tool MapMan to allow statistical analysis of arrays, display of coresponding genes, and comparison with known responses, **Plant Physiol**. 138, 1195–1204, 2005.

[38] MURGIA, D. TARANTINO, C. VANNINI, M. BRACALE, S. CARRAVIERI, C. SOAVE, *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death, **Plant J**. 38, 940–953, 2004.

[39] MILLER, N. SUZUKI, L. RIZHSKY, A. HEGIE, S. KOUSSEVITZKY, R. MITTLER, Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses., **Plant Physiol**. 144, 1777–1785, 2007.

[40] D'AUTRÉAUX, M.B. TOLEDANO. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis, **Nat. Rev. Mol. Cell Biol.** 8, 813–824, 2007.

[41] WU, M. MIRZAEI, D. PASCOVICI, J.M. CHICK, B.J. ATWELL, P.A. HAYNES, Quantitative proteomic analysis of two different rice varieties reveals that drought tolerance is correlated with reduced abundance of photosynthetic machinery and increased abundance of ClpD1 protease, **J. Proteomics**. 143, 73–82, 2016.

[42] MULLINEAUX, Spatial dependence for hydrogen peroxide-directed signaling in light-stressed plants, **Plant Physiol**. 141, 346–350, 2006.

[43] FLEXAS, J. BOTA, F. LORETO, G. CORNIC, T.D. SHARKEY, Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants, **Plant Biol.** 6, 269–279, 2004.

[44] LIMA NETO, M.C., A.K.M. LOBO, M.O. MARTINS, A.V. FONTENELE, J.A.G. SILVEIRA, Dissipation of excess photosynthetic energy contributes to salinity tolerance: A comparative study of salt-tolerant *Ricinus communis* and salt-sensitive *Jatropha curcas*, **J. Plant Physiol.** 171, 23–30, 2014.

[45] HEBER, R. BLIGNY, P. STREB, R. DOUCE, Photorespiration is essential for the protection of the photosynthetic apparatus of C3 plants against photoinactivation under sunlight, **Bot. Acta**. 109, 307–315, 1996.

[46] LÓPEZ-CALCAGNO, T.P. HOWARD, C.A. RAINES, The CP12 protein family: a thioredoxin-mediated metabolic switch? **Front. Plant Sci.** 5, 9, 2014.

[47] M.J. GARCÍA-MURRIA, H.P.K. SUDHANI, J. MARÍN-NAVARRO, M.M. SÁNCHEZ DEL PINO, J. MORENO, Dissecting the individual contribution of conserved cysteines to the redox regulation of RubisCO, **Photosynth. Res.** 137, 251–262, 2018.

[48] SILVEIRA, J.A.G., F.E.L. CARVALHO, Proteomics, photosynthesis and salt resistance in crops: An integrative view, **J. Proteomics**. 143, 24–35, 2016.

[49] FENG, Y. HAN, G. LIU, B. AN, J. YANG, G. YANG, Y. LI, Y. ZHU, Overexpression of sedoheptulose-1,7-bisphosphatase enhances photosynthesis and growth under salt stress in transgenic rice plants, **Funct. Plant Biol.** 34, 822–834, 2007.

[50] JIMBO, R. YUTTHANASIRIKUL, T. NAGANO, T. HISABORI, Y. HIHARA, Y. NISHIYAMA, Oxidation of translation factor EF-Tu inhibits the repair of photosystem II, Plant Physiol. 176, 2018.

[51] PULIDO, E. LLAMAS, M. RODRIGUEZ-CONCEPCION, Both Hsp70 chaperone

and Clp protease plastidial systems are required for protection against oxidative stress, Plant Signal. Behav. 12, 1–4, 2017.

[52] FINAZZI, G.N. JOHNSON, Cyclic electron flow: facts and hypotheses, **Photosynth. Res.** 129, 227–230, 2016.

[53] VOSS, B. SUNIL, R. SCHEIBE, A.S. RAGHAVENDRA, Emerging concept for the role of photorespiration as an important part of abiotic stress response, **Plant Biol**. 15, 713–722, 2013.

[54] SHAN, S. ZHANG, X. OU, The roles of H_2S and H_2O_2 in regulating AsA-GSH cycle in the leaves of wheat seedlings under drought stress, **Protoplasma**, 2018.

[55] NARANJO, A. DIAZ-ESPEJO, M. LINDAHL, F.J. CEJUDO, Type-f thioredoxins have a role in the short-term activation of carbon metabolism and their loss affects growth under short-day conditions in *Arabidopsis thaliana*, **J. Exp. Bot**. 67, 1951–1964, 2016.

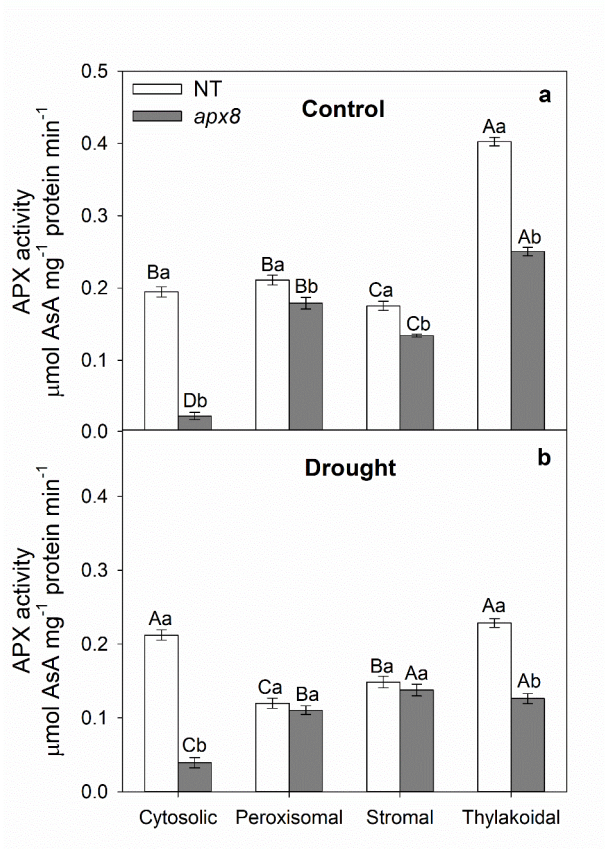


Figure 1. Ascorbate peroxidase activity in cytosolic, stromal, thylakoidal and peroxisomal fractions measured in leaves of rice plants exposed to control (a) or 4 days of drought stress (b). Different capital letters represent significant differences between the treatments within genotypes (NT and *apx8*), and different lowercase letters represent significant differences between the genotypes within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test, ($p \leq 0.05$).

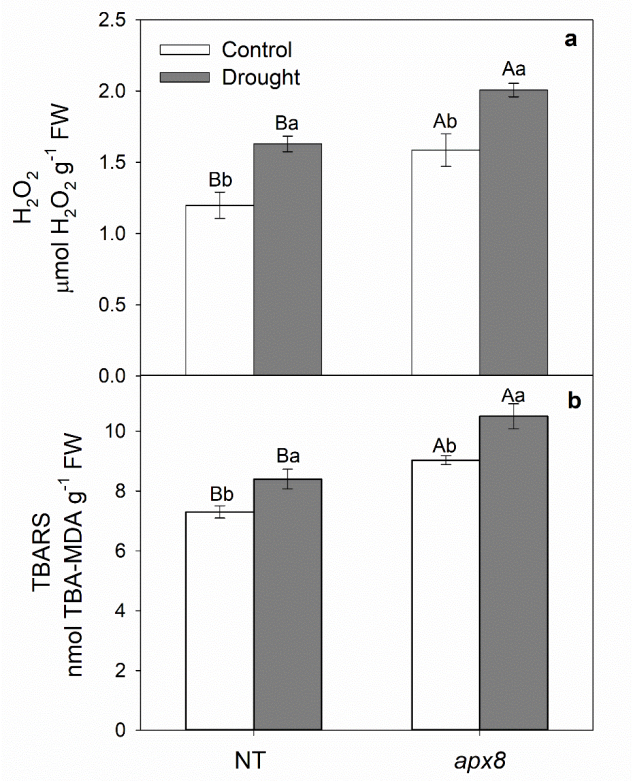


Figure 2. H_2O_2 (a) and thiobarbituric acid-reactive-substances, TBARS (b) contents of NT and *apx8* rice plants exposed to control or drought stress for 4 days. Different capital letters represent significant differences between the treatments within genotypes (NT and *apx8*), and different lowercase letters represent significant differences between the genotypes within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test, ($p \leq 0.05$).

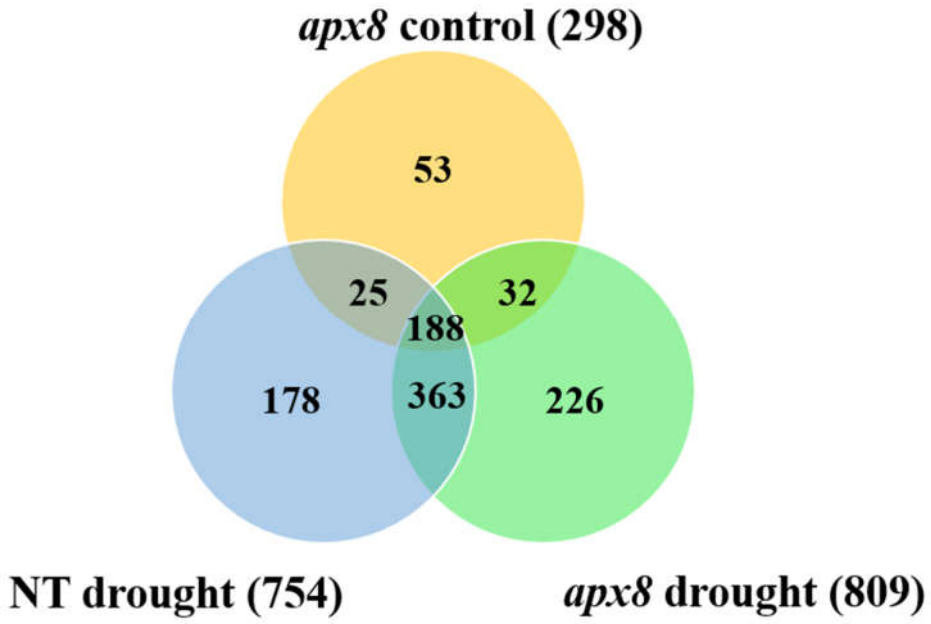


Figure 3. Venn diagram depicting protein species of NT and *apx8* rice leaves after control or 4 days of drought stress. The Venn diagram was constructed in the Pangloss Venn diagram generator (http://www.pangloss.com/seidel/Protocols/venn.cgi).

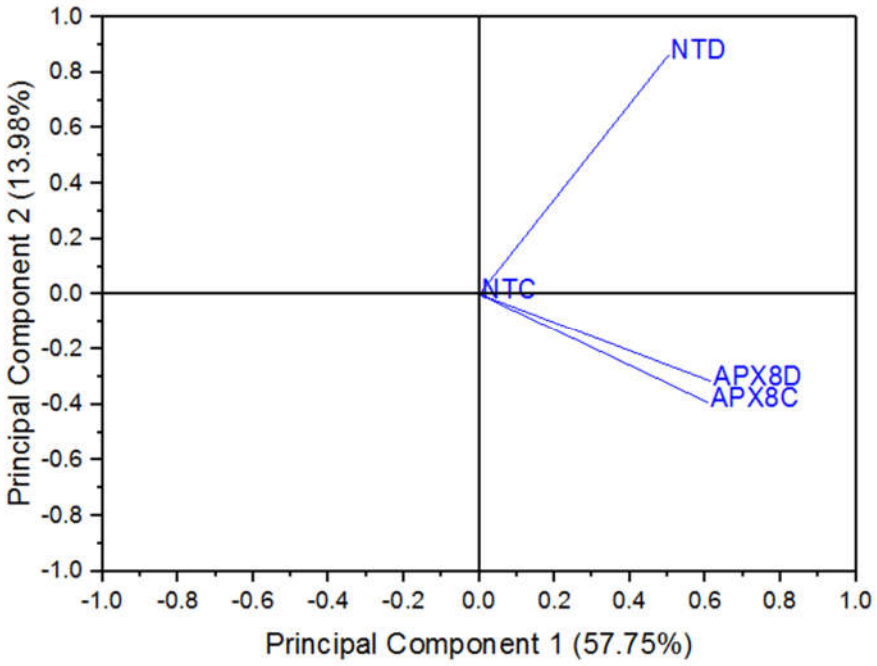


Figure 4. Loading plot based on the principal component analysis (PCA) results from proteomic changes of data of rice NT and *apx8* leaves subjected to control (NTC and APX8C, respectively) and 4 days of drought stress (NTD and APX8D, respectively). The PCA was performed employing normalized protein species ratios, based on NTC abundance levels as reference. The principal components (PC) 1 and 2 explain 71.73% of the variance.

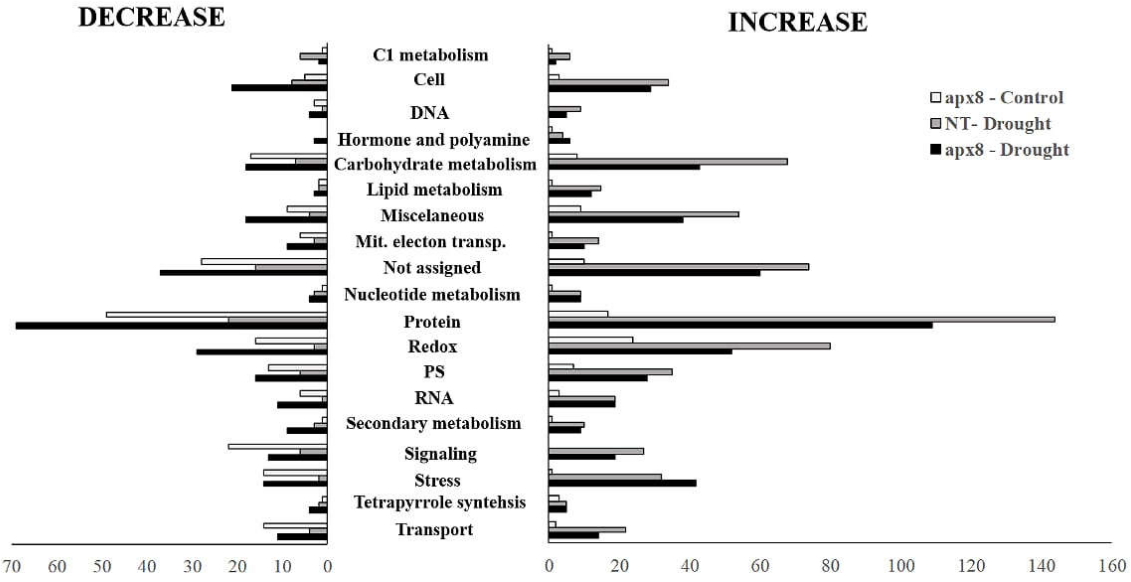


Figure 5. Proteome changes of rice NT and *apx8* leaves after control or 4 days of drought stress. Proteins were extracted from rice leaves using the TCA method. After digestion with trypsin and lysyl endopeptidase, peptides were compared using LC−MS/MS-based proteomic analysis. Protein species abundance ratios were estimated using SIEVE software. One-way ANOVA, followed by Student's t-test, were performed with GraphPad software ($p \leq 0.05$). Significantly changed protein species ($p \leq 0.05$) were analyzed by ratio of protein levels > 1.0 (increased) or ≤ 1.0 (decreased). Functional categories of significantly changed proteins were performed using MapMan ontology.

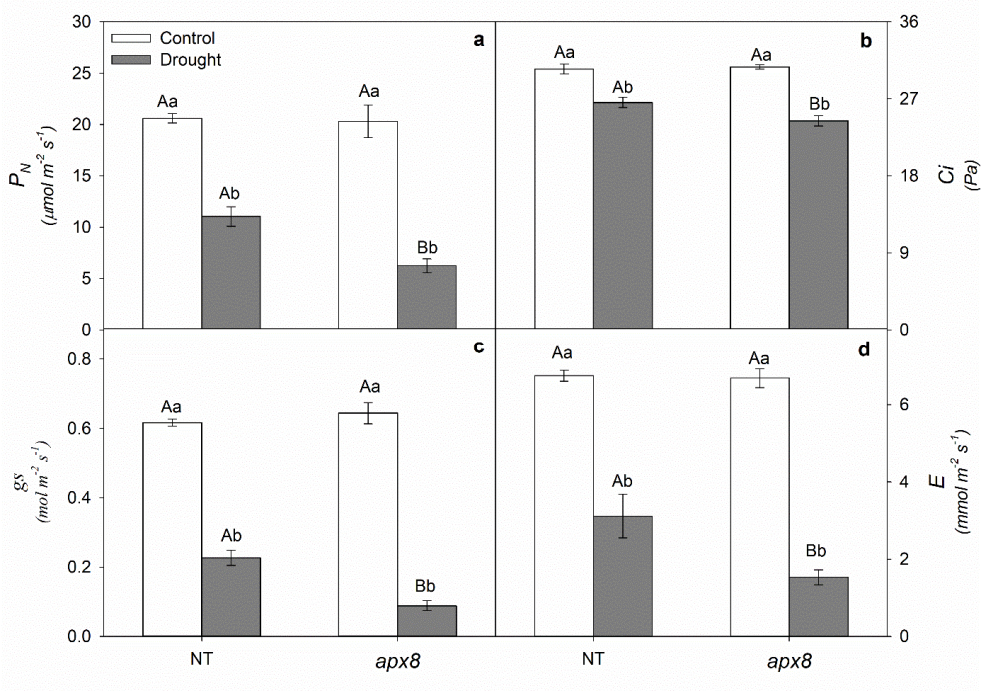


Figure 6. Photosynthesis, P_N (a), intercellular CO_2 partial pressure, Ci (b), stomatal conductance, g_S (c) and transpiration, E (d) of NT and apx8 rice plants exposed to control or drought stress for 4 days. Different capital letters represent significant differences between the treatments within genotypes (NT and apx8), and different lowercase letters represent significant differences between the genotypes within treatments (control and drought). Data are means of four replicates and the averages were compared using one-way ANOVA, followed by Tukey's test ($p \leq 0.05$).

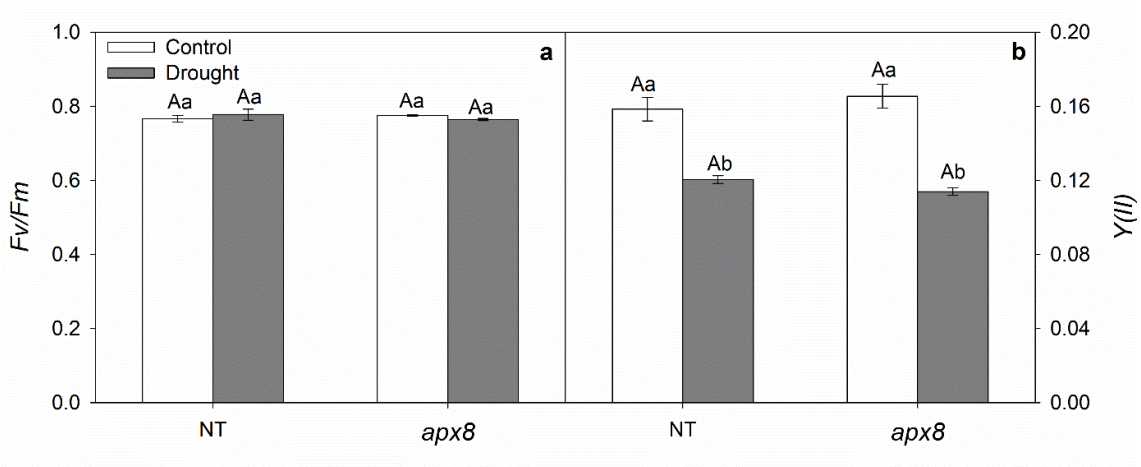


Figure 7. Potential quantum yield (Fv/Fm) (a) and effective quantum yield of PSII [Y(II)] (b) of NT and apx8 rice plants exposed to control or drought stress for 4 days. Different capital letters represent significant differences between the treatments within genotypes (NT and apx8), and different lowercase letters represent significant differences between the genotypes within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test, ($p \leq 0.05$).

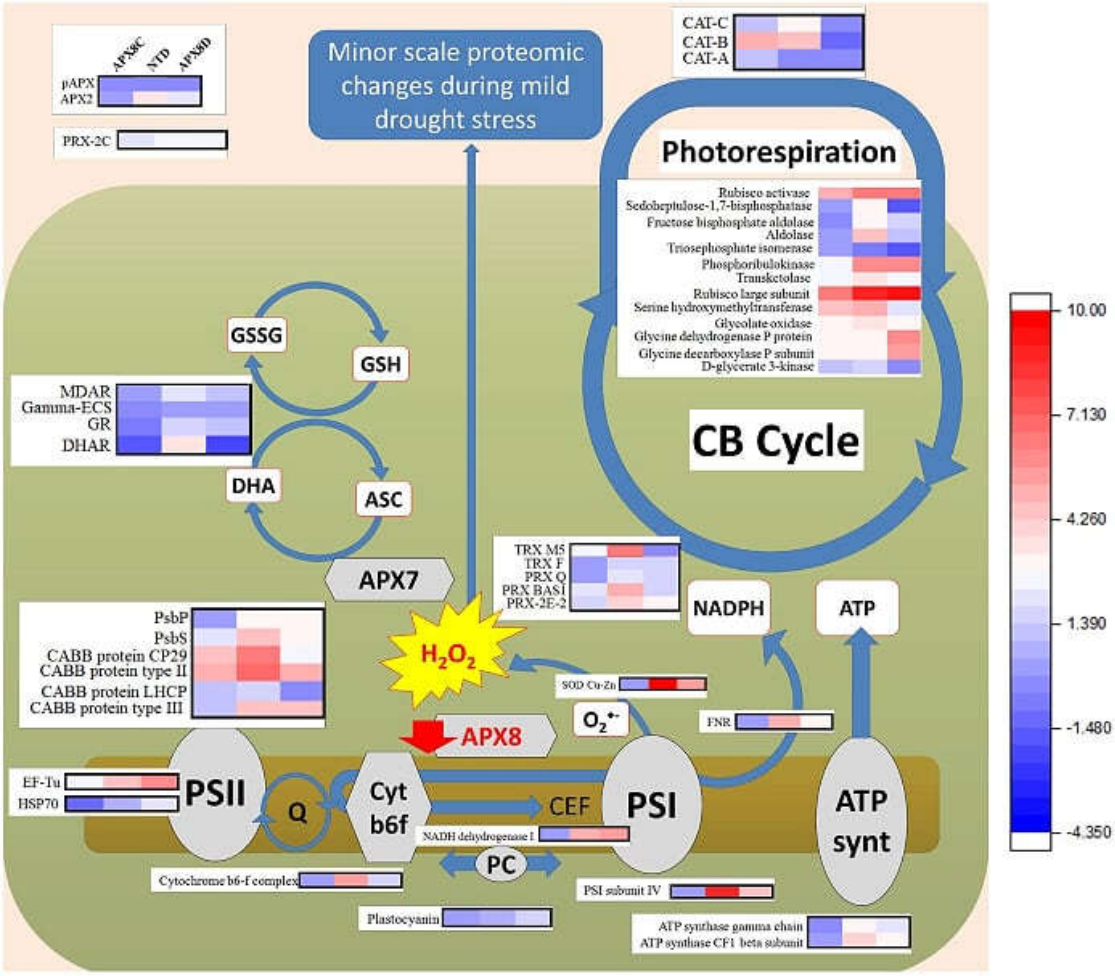


Figure 8. Scheme summarizing some of the main proteomic contrasts observed between *apx8* and NT plants under mild drought stress. The H_2O_2 content is increased in *apx8* plants, which probably have contributed for intense changes in the proteomic profile before water deficit. After mild drought stress, the level of this ROS is increased in both plant genotypes, but these plants responded differentially in terms of proteomics and physiological alterations. Despite both NT and silenced plants have exhibited great changes in their proteomic profile, these changes reached a minor intensity in *apx8*. Probably, as a consequence, *apx8* displayed greater decreases in photosynthetic capacity and exhibited physiological signs related to higher drought sensitivity. In the scheme, heat maps representing the changes in the abundance of important protein species involved in photosynthesis, photorespiration and redox metabolism are highlighted. Protein species abundance ratios were expressed as log2 basis and heatmaps were plotted using the OriginPro 2017 software (Origin Lab Corporation, Northampton, USA). Abbreviations: ASC – ascorbate, CABB – chlorophyll *a*/*b* binding, CB cycle – Calvin-Benson cycle, CAT – catalase, DHAR – dehydroascorbate reductase, MDHAR – monodehydroascorbate reductase, GSH – glutathione, PRX – peroxiredoxin, TRX – thioredoxin, pAPX – peroxisomal APX, CEF – cyclic electron flow and HSP – heat shock protein.

Table 1. Summary of differentially accumulated proteins of NT and *apx8* rice plants exposed to control or drought stress for 4 days. The relative abundance of each protein was expressed as ratio in relation to control NT plants and it was considered statically significant when $p \leq 0.05$. *ND: not detected.*

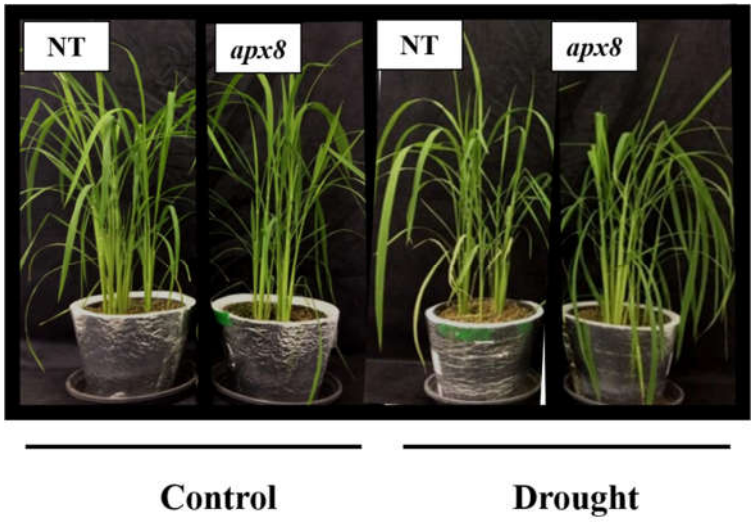
Function	Acession*	Nº	Protein name	*p-value*	NT-D x NT-C	*p-value*	*apx8*-D x NT-C	*p-value*	*apx8*-C x NT-C
PS	Os02t0197600	1	Chlorophyll a/b binding protein type III	0.01	26.40	0.00	20.63	0.04	2.40
	Os01t0600900	2	Chlorophyll a/b binding protein LHCP	0.00	2.90	0.00	0.89	0.01	2.43
	Os03t0592500	3	Chlorophyll a/b binding protein type II	0.00	114.13	0.00	35.35	0.00	27.87
	Os07t0558400	4	Chlorophyll a/b binding protein CP29	0.00	97.42	0.00	7.07	0.00	25.31
	Os01t0869800	5	PsbS	0.00	25.15	0.00	7.41	0.01	3.72
	Os07t0141400	6	PsbP	0.00	8.31	0.00	7.49	0.00	ND
	Os07t0435300	7	Photosystem I reaction center subunit IV	0.00	505.73	0.00	20.66	0.00	ND
	Os07t0556200	8	Cytochrome b6-f complex	0.01	49.00	0.00	3.54	0.00	ND
	Os02t0103800	9	Ferredoxin NADP reductase (FNR)	0.00	32.53	0.00	7.80	0.00	ND
	Os06t0101600	10	Plastocyanin	0.00	1.64	0.00	2.74	0.00	ND
	Os10t0355800	11	ATP synthase CF1 beta subunit.	0.00	16.35	0.00	7.43	0.00	ND
	Os07t0513000	12	ATP synthase gamma chain	0.00	9.48	0.00	3.96	0.00	0.91
	Os01t0682500	13	D-glycerate 3-kinase	0.00	3.53	0.00	0.81	0.01	2.26
	Os06t0611900	14	Glycine decarboxylase P subunit	0.00	7.34	0.00	45.97	0.00	7.99
	Os01t0711400	15	Glycine dehydrogenase P protein	0.00	8.08	0.00	58.27	0.00	8.29
	Os07t0152900	16	Glycolate oxidase	0.00	9.97	0.00	7.82	0.01	8.30
	Os03t0738400	17	Serine hydroxymethyltransferase	0.00	33.22	0.00	4.05	0.00	20.10
	Os12t0207600	18	Rubisco large subunit	0.00	382.39	0.00	707.89	0.00	92.69
	Os06t0133800	19	Transketolase	0.00	11.32	0.00	5.59	0.00	6.40
	Os02t0698000	20	Phosphoribulokinase	0.00	54.03	0.00	60.22	0.00	5.56
	Os09t0535000	21	Triosephosphate isomerase	0.00	0.50	0.01	0.30	0.00	ND
	Os01t0905800	22	Aldolase	0.00	25.66	0.00	2.40	0.00	ND
	Os11t0171300	23	Fructose bisphosphate aldolase	0.00	9.78	0.00	2.70	0.00	0.84
	Os04t0234600	24	Sedoheptulose-1,7-bisphosphatase	0.00	8.53	0.00	0.30	0.00	ND
	Os11t0707000	25	Ribulose bisphosphate carboxylase activase	0.00	87.87	0.00	89.50	0.00	27.01
	Os01t0882500	26	NADH dehydrogenase I	0.03	29.52	0.00	46.00	0.00	ND
Stress	Os06t0197550	27	Heat shock protein 10	0.00	5.76	0.01	6.84	0.00	ND
	Os01t0840100	28	Heat shock protein 70	0.00	3.47	0.00	5.00	0.03	0.00
	Os12t0244100	29	Heat shock protein 70	0.00	1.62	0.00	3.69	0.01	0.43
	Os06t0716700	30	Heat shock protein 90	0.03	0.52	0.00	2.68	0.02	0.46
	Os09t0474300	31	Heat shock protein 90	0.00	1.56	0.00	2.10	0.03	0.05
Redox	Os11t0199200	32	Protein disulfide isomerase	0.01	12.65	0.00	0.97	0.01	0.54
Signaling	Os04t0402100	33	Calnexin	0.01	10.08	0.01	2.36	0.00	ND
DNA	Os06t0701100	34	Eukaryotic initiation factor 4A	0.00	6.48	0.00	2.70	0.03	0.60
Protein	Os12t0507200	35	Eukaryotic translation initiation factor 5A	0.00	1.76	0.00	2.67	0.00	ND
	Os03t0577000	36	Ribosomal protein S3	0.00	2.56	0.00	0.09	0.01	0.79
	Os11t0482000	37	40S ribosomal protein S5.	0.00	11.43	0.00	7.74	0.00	ND
	Os05t0388500	38	50S ribosomal protein L1.	0.00	11.60	0.05	0.73	0.00	ND
	Os05t0568300	39	50S ribosomal protein L12	0.00	17.94	0.00	0.88	0.00	ND

	Os04t0538100	40	Elongation factor G	0.00	3.44	0.00	2.37	0.04	0.05
	Os02t0519900	41	Elongation factor EF-2	0.00	4.15	0.00	0.90	0.04	2.40
	Os04t0118400	42	Elongation factor EF-2	0.00	4.18	0.00	0.90	0.04	2.40
	Os07t0662500	43	Elongation factor 1-beta	0.00	5.59	0.00	4.54	0.00	ND
	Os02t0595700	44	Chloroplast translational elongation factor Tu	0.00	26.33	0.00	57.42	0.01	6.23
	Os05t0373700	45	Nascent polypeptide associated complex	0.00	26.54	0.00	2.86	0.00	ND
	Os12t0624000	46	Methionine synthase protein	0.00	3.34	0.00	5.27	0.02	0.05
	Os01t0323600	47	S-adenosylmethionine synthase 2	0.00	1.90	0.00	3.09	0.00	ND
	Os05t0135700	48	S-adenosylmethionine synthetase 1	0.00	1.89	0.00	3.07	0.00	ND
Hormone and polyamine synthesis	Os07t0408700	49	Spermidine synthase 2	0.00	ND	0.03	9.47	0.00	ND
Carbohydrate metabolism	Os06t0136600	50	Enolase 1	0.00	8.46	0.00	6.48	0.03	3.62
	Os10t0167300	51	Enolase 2	0.00	14.94	0.00	6.61	0.00	ND
	Os04t0677500	52	Pyruvate kinase	0.01	5.20	0.00	2.58	0.01	0.58
	Os05t0522500	53	Hexokinase 1	0.03	2.13	0.00	ND	0.00	ND
	Os01t0742500	54	Hexokinase	0.00	ND	0.01	0.30	0.01	0.61
	Os06t0256500	55	Glucose-6-phosphate isomerase	0.00	1.85	0.00	2.28	0.00	ND
	Os04t0486600	56	Glyceraldehyde-3-phosphate dehydrogenase	0.00	20.75	0.00	30.61	0.00	ND
	Os03t0712700	57	Phosphoglucomutase	0.00	1.63	0.00	2.35	0.02	0.98
	Os01t0639900	58	Carbonic anhydrase	0.00	18.46	0.00	4.34	0.01	2.04
	Os02t0739600	59	Pyruvate dehydrogenase E1	0.00	16.60	0.00	3.30	0.00	ND
	Os09t0509200	60	Pyruvate dehydrogenase E1 beta	0.00	3.85	0.01	0.30	0.00	ND
	Os07t0410100	61	Dihydrolipoamide S-acetyltransferase	0.02	3.11	0.00	ND	0.00	ND
Redox	Os02t0553200	62	Thylakoid bound ascorbate peroxidase	0.00	4.96	0.00	0.60	0.00	0.06
	Os07t0694700	63	Ascorbate peroxidase 2	0.00	10.92	0.00	4.05	0.00	ND
	Os08t0549100	64	Peroxisome type ascorbate peroxidase	0.00	0.84	0.00	0.80	0.00	0.70
	Os06t0232600	65	Dehydroascorbate reductase.	0.00	12.70	0.03	0.20	0.02	0.26
	Os02t0813500	66	Glutathione reductase	0.00	3.08	0.00	2.26	0.01	0.50
	Os07t0462000	67	Glutamate cysteine ligase	0.00	1.08	0.00	ND	0.01	0.81
	Os01t0675100	68	Peroxiredoxin-2C	0.00	6.03	0.00	6.81	0.04	4.73
	Os02t0192700	69	Peroxiredoxin-2E-2	0.00	14.95	0.00	7.37	0.00	2.98
	Os02t0537700	70	2-cys peroxiredoxin BAS1	0.00	28.63	0.00	2.75	0.05	3.96
	Os02t0115700	71	Catalase isozyme A	0.00	0.75	0.00	0.90	0.04	2.21
	Os06t0727200	72	Catalase isozyme B	0.00	20.09	0.00	0.45	0.03	28.00
	Os03t0131200	73	Catalase isoenzyme C	0.00	9.62	0.00	0.71	0.01	2.43
	Os05t0323900	74	Superoxide dismutase Mn	0.00	9.92	0.00	3.64	0.01	0.83
	Os09t0567300	75	Monodehydroascorbate reductase	0.00	5.01	0.00	2.55	0.00	ND
	Os06t0196300	76	Peroxiredoxin Q	0.02	3.91	0.00	2.89	0.00	ND
	Os01t0913000	77	Thioredoxin F	0.01	3.65	0.01	3.61	0.00	ND
	Os08t0561700	78	Superoxide dismutase Cu-Zn	0.00	1001.10	0.00	40.69	0.00	ND
	Os12t0188700	79	Thioredoxin M5	0.00	78.74	0.01	0.78	0.02	5.23

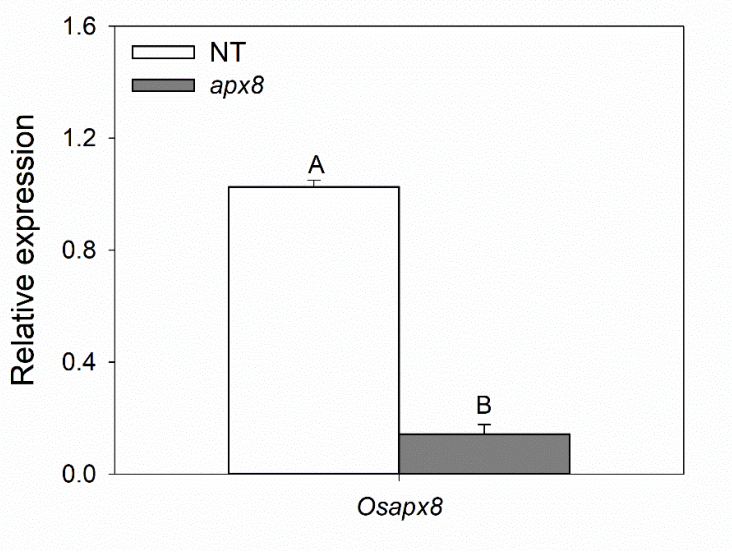
*Acession: protein identification according to the The Rice Annotation Project Database (RAP-DB).

Table 2. V_{cmax} (in vivo Rubisco carboxylation rate), J_{max} (maximum electron transport rate), P_N (the maximum CO_2 assimilation rate), P_R (photorespiration) and gm (mesophyll conductance) of NT and *apx8* rice plants exposed to control or drought stress for 4 days. Different capital letters represent significant differences between the treatments within lines (NT and *apx8*), and different lowercase letters represent significant differences between the lines within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test ($P<0.05$).

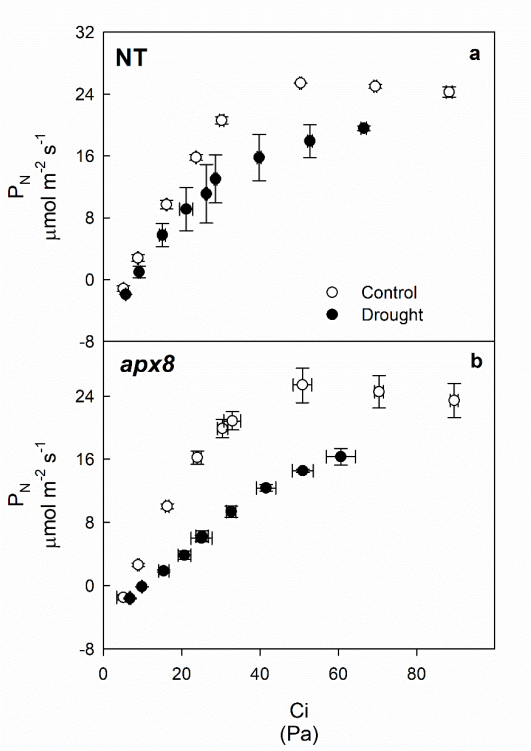
		V_{cmax}		J_{max}		P_{Nmax}		P_R		gm	
NT	**Control**	156.1	Aa	111.8	Aa	24.9	Aa	20.2	Bb	10.2	Aa
	Drought	122.3	Bb	90.1	Bb	17.7	Bb	26.9	Ba	7.5	Ab
apx8	**Control**	144.8	Aa	109.5	Aa	24.4	Aa	27.6	Ab	11.3	Aa
	Drought	70.3	Cb	80.4	Cb	12.0	Cb	50.9	Aa	4.4	Bb



Supplementary Figure 1. Aspects of shoots from NT and *apx8* rice plants exposed to control or drought stress for 4 days. Plants are the most representative from four biological replicates.



Supplementary Figure 2. Transcript levels of *OsApx8* in plants grown in control conditions. Different capital letters represent significant differences among the NT and *apx8* plants. The relative expression of APX locus was normalized by the average value obtained from the NT and *apx8* plants. The relative expression of APX locus was normalized by the average value obtained from the NT control plants.



Supplementary Figure 3. Photosynthesis response to increasing internal CO_2 partial pressure (P_N/Ci curve) in NT and *apx8* rice plants exposed to control or drought stress for 4 days. Data are the means of four replications ± SD.

Supplementary Table 1. Minimum and maximum changes in air temperature, humidity, vapor pressure deficit (VPD) and light intensity of greenhouse conditions measured in a typical day. Data are means of four days.

	Air temperature (ºC)	**Humidity (%)**	**VPD (kPa)**	**Light intensity (μmol m^{-2} s^{-1})**
Minimum	24.3 ± 0.5	42.7 ± 3.2	0.7 ± 0.1	0
Maximum	33.7 ± 0.6	80.2 ± 5.2	1.5 ± 0.2	500.3 ± 5.8

Supplementary Table 2. Number of leaves, tillers, shoot length, shoot biomass and membrane damage of NT and *apx8* rice plants exposed to control or drought stress for 4 days. Different capital letters represent significant differences between the treatments within lines (NT and *apx8*), and different lowercase letters represent significant differences between the lines within treatments (control and drought). Data are means of four replicates and the averages were compared using the Tukey's test (P<0.05).

		Number of leaves[1]		**Number of tillers[1]**		**Shoot lenght[2]**		**Shoot biomass[3]**		**Membrane damage[4]**	
NT	**Control**	14.8	Aa	3.5	Aa	86.8	Aa	30.2	Aa	26.5	Bb
	Drought	14.0	Aa	3.0	Aa	85.8	Aa	24.3	Ab	36.5	Ba
apx8	**Control**	13.6	Aa	2.8	Ba	83.3	Ba	26.7	Ba	34.4	Ab
	Drought	13.4	Aa	2.6	Ba	82.3	Ba	21.2	Bb	44.6	Aa

[1] plant^{-1}; [2] cm; [3] g plant^{-1}; [4] %

Supplementary Table 3. Differentially accumulated proteins in leaves of NT and *apx8* rice plants exposed to control or drought stress for 4 days. Proteins were identified by label-free proteomics and compared to NT control treatment. (Excel file)

(Unpublished manuscript)

7 CYCLIC ELECTRON FLOW IS TRIGGERED TO COMPENSATE THE EFFECTS OF THE DISRUPTED WATER-WATER CYCLE ON THYLAKOIDAL APX KNOCKDOWN RICE PLANTS

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Abstract

In higher plants, photoprotector mechanisms are important to avoid reactive oxygen species over-accumulation that can result on photoinhibition of both PSII and PSI. PSII is more sensible to photoinhibition than PSI, however the recovery of photoinhibited PSI is much slower compared with PSII. In fact, the specific role of some of these photoprotector mechanisms such as the water-water cycle and the cyclic electron flow is not well known. Addressing these questions, non-transformed plants (NT) and thylakoid ascorbate peroxidase silenced rice plants (*apx8*), with reduced water-water cycle, were treated in the presence or absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). DCMU blocks the electron transport from PSII to PSI, and it was used as one of the tools in this research to estimate the cyclic electron flow (CEF). In addition, two different light regimes (400 or 1000 μmol m^{-2} s^{-1}) were applied in both genotypes. *apx8* plants showed different regulation of the photosynthetic electron transport rate in moderate light, as lower Y(II), ETR(II) and higher NPQ compared with NT plants. Under high light in presence of DCMU, *apx8* plants showed higher NPQ

compared with NT plants, probably by the higher efficiency of CEF producing ΔpH under this condition. $P700^+$ was increased in *apx8* plants under moderate light in absence of DCMU. In contrast, *apx8* plants showed higher $P700^+$ compared with NT in presence of DCMU and high light. The regulation of Y(NA) and Y(ND) between the genotypes, light regimes and DCMU suggests that *apx8* plants triggered higher CEF under both light regimes. These findings were corroborated by estimating CEF by different approaches. These results suggest that *apx8* plants triggers the cyclic electron flow under moderate or high light as a compensatory photoprotector mechanisms as these plants are not able to perform the water-water cycle efficiently due to the reduced activity of thylakoid APX.

Keywords: hydrogen peroxide; *Oryza sativa*; photoprotection; thylakoidal ascorbate peroxidase; chloroplast

Introduction

Light drives photosynthesis. Nevertheless, excess light causes an over-reduction of the electrons transporters, leading to the overproduction of reactive oxygen species (ROS) that can cause photoinhibition in both photosystems (Takagi et al., 2016). Photosystem II (PSII) is very sensitive to environmental stresses and its photoinhibition has been shown to be caused by singlet oxygen, which is produced through charge recombination of triplet state of P680. Singlet oxygen degrades D1 protein into the core of PSII through the oxidative inactivation of the thioredoxin-regulated elongation factor G. In addition, ROS suppress the *de novo* synthesis of D1 protein (Nishiyama et al., 2011). Recent studies revealed that superoxide radical is also produced by PSII, which can cause photoinhibition (Takagi et al., 2016). PSI can also be photoinhibited by ROS, primarily by superoxide and singlet oxygen (Sejima et al., 2014; Takagi et al., 2016). In fact, PSI photoinhibition occurs when the PSI electron carriers become reduced, impairing net photosynthesis and plant growth (Kono and Terashima, 2014). However, the recovery of PSI photoinhibition is very slow compared with the recovery of PSII (Kono and Terashima, 2016). Therefore, PSI photoinhibition could have more severe consequences then PSII photoinhibition to plant growth and productivity.

Plants have prompt responsive mechanisms, within seconds and minutes, to photon excess to avoid photoinhibition, that can safely dissipate energy excess electrons generated in thylakoids to suppress the production of ROS (Asada, 2000). The non-photochemical quenching, associated with the de-epoxidation of violaxanthin (Foyer et al., 2017); photorespiration, dissipating excess photons and electrons by consumption of NADPH and ATP (Voss et al., 2013); plastid terminal oxidase (Johnson and Stepien, 2016), the water-water cycle (Asada, 1999) and the cyclic electron flow (Joliot and Joliot, 2005) are some of these mechanisms commonly triggered by plants to avoid photoinhibition (Goh et al., 2011). However, despite the energetics of photosynthesis has been studied for decades, the regulation of the electron transport fluxes and their interconnectivities are still unclear (Labs et al., 2016). In fact, PSI photoinhibition and its photoprotection mechanisms remains poorly understood (Chaux et al., 2015). It was previously shown that superoxide radicals act on the PSI-bound iron-sulfur centers, promoting PSI photoinhibition (Sonoike, 2011). In addition, superoxide and hydrogen peroxide could lead, via Haber-Weiss reaction, to the production of radical hydroxyl. This radical is very reactive, with a short half-life causing PSI photoinhibition as well (Huang et al., 2018; Takagi et al., 2016). However, under optimum conditions, ROS are scavenged by enzymatic and non-enzymatic antioxidants (Asada, 2000). When ROS production exceeds detoxifying mechanisms, these ROS irreversibly damages PSI primary acceptors, preventing the accumulation of $P700^{+}$ in high light, resulting in decreases on the oxidizable P700, a common measurement for probing PSI photoinhibition (Chaux et al., 2015).

Currently, it is assumed that the CEF is important to balance the ATP/NADPH ratio, by increasing the ATP supply, providing a proton motive force to trigger photoprotective mechanisms (Alric and Johnson, 2017; Johnson, 2005). The lack of a generally accepted approach to measure CEF directly and the existence of divergent CEF pathways in different

photosynthetic organisms have further complicated the overall picture (Labs et al., 2016). One of the principal problems in studying CEF has been the difficulty in assaying it. It is necessary to be able to determine the relative contribution of linear electron flow (LEF) to the overall flux. Perhaps, the simplest assay that can be performed to measure CET is to illuminate a leaf with far-red light or in the presence of 3-(3,4-cichlorophynyl)-1,1-dimethylurea (DCMU) and measure the electron flux through PSI. DCMU inhibits electron transfer from Q_A to Q_B blocking LEF. CEF could be measured *in vivo* and the use of DCMU led to identification of a key player (Labs et al., 2016). Alternatively, one can measure redox changes related to electron flow spectroscopically. Typically measuring the post-illumination reduction rates of $P700^+$ or the oxidation rate of P700 oxidation upon exposure to far red light (avoiding electron flow from PSII). Overall it is strongly recommended that once a particular phenotype is likely related to changes in CEF, several complementary techniques are used at the same time to substantiate conclusions (Finazzi and Johnson, 2016).

The water-water cycle (WWC) photoreduces dioxygen to water without release of superoxide and hydrogen peroxide from their generations sites. In the molecular level, the electrons coming from PSII can reduce dioxygen to superoxide in PSI. This radical is promptly disprotonated by superoxide dismutase (SOD) to hydrogen peroxide. Thereafter, SODs are found in the vicinity of PSI (Asada, 1999). Then, ascorbate peroxidase (APX) catalyzes the reduction of H_2O_2 to water, using ascorbate as the specific electron donor (Asada, 1999). The thylakoidal APX (tAPX) is localized in the vicinity of PSI in thylakoid membrane, where O_2 is photoreduced (Mano et al., 1997). This mechanism is important for suppressing the photoinhibition by reducing the production of ROS in chloroplasts. In addition, the water-water cycle participates in the dissipation of excess photons and electrons as an alternative electron flux (Asada, 2000). In fact, the CEF and the WWC contribute to

production of additional ATP and enhancement of NPQ in PSII antenna system, through generation of a proton motive force (Miyake, 2010).

In plants, APXs (EC 1.11.1.11) are important enzymes controlling the H_2O_2 level in different compartments of the cell, using ascorbate as the specific electron donor (Nakano and Asada, 1981). The detoxification of H_2O_2 by APX is followed by a set of reactions catalyzed by monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). These reactions, in consonance, comprise the Halliwell-Asada or ascorbate-glutathione cycle (Foyer and Halliwell, 1976). Rice plants (*Oryza sativa* L.) have 8 different isoforms distributed in almost every compartment (cytosol, mitochondria, chloroplast stroma and thylakoid, and peroxisomes) of the plant cell (Teixeira et al., 2004). Due to the lack of catalase and guaiacol peroxidase in chloroplasts, APXs are important in this organelle to remove the H_2O_2 produced by the activity of SOD (Asada, 2000). Therefore, the use of mutants lacking the activity of this important H_2O_2 scavenger is an important tool to clarify the regulation of ROS on the efficiency of PSI and the possible photoprotective mechanisms of the water-water cycle. Rice plants have two APX isoforms in chloroplasts, APX7 (soluble) and APX8 (thylakoid-bound) (Teixeira et al., 2006). Recently, our group demonstrated that plants silenced to *OsApx8* (RNAi*OsApx8*) have lower growth rate and higher H_2O_2 content compared with NT plants (Jardim-Messeder et al., 2018).

In this article we examine the physiological contribution of the regulation of the photosynthetic electron flow in plants lacking tAPX isoform. These plants presented disrupted WWC with higher levels of H_2O_2 in chloroplasts compared with NT plants (Jardim-Messeder et al., 2018). Using different approaches, we estimated the CEF in leaves in presence or absence of DCMU in two light regimes (400 and 1000 μmol m^{-2} s^{-1}). It was clearly shown that *apx8* plants display a different compensatory mechanism to avoid excess energy in chloroplasts compared with NT plants under both light regimes. In particular, we focused on

the importance of the CEF, as a compensatory mechanism, in plants lacking the WWC to avoid ROS burst and photoinhibition. The importance of these findings could be central for the further understanding of PSI photoinhibition, proposed as a major determinant in crop productivity (Tikkanen et al., 2014), allowing a rational modification of photosynthesis to improve the efficiency of plant crops and the production of renewable bioenergy (Chaux et al., 2015).

Material and methods

Construction of the plant vector and plant transformation

A chimeric gene producing mRNA with a hairpin structure (hpRNA) was constructed based on the sequence of the *OsApx8* (LOC_Os02g34810) gene. The following primer pairs were used to amplify a 227 bp *RNAiOsApx8* sequence: 5'-CTCGAGGCTGCGAAATACTCCTACGG-3' and 5'-GGTACCTCGAGAGGAGGTCATCAGACCATCG-3'. PCR product was cloned into the Gateway vector pANDA, in which hairpin RNA is driven by the maize ubiquitin promotor and an intron placed upstream of the inverted repeats (Miki and Shimamoto, 2004). Rice plants with knockdown of *OsApx8* were obtained by *Agrobacterium tumefaciens*-mediated transformation of rice embryogenic calli (*Oryza sativa* L. ssp japonica cv. Nipponbare) induced from seeds and cultivated in NB medium at 28°C in the dark (Upadhyaya et al., 2000).

Plant material and growth conditions

NT and *apx8* plants were grown in a 2L vase containing half-strength Hoagland-Arnon nutritive solution at pH 6.0 (Hoagland and Arnon, 1950). Plants were grown for 25 days in a greenhouse under natural conditions as follow: day/night mean temperature of 29/24 °C, mean relative humidity of 68%, and a photoperiod of 12 h. The light intensity inside the

greenhouse varied as a typical day from 6:00 a.m. to 6:00 p.m., reaching an average of maximum PPFD equals to 820 μmol m^{-2} s^{-1} at noon.

2.3 Leaf gas exchange, chlorophyll a fluorescence and P700 redox state measurements

For the assessment of gas exchange and photochemical parameters, plants were transferred to a growth chamber, with controlled conditions of 29 °C, RH 70% and PPFD of 700 μmol m^{-2} s^{-1}. After one hour of plant acclimation to these conditions, the measurements were performed in the third fully-expanded leaf.

In vivo chlorophyll *a* fluorescence was measured using a Dual-PAM 100 (Walz, Effelrich, Germany). Dark adapted leaves (30 min) were exposed to different light regimes of actinic light (AL) (400 or 1000 μmol m^{-2} s^{-1}). The intensity and duration of the saturation pulse (SP) were 8,000 μmol m^{-2} s^{-1} and 0.7 seconds, respectively. For the measurements of chlorophyll *a* fluorescence the following parameters were assessed: the maximum quantum efficiency of PSII [$F_v/F_m=(F_m-F_o)/F_m$], the effective quantum efficiency of PSII [$YII=(F_m'-F_s)/F_m'$], the non-photochemical quenching [$NPQ=(F_m-F_m')/F_m'$] and the apparent electron transport rate through the photosystem II [ETR_{II} = (YII x PPFD x 0.5 x 0.84)]. To estimate ETR_{II}, 0.5 was used assuming the fraction of excitation energy distributed to PSII, and 0.84 was used assuming as the fraction of incoming light absorbed by the leaves. The F_m and F_o are the maximum and minimum fluorescence of dark-adapted leaves, respectively; F_m', F_o' and F_s are the maximum, the minimum and the steady-state fluorescence in the light-adapted samples (Maxwell and Johnson, 2000).

Photochemical activity of PSI was measured under the same conditions described previously for the measurements of PSII activity. The redox state of P700 was assessed using a Dual PAM 100 (Walz, Effelrich, Germany). $P700^+$ was monitored as the absorption difference between 830 and 875 nm in transmission mode. The quantum yields of PSI were determined using the saturation pulse (SP) method (Klughammer and Schreiber, 1994). The

maximum P700 oxidable signal ($P700^+$) was determined by the application of the SP in presence of far-red light (FR) (720 nm). The P700 parameters were calculated as follow: effective quantum yield of PSI [Y(I)=(Pm´-P)/Pm]; the electron transport rate at PSI (assuming equal distribution of light energy between PSII and PSI and the leaf absorbance of 0.84) [ETRI = Y(I) x PPFD x 0.5 x 0.84]. The complementary quantum yields are defined as Y(I) + Y(ND) +Y(NA) = 1. The quantum yield of non-photochemical energy dissipation due to donor-side limitation of PSI [Y(ND)] and the quantum yield of non-photochemical energy dissipation due to acceptor-side limitation of PSI [Y(NA)] (Klughammer and Schreiber, 1994; Zivcak et al., 2015). The cycle electron flow (CEF) was estimated by the ETRI/ETRII ratio (Yamori et al., 2011).

Rapid light response curves were performed after steady-state of photosynthesis was reached, a rapid light curve was triggered with different light intensities for 30 s at each light intensity with saturation pulse and far-red pulse determinations after 30 s at each light intensity (Zivcak et al., 2014).

$P700^+$ fast kinetics

The normalized P700 kinetics at far-red illumination was monitored in light adapted leaves (400 or 1000 μmol $m^{-2}s^{-1}$) for thirty minutes. After illumination time, lights were turned off. Immediately after, dark points were saved in a time resolution of 50 μs. The leaves were kept in dark for 100 ms. Then, the P700 pool was oxidized by far red (FR) light for 40 s. After that, the FR light was turn off and points were saved during 30 s to follow the re-reduction of P700 (Joliot and Joliot, 2006; Zivcak et al., 2014).

Vacuum infiltration with DCMU

Leaves segments were used for these experiments. First, plants were dark adapted and then detached leaves were vacuum infiltrated with 100 μM DCMU in 300 mM Hepes or only in Hepes (mock) for 30 min in dark condition (Trubitsin et al., 2014).

2.6 Statistical analysis and experimental design

The experiments were arranged in a completely randomized design in a 2 × 2 × 2 factorial (NT and *apx8* plants × 0 and 100 μM DCMU x 400 and 1000 μmol $m^{-2}s^{-1}$) and six replicates, each consisting of an individual plant per pot. Data were analyzed using ANOVA, and the means were compared using Tukey's ($P<0.05$).

Results

In order to understand how *apx8* plants deal with the reduction of tAPX isoform activity on the PSI redox state, the following experiments were performed: NT and *apx8* plants were exposed to different light treatments (400 or 1000 μmol $m^{-2}s^{-1}$, moderate and high light, respectively) in the presence or absence of DCMU, an inhibitor of the electron transport from PSII to PSI. We have previously demonstrated that gas-exchange measures and PN-Ci curves performed in both NT and *apx8* plants showed no significative differences among these plants, suggesting that the knockdown of tAPX isoform did not affected photosynthesis under optimum conditions (Cunha et al., 2018 – Chapter III).

In relation to PSII photochemistry, *apx8* plants under moderate light (400 μmol m^{-2} s^{-1}), displayed a slightly lower effective quantum yield of PSII [Y(II)] compared with NT plants. This same trend was followed by the electron transport rate of PSII [ETR(II)] (Figure 1a-c). In contrast, increasing the irradiance to 1000 μmol m^{-2} s^{-1} these differences were extinguished, and both genotypes, as expected, displayed lower Y(II) and ETR(II) compared with moderate light (Figure 1b-d). The non-photochemical quenching (NPQ) was slightly higher in *apx8* plants under moderate light, corroborating the lower Y(II) and ETR(II) in this

genotype at 400 μmol m^{-2} s^{-1} (Figure 1e). Both genotypes did not display photoinhibition through Fv/Fm (Figure 1g-h). In general, increasing light to 1000 μmol $m^{-2}s^{-1}$ these differences were extinguished with both genotypes displaying no significant differences in any parameters (Figure 1b-h). In addition, DCMU was used to limit the electron flow to PSI, by blocking LEF (Huang et al., 2016). Under these conditions, it was clearly shown that the DCMU treatment was efficient, as Y(II) and ETR(II) was close to zero with decreases in Fv/Fm for both light treatments and both genotypes (Figure 1). However, under high light, *apx8* plants showed a significant higher NPQ compared with NT plants in presence of DCMU (Figure 1f).

Regarding the efficiency of PSI under moderate light (400 μmol m^{-2} s^{-1}), there was no significative difference on the effective quantum yield of PSI [Y(I)] for both genotypes in the absence of DCMU (Figure 2a). The same trend was followed by ETR(I) (Figure 2c). However, *apx8* plants exhibited a significative lower $P700^{+}$ compared with NT plants (Figure 2e). When plants were exposed to high light Y(I) was decreased for both genotypes, compared with moderate light, as expected. However, Y(I), ETR(I) and $P700^{+}$ were practically the same for both genotypes under high light (Figure 2bf). Treating the leaves with DCMU significantly decreased Y(I), ETR(I) and $P700^{+}$ for both genotypes and light treatments, with no significative differences between NT and *apx8* plants (Fig 2a-f). However, under high light in presence of DCMU, *apx8* plants displayed lower $P700^{+}$ compared with NT plants (Figure 2f).

Under moderate light, the quantum yield of non-photochemical energy dissipation due to donor-side limitation of PSI [Y(ND)] and the quantum yield of non-photochemical energy dissipation due to acceptor-side limitation of PSI [Y(NA)] displayed no significant differences between NT and *apx8* plants in absence of DCMU (Figure 3a-c). Under high light, Y(ND) was increased in both genotypes compared with moderate light (Figure 3a-b). In

contrast, Y(NA) was slightly lower in *apx8* plants exposed to high light compared with NT plants (Figure 3d). In the presence of DCMU, Y(ND) and Y(NA) were increased for both genotypes and both light regimes (Figure 3a-d). Under both light regimes, *apx8* plants displayed higher Y(ND) and lower Y(NA) compared with NT plants in presence of DCMU (Figure 3a-d).

Due to the modulation of the previous parameters we estimate the CEF (Yamori et al., 2011). Under moderate light in absence of DCMU, *apx8* plants displayed a slightly higher CEF compared with NT plants. However, increasing light to 1000 μmol m^{-2} s^{-1} this difference was extinguished (Figure 4). In contrast, in the presence of DCMU, under moderate light, *apx8* plants presented 3 times higher CEF than NT plants. This enormous difference between genotypes was decreased under high light. Nevertheless, in the presence of DCMU and high light, *apx8* displayed higher CEF compared with NT plants (Figure 4). To corroborate the previous results, we performed re-reduction kinects of $P700^{+}$. This is one common assay to measure the CEF, stopping the illumination abruptly and measuring the rate at which oxidized P700 is re-reduced (Johnson, 2005). Under moderate light, in the absent of DCMU, *apx8* plants displayed a slightly higher $P700^{+}$ level compared with NT. In addition, *apx8* plants displayed a slightly faster re-reduction compared with NT (Figure 5a). Under high light, NT plants displayed higher $P700^{+}$ signal compared with *apx8* plants, and *apx8* plants presented a faster re-reduction compared with NT (Figure 5b). In the presence of DCMU, and moderate light, *apx8* continued to display a slightly higher $P700^{+}$ level, compared with NT. In contrast, under high light *apx8* plants displayed lower $P700^{+}$ compared with NT and presented a faster re-reduction (Figure 5 c-d).

Rapid light curves responses were performed to understand the modulation of PSI yields in response to increasing light. The data from the light curves corroborate the previous results. ETR(II) in the presence or absence of DCMU displayed no significative differences

between the genotypes (Supplementary figure 1). However, *apx8* plants presented slightly higher ETR(I) in presence and absence of DCMU compared with NT plants (Supplementary figure 2). DCMU decreased Y(I) in both genotypes, and *apx8* plants displayed higher Y(ND) and lower Y(NA) compared with NT plants in response to increasing light (Figure 6c-d). Estimating the CEF from the rapid light curve, we clearly show again, that *apx8* plants display higher CEF from 500 μmol m^{-2} s^{-1} compared with NT plants (Figure 7a). In the presence of DCMU, *apx8* plants displayed a strongly increase in the CEF compared with NT from 300 μmol m^{-2} s^{-1} (Figure 7b).

Altogether, our data clearly show that rice plants silenced for tAPX display different regulation mechanisms of the photosynthetic electron transport rate even in moderate light, in special regarding PSI yields. One of these mechanisms could enable the activation of CEF, measured in this manuscript in different manners, as a compensatory photoprotector mechanism for knockdown rice plants with disrupted WWC.

Discussion

In this study we show that the CEF is triggered as a possible photoprotection mechanism to alleviate the lack of tAPX in knockdown rice plants. In other words, the CEF is triggered as a potential photoprotective mechanism in plants lacking the WWC. Our group recently reported that *apx8* plants have a slightly lower growth rate and higher H_2O_2 content compared with NT plants, with no differences on gas exchange parameters when well irrigated (Jardim-Messeder et al., 2018), which is in accordance with our data from P_N-Ci curves. In contrast, we show that the photosynthetic electron transport is differently regulated in *apx8* plants to maintain the same rate of photosynthesis of NT plants when well hydrated.

Under moderate light, *apx8* plants displayed lower Y(II) and ETR(II) compared with NT plants. This regulation is possibly explained by the increase in NPQ in *apx8* plants,

reflecting the electron transport rate of PSII (Ruban, 2017). In addition, NT and *apx8* plants did not display photoinhibition through Fv/Fm. NPQ of PSII avoids excessive electron transport to PSI via LEF, preventing photoinhibition (Sonoike, 2011). Interesting to note that increasing the electron pressure on the photosynthetic electron transport chain (ETC) these differences were extinguished. It seems that under moderate light, *apx8* plants regulates the electron transport at PSII level, avoiding the over-reduction of the photosynthetic electron transport chain, to mitigate the overproduction of ROS and consequently photoinhibition. This should be an important mechanism, even under moderate light, since reducing the tAPX isoform increases H_2O_2 on the chloroplast (Jardim-Messeder et al., 2018). In presence of DCMU, that blocks the LEF, and high light, *apx8* plants displayed higher NPQ compared with NT. The increase in NPQ in presence of DCMU could be related with the increase of the formation of ΔpH by the CEF, since the electron transport from PSII to PSI is blocked. Therefore, this was the first evidence of the increase in CEF in *apx8* plants.

Under moderate light, *apx8* plants displayed lower $P700^+$ compared with NT plants (Figure 2e). In contrast, under high light, NT plants and *apx8* presented no differences in $P700^+$. DCMU decreased $P700^+$ level compared with absence of DCMU for both genotypes and light regime. It was previously shown that DCMU incompletely prevent PSI photinhibition by blocking electron transfer from PSII to PSI (Chaux et al., 2015; Huang et al., 2018). However, this response on PSI photoinhibition by DCMU is controversial on the literature. In *Arabdopsis*, DCMU did not result in a significant increase in the oxidation of P700 (Hald et al., 2008; Suorsa et al., 2012). In *Psychotri rubra,* PSI photoinhibition was mainly controlled by electron transfer from PSII to PSI rather than PSI redox state (Huang et al., 2016). The excess electron transfer from PSII to PSI induces the production and accumulation of hydroxyl radicals at the acceptor side of PSI and then causes PSI photoinhibition in *P. rubra*, irrespective of P700 oxidation ratio. Therefore, the major

mechanisms of PSI photoinhibition is probably different in *P. rubra* compared with *Arabidopsis* and cucumber (Huang et al., 2016).

Under moderate light, in absence of DCMU, NT and *apx8* displayed the same pattern of Y(ND) and Y(NA). However, in presence of DCMU in the same light intensity (400 μmol $m^{-2}s^{-1}$), *apx8* plants increased Y(ND) and decreased Y(NA) compared with NT plants. Increases in Y(ND) in presence of DCMU is pertinent due to the blockage of electron transport from PSII to PSI. In addition, these patterns were maintained under high light. Nevertheless, the balance between Y(ND) and Y(NA) displayed by *apx8* plants in presence of DCMU is another evidence of the triggering of CEF in this genotype under moderate light. As reported previously, *pgr5*-mutants of *A. thaliana* showed high levels of Y(NA) under high light because the reduced formation of the proton motive force (Wood et al., 2018; Yamori et al., 2016). In contrast, the alleviation of Y(NA) by CEF was shown to suppress PSI photoinhibition (Kono and Terashima, 2014). In addition, the increase in Y(ND), by suppressing the electron flow into PSI is also important for the protection of PSI from photoinhibition (Kono and Terashima, 2016, 2014). Therefore, the alleviation of Y(NA) and acceleration of Y(ND) are indispensable for the regulation of CEF-PSI (Huang et al., 2018, 2016; Kono and Terashima, 2014).

The ratio ETR(I)/ETR(II), that estimate the cyclic electron flow corroborated the previous results. In addition, the post-illumination reduction rates of $P700^+$, showed faster re-reduction of $P700^+$ in *apx8* plants in all experimental conditions. After the oxidation of P700 upon exposure to far-red light, in light adapted plants abruptly exposed to dark, the re-reduction of P700 is a reliable estimate of electron transport flow around PSI (Finazzi and Johnson, 2016; Johnson, 2005). Primarily, the estimation of CEF must be assessed with far-red light or with PSII poison with DCMU, to distinguish PSI turnover from PSII (Finazzi and Johnson, 2016). On the other hand, these methods could lead to possible artefacts, in general

an underestimation of the CEF. In *Arabdopsis* and rice, the $P700^+$ level was increased with increasing light from 200 to 1900 μmol m^{-2} s^{-1} (Munekage et al., 2002; Nishikawa et al., 2012). Our data suggest that the reduction in tAPX increased the CEF in *apx8* plants in presence or absence of DCMU since 500 μmol m^{-2} s^{-1} of light.

The cyclic electron flow may act as a safety valve to the excess of electrons in chloroplasts. However, the physiological function of CEF around PSI remains to be clarified (Yamori and Shikanai, 2016). The impairment of CEF reduced the photosynthetic rate under fluctuating light, leading to photoinhibition of PSI and consequently a reduction in plant biomass in rice (Yamori and Shikanai, 2016). CEF can be seen as limiting ROS production under acceptor side limitations. It has been suggested that an interplay between CEF and O_2 photoreduction acts as a buffer to poise electron flow toward carbon fixation (Chaux et al., 2015). It was previously shown that CEF was strongly increased by H_2O_2. However, this H_2O_2-dependent CEF is probably not involved with the PGR5/PGRL1 pathway, but could be involved with the NADPH:PQ reductase (NDH) complex CEF dependent (Strand et al., 2015). In fact, the action of H_2O_2 as an activator of NDH-CEF in *Arabidopsis* provides further evidence that O_2 photoreduction pathways and CEF are working in tandem, and H_2O_2 is a missing link between environmental stress, metabolism and redox regulation of CEF in higher plants (Strand et al., 2015). In addition, it was demonstrated that increases in H_2O_2 level inactive several Calvin-Benson cycle enzymes and inhibits the CO_2 fixation (Asada, 2000). This is due to the oxidative inactivation of the thiol groups in the amino acids of these enzymes, resulting in the formation of disulfide bridges between the groups (Miyake, 2010). Furthermore, the accumulated H_2O_2 oxidatively degrades APX in chloroplasts, resulting in the stimulated accumulation of H_2O_2 and enhanced inactivation of photosynthesis (Makino et al., 2002).

The excess of electron pressure on the thylakoid could reduce the molecular oxygen to superoxide radical. Superoxide radical and singlet oxygen produced within the thylakoid membrane cause photoinhibition of PSI (Takagi et al., 2017, 2016). In normal conditions, SOD and APX activities, by the WWC, scavenge these ROS protecting PSI from photoinhibition (Asada, 2006; Makino et al., 2002). In *apx8* plants, the H_2O_2 produced by SOD in chloroplast is maintained, since the reduction of the tAPX isoform. So, the WWC is disrupted (Asada, 2000). It was previously demonstrated that the electron sink ability of the WWC increases with the saturation of photosynthesis against light intensity. Thus, the WWC is an important photoprotector mechanism to avoid photoinhibition. As previously discussed, the increase in H_2O_2 content in chloroplasts can act as a signalling agent to activate CEF in higher plants *in vivo* (Strand et al., 2015). The H_2O_2 in excess in chloroplasts can lead the production or more harmful ROS as radical hydroxyl by the transition metal-catalyzed Haber-Weiss reaction (Asada, 2006). Furthermore, the radical hydroxyl decomposes the PSI complex in thylakoid membranes (Sonoike, 2011). It is important to note, that H_2O_2 in excess decreases the efficiency of APX, resulting in a higher probability of the inactive forms of APXs since excess H_2O_2 can leak from chloroplasts, where APX is inactivated (Miyake and Asada, 1996).

The model that emerges is that the regulation of temporary excesses reductants at the acceptor side of PSI is controlled by an interplay among CEF, Mehler reaction and malate valve with another level of control by redox regulators as thioredoxins (Scheibe and Dietz, 2012). These pathways likely form a set of communicating reactions to balance NADPH/$NADP^+$ ratios avoiding PSI photoinhibition (Chaux et al., 2015). It was suggested that the activity of the WWC competes with that of CEF. The WWC oxidizes the PQ pool for the turnover of CEF and its requirement for PQ pool oxidation increases under suppressed conditions of photosynthesis. So, unless the WWC functions, CEF cannot be active (Miyake,

2010). Our data go against this assumption. In this manuscript, in conclusion, our data suggest that tAPX knockdown rice plants, with disturbing WWC and increased H_2O_2 content, triggers CEF as a compensatory mechanism to avoid PSI photoinhibition (Figure 8). In other words, CEF is triggered to compensate the decrease in the efficiency of the WWC in rice plants under moderate or high light.

References

ALRIC, J., JOHNSON, X. Alternative electron transport pathways in photosynthesis: a confluence of regulation. **Curr. Opin. Plant Biol.** 37, 78–86, 2017.

ASADA, K. Production and scavenging of reactive oxygen species in chloroplasts and their functions. **Plant Physiol.** 141, 391–396, 2006.

ASADA, K. The water-water cycle as alternative photon and electron sinks. **Philos. Trans. R. Soc. Lond. B. Biol. Sci.** 355, 1419–31, 2000.

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

CHAUX, F., PELTIER, G., JOHNSON, X. A security network in PSI photoprotection: regulation of photosynthetic control, NPQ and O2 photoreduction by cyclic electron flow. **Front. Plant Sci.** 6, 1–7, 2015.

FINAZZI, G., JOHNSON, G.N. Cyclic electron flow : facts and hypotheses. **Photosynth. Res.** 129, 227–230, 2016.

FOYER, C.H., HALLIWELL, B. The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. **Planta** 133, 21–25, 1976.

FOYER, C.H., RUBAN, A. V., NOCTOR, G. Viewing oxidative stress through the lens of oxidative signalling rather than damage. **Biochem. J.** 474, 877–883, 2017.

GOH, C.-H., KO, S.-M., KOH, S., KIM, Y.-J., BAE, H.-J. Photosynthesis and Environments: Photoinhibition and Repair Mechanisms in Plants. **J. Plant Biol.** 93–101, 2011.

HALD, S., NANDHA, B., GALLOIS, P., JOHNSON, G.N. Feedback regulation of photosynthetic electron transport by NADP(H) redox poise. **Biochim. Biophys. Acta** 1777, 433–40, 2008.

HOAGLAND, D.R., ARNON, D.J. The water culture method for growing plants without soil. **Agric Exp Cir** 347, 1950.

HUANG, W., TIKKANEN, M., ZHANG, S.-B. Photoinhibition of photosystem I in Nephrolepis falciformis depends on reactive oxygen species generated in the chloroplast stroma. **Photosynth. Res.** 137, 129–140, 2018.

HUANG, W., YANG, Y.-J., ZHANG, J.-L., HU, H., ZHANG, S.-B. PSI photoinhibition is more related to electron transfer from PSII to PSI rather than PSI redox state in Psychotria rubra. **Photosynth. Res.** 129, 85–92, 2016.

JARDIM-MESSEDER, D., CAVERZAN, A., RAUBER, R., RIBEIRO, J., CARVALHO, F.E.L., GAETA, M.L., DA FONSECA, G.C., COSTA, J.M., FREI, M., DA SILVEIRA, J.A., MARGIS, R., SAIBO, N.J.M., MARGIS-PINHEIRO, M. Thylakoidal APX modulates hydrogen peroxide content and stomatal closure in rice (Oryza sativa L.). **Environ. Exp. Bot.** 150, 46-56, 2018.

JOHNSON, G.N. Cyclic electron transport in C3 plants: fact or artefact? **J. Exp. Bot.** 56, 407–16, 2005.

JOHNSON, G.N., STEPIEN, P. Plastid Terminal Oxidase as a route to improving plant stress tolerance - Known Knowns and Known Unknowns. **Plant Cell Physiol.** 57, 1387–1396, 2016.

JOLIOT, P., JOLIOT, A. Cyclic electron flow in C3 plants. **Biochim. Biophys. Acta** 1757, 362–8, 2006.

JOLIOT, P., JOLIOT, A. Quantification of cyclic and linear flows in plants. **Proc. Natl. Acad. Sci. U. S. A.** 102, 4913–8, 2005.

KLUGHAMMER, C., SCHREIBER, U. An improved method, using saturating light pulses, for the determination of photosystem I quantum yield via P700+-absorbance changes at 830 nm. **Planta** 192, 261–268, 1994.

KONO, M., TERASHIMA, I. Elucidation of Photoprotective Mechanisms of PSI Against Fluctuating Light photoinhibition. **Plant Cell Physiol**. 57, 103, 2016.

KONO, M., TERASHIMA, I. Long-term and short-term responses of the photosynthetic electron transport to fluctuating light. **J. Photochem. Photobiol. B.** 137, 89–99, 2014.

LABS, M., RUHLE, T., LEISTER, D. The antimycin A-sensitive pathway of cyclic electron flow: from 1963 to 2015. **Photosynth. Res.** 1–8, 2016.

LIMA NETO, M.C., CERQUEIRA, J.V.A., CUNHA, J.R., RIBEIRO, R.V., SILVEIRA, J.A.G. Cyclic electron flow, NPQ and photorespiration are crucial for the establishment of young plants of Ricinus communis and Jatropha curcas exposed to drought. **Plant Biol.** 19, 650–659, 2017.

MAKINO, A., MIYAKE, C., YOKOTA, A. Physiological Functions of the Water–Water Cycle (Mehler Reaction) and the Cyclic Electron Flow around PSI in Rice Leaves. **Plant Cell Physiol.** 43, 1017–1026, 2002.

MANO, J., USHIMARU, T., ASADA, K. Ascorbate in thylakoid lumen as an endogenous electron donor to Photosystem II : Protection of thylakoids from photoinhibition and regeneration of ascorbate in stroma by dehydroascorbate reductase. **Photosynthesis Research**, 197–204, 1997.

MAXWELL, K., JOHNSON, G.N. Chlorophyll fluorescence--a practical guide. **J. Exp. Bot.** 51, 659–68, 2000.

MIYAKE, C. Alternative electron flows (water-water cycle and cyclic electron flow around PSI) in photosynthesis: Molecular mechanisms and physiological functions. **Plant Cell Physiol.** 51, 1951–1963, 2010.

MIYAKE, C., ASADA, K. Inactivation Mechanism of Ascorbate Peroxidase at Low Concentrations of. **Plant Cell Physiol.** 37, 423–430, 1996.

MUNEKAGE, Y., HOJO, M., MEURER, J., ENDO, T., TASAKA, M., SHIKANAI, T., PGR5 Is Involved in Cyclic Electron Flow around Photosystem I and Is Essential for Photoprotection in Arabidopsis. **Cell** 110, 361–371, 2002.

NAKANO, Y., ASADA, K. Hydrogen peroxide is scavenged by ascorbato specific peroxidase in spinach chloroplasts. **Plant Cell Physiol** 22, 867–880, 1981.

NISHIKAWA, Y., YAMAMOTO, H., OKEGAWA, Y., WADA, S., SATO, N., TAIRA, Y., SUGIMOTO, K., MAKINO, A., SHIKANAI, T. PGR5-dependent cyclic electron transport around PSI contributes to the redox homeostasis in chloroplasts rather than CO_2 fixation and biomass production in rice. **Plant Cell Physiol**. 53, 2117–2126, 2012.

NISHIYAMA, Y., ALLAKHVERDIEV, S.I., MURATA, N. Protein synthesis is the primary target of reactive oxygen species in the photoinhibition of photosystem II. **Physiol. Plant.** 142, 35–46, 2011.

RUBAN, A. V. Quantifying the efficiency of photoprotection. **Philos. Trans. R. Soc. Lond. B. Biol.** Sci. 372, 201, 2017.

SCHEIBE, R., DIETZ, K.J. Reduction-oxidation network for flexible adjustment of cellular metabolism in photoautotrophic cells. **Plant. Cell Environ**. 35, 202–216, 2012.

SEJIMA, T., TAKAGI, D., FUKAYAMA, H., MAKINO, A., MIYAKE, C. Repetitive short-pulse light mainly inactivates photosystem i in sunflower leaves. **Plant Cell Physiol.** 55, 1184–1193, 2014.

SHARKEY, T.D., BERNACCHI, C.J., FARQUHAR, G.D., SINGSAAS, E.L. Fitting photosynthetic carbon dioxide response curves for C(3) leaves. **Plant. Cell Environ.** 30, 1035–40, 2007.

SONOIKE, K. Photoinhibition of photosystem I. **Physiol. Plant.** 142, 56–64, 2011.

STRAND, D.D., LIVINGSTON, A.K., SATOH-CRUZ, M., FROEHLICH, J.E., MAURINO, V.G., KRAMER, D.M. Activation of cyclic electron flow by hydrogen peroxide in vivo. **Proc. Natl. Acad. Sci.** 112, 5539–5544, 2015.

SUORSA, M., JÄRVI, S., GRIECO, M., NURMI, M., PIETRZYKOWSKA, M., RANTALA, M., KANGASJÄRVI, S., PAAKKARINEN, V., TIKKANEN, M., JANSSON, S., ARO, E.-M. PROTON GRADIENT REGULATION5 is essential for proper acclimation of Arabidopsis photosystem I to naturally and artificially fluctuating light conditions. **Plant Cell** 24, 2934–48, 2012.

TAKAGI, D., ISHIZAKI, K., HANAWA, H., MABUCHI, T., YAMAMOTO, H., MIYAKE, C. Diversity of strategies for escaping reactive oxygen species production within photosystem I among land plants: P700 oxidation system is prerequisite for alleviating photoinhibition in photosystem I. **Physiol Plant.** 161(1):56-74, 2017.

TAKAGI, D., TAKUMI, S., HASHIGUCHI, M., SEJIMA, T., MIYAKE, C. Superoxide and Singlet Oxygen Produced within the Thylakoid Membranes Both Cause Photosystem I Photoinhibition. **Plant Physiol.** 171, 1626–1634, 2016.

TEIXEIRA, F.K., MENEZES-BENAVENTE, L., GALVÃO, V.C., MARGIS, R., MARGIS-PINHEIRO, M. Rice ascorbate peroxidase gene family encodes functionally diverse isoforms localized in different subcellular compartments. **Planta** 224, 300–314, 2006.

TIKKANEN, M., MEKALA, N.R., ARO, E.-M. Photosystem II photoinhibition-repair cycle protects Photosystem I from irreversible damage. **Biochim. Biophys. Acta** 1837, 210–5, 2014.

TRUBITSIN, B. V, MAMEDOV, M.D., SEMENOV, A.Y., TIKHONOV, A.N. Interaction of ascorbate with photosystem I. **Photosynth. Res.** 122, 215–231, 2014.

UPADHYAYA, N.M., SURIN, B., RAMM, K., GAUDRON, J., SCHÜNMANN, P.H.D., TAYLOR, W., WATERHOUSE, P.M., WANG, M.-B. Agrobacterium-mediated transformation of Australian rice cultivars Jarrah and Amaroo using modified promoters and selectable markers. **Funct. Plant Biol.** 27, 201, 200.

VOSS, I., SUNIL, B., SCHEIBE, R., RAGHAVENDRA, S. Emerging concept for the role of photorespiration as an important part of abiotic stress response. **Plant Biol. (Stuttg).** 15, 713–722, 2013.

WOOD, W.H.J., MACGREGOR-CHATWIN, C., BARNETT, S.F.H., MAYNEORD, G.E., HUANG, X., HOBBS, J.K., HUNTER, C.N., MATTHEW, P. Dynamic thylakoid stacking regulates the balance between linear and cyclic photosynthetic electron transfer. **Nat. Plants** 4, 2018.

YAMORI, W., MAKINO, A., SHIKANAI, T. A physiological role of cyclic electron transport around photosystem I in sustaining photosynthesis under fluctuating light in rice. **Sci. Rep.** 6, 1–12, 2016.

YAMORI, W., SAKATA, N., SUZUKI, Y., SHIKANAI, T., MAKINO, A. Cyclic electron flow around photosystem I via chloroplast NAD(P)H dehydrogenase (NDH) complex performs a significant physiological role during photosynthesis and plant growth at low temperature in rice. **Plant J.** 68, 966–76, 2011.

YAMORI, W., SHIKANAI, T. Physiological Functions of Cyclic Electron Transport Around Photosystem I in Sustaining Photosynthesis and Plant Growth. **Annu. Rev. Plant Biol.** 67, 81–106, 2016.

ZIVCAK, M., BRESTIC, M., KUNDERLIKOVA, K., SYTAR, O. Repetitive light pulse-induced photoinhibition of photosystem I severely affects CO2 assimilation and photoprotection in wheat leaves. **Photosynth. Res.** 449–463, 2015.

ZIVCAK, M., KALAJI, H.M., SHAO, H.-B., OLSOVSKA, K., BRESTIC, M. Photosynthetic proton and electron transport in wheat leaves under prolonged moderate drought stress. **J. Photochem. Photobiol. B.** 137, 107–15, 2014.

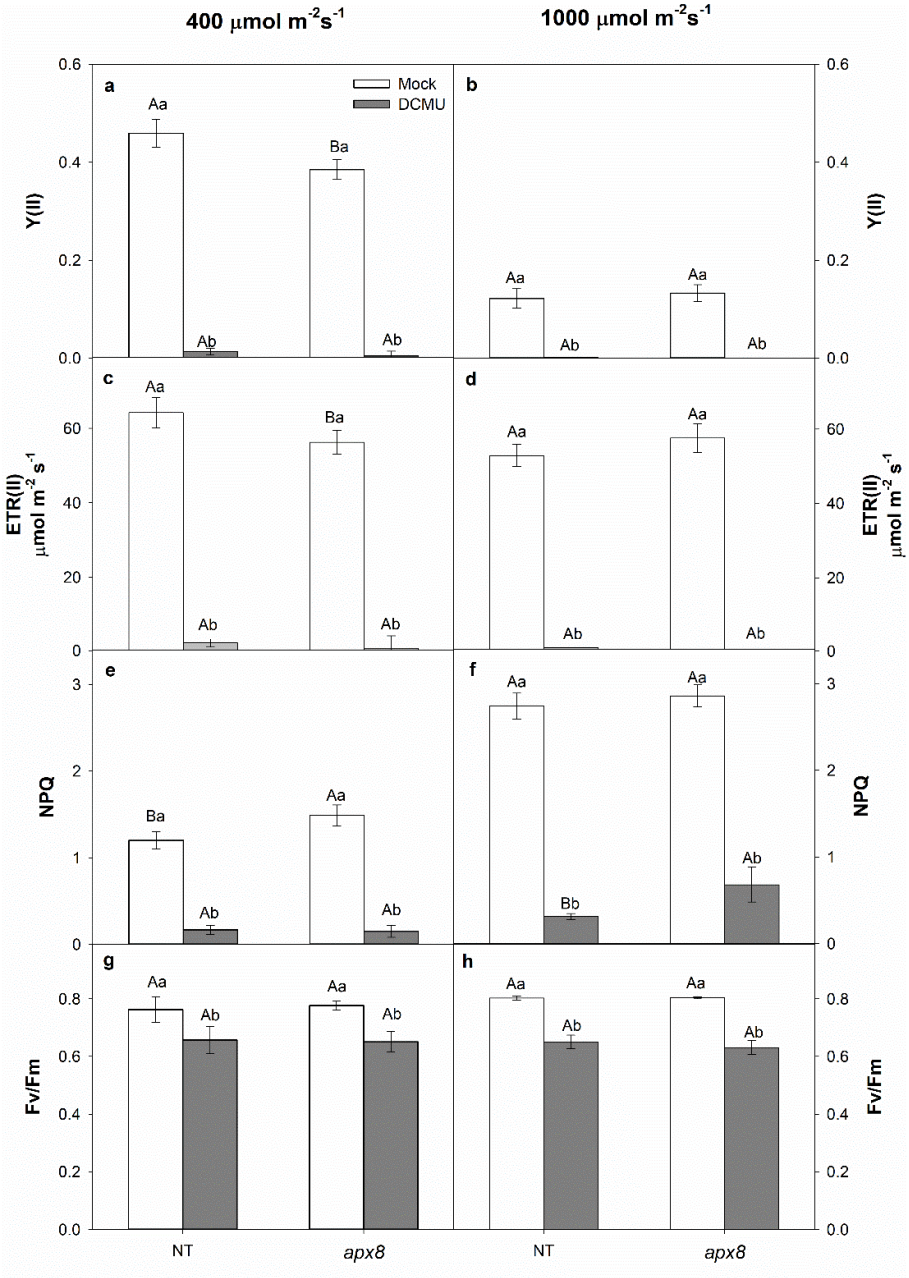


Figure 1. Left panel: NT and *apx8* rice plants were exposed to moderate light in absence (white bars) or presence of 100 μM DCMU in dark for 30 minutes (dark bars). Right panel: NT and *apx8* plants were exposed to high light in absence or presence of DCMU. After infiltration with mock of DCMU for thirty minutes, Fv/Fm were measured in the dark. Then, the leaves were light adapted for thirty minutes, under the different light treatments to achieve steady-state after the photosynthetic induction. The following parameters were measured: the effective quantum yield of PSII (a,b); the apparent electron transport rate at PSII (c,d); the non-photochemical quenching of PSII (e,f) and maximum quantum yield of PSII (g,h). Data are the means of five replicates ± SD. Different capital letters represent significant differences between genotypes. Different lowercase letters represent significant differences between DCMU treatments for the same genotype by Tukey´s test ($P<0.05$).

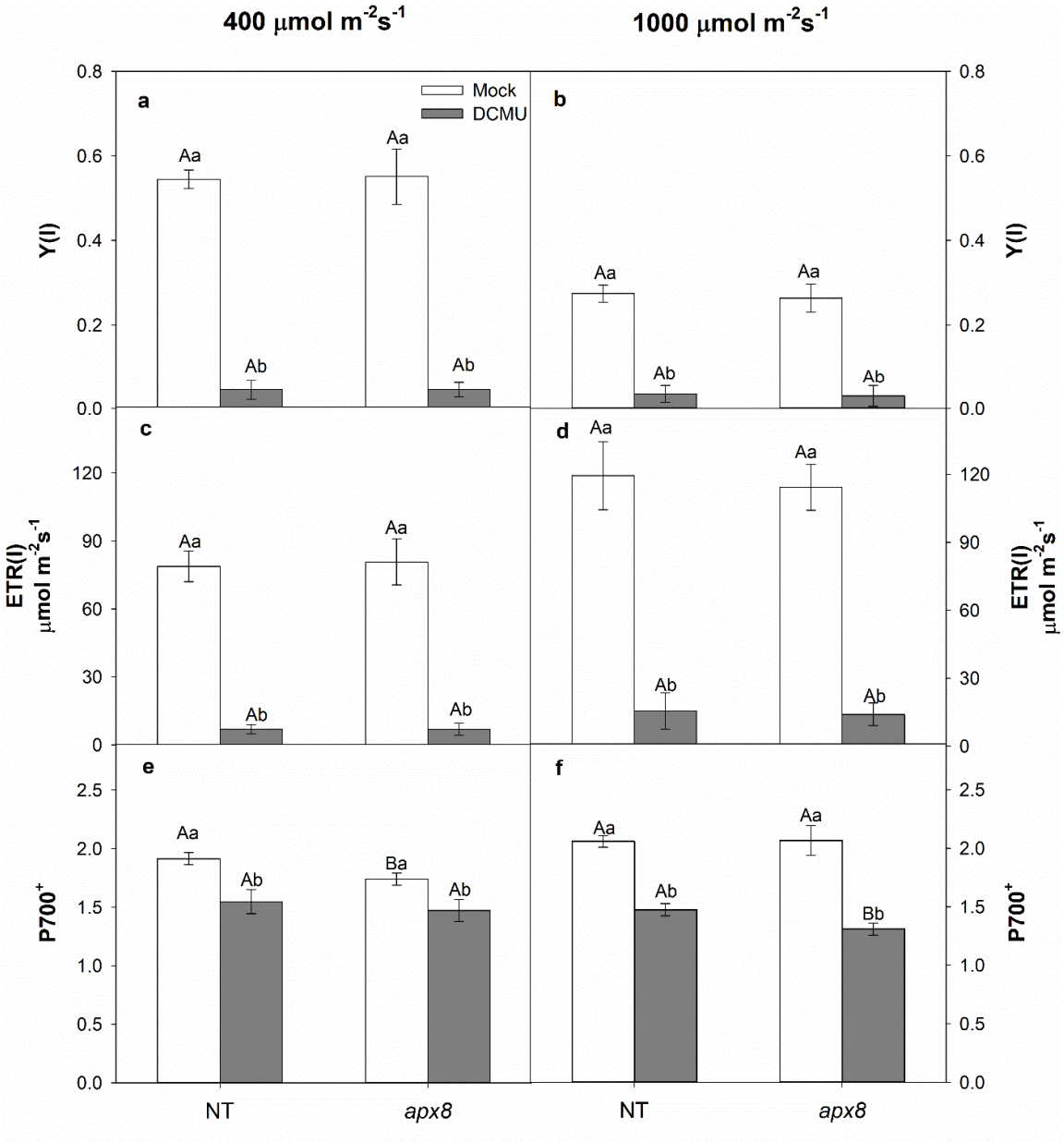


Figure 2. Left panel: NT and *apx8* rice plants were exposed to moderate light in absence (white bars) or presence of 100 μM DCMU in dark for 30 minutes (dark bars). Right panel: NT and *apx8* plants were exposed to high light in absence or presence of DCMU. After infiltration with mock of DCMU for thirty minutes The following parameters were measured: the effective quantum yield of PSI (a,b); the apparent electron transport rate at PSI (c,d) and the maximum P700 oxidable signal (e,f). Data are the means of five replicates ± SD. Different capital letters represent significant differences between genotypes. Different lowercase letters represent significant differences between DCMU treatments for the same genotype by Tukey´s test ($P<0.05$).

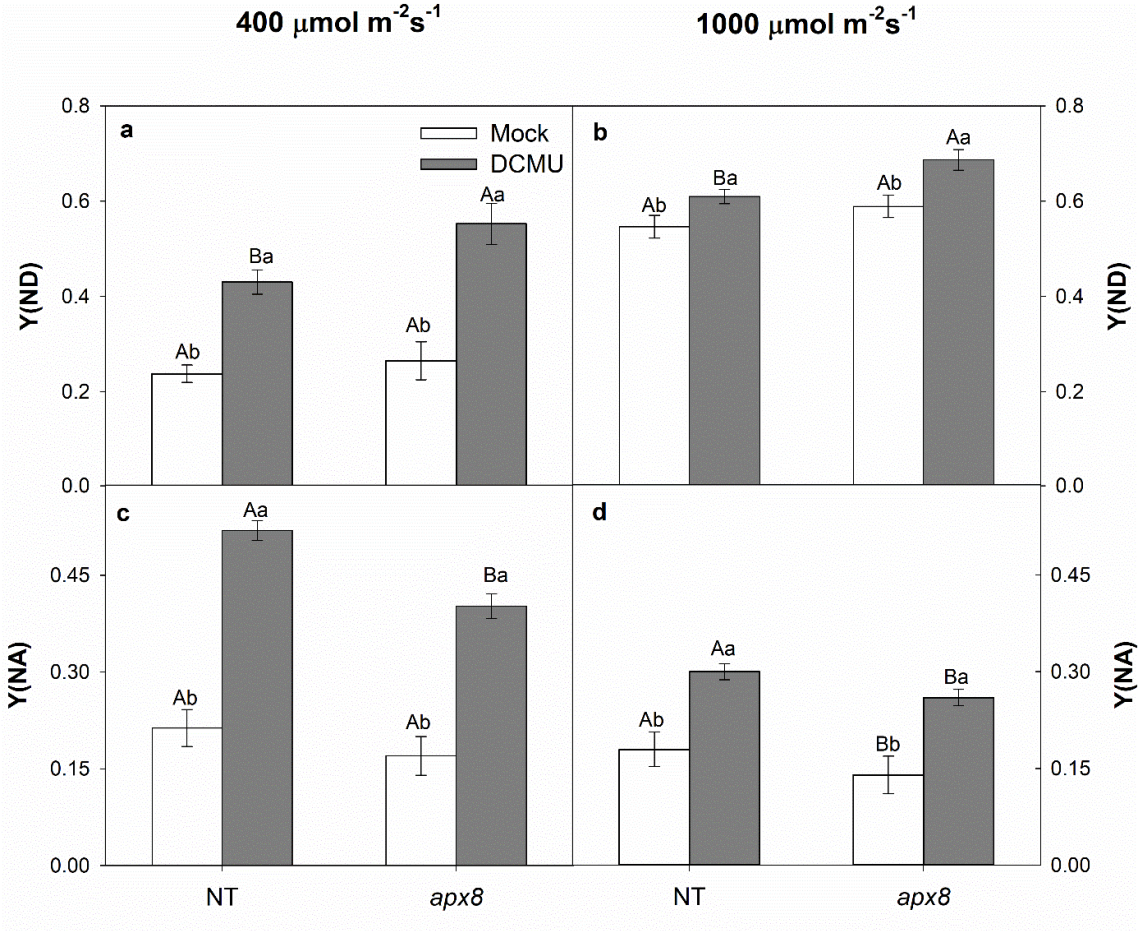


Figure 3. The quantum yield of non-photochemical energy dissipation due to donor-side limitation of PSI under moderate light (a) and high light (b) and the quantum yield of non-photochemical energy dissipation due to acceptor-side limitation of PSI under moderate light (c) and high light (b) in NT and *apx8* rice plants. White bar represents the absence of DCMU and dark bars the vacuum infiltration of 100 μM DCMU in dark for 30 minutes. Data are the means of five replicates ± SD. Different capital letters represent significant differences between genotypes. Different lowercase letters represent significant differences between DCMU treatments for the same genotype by Tukey´s test ($P<0.05$).

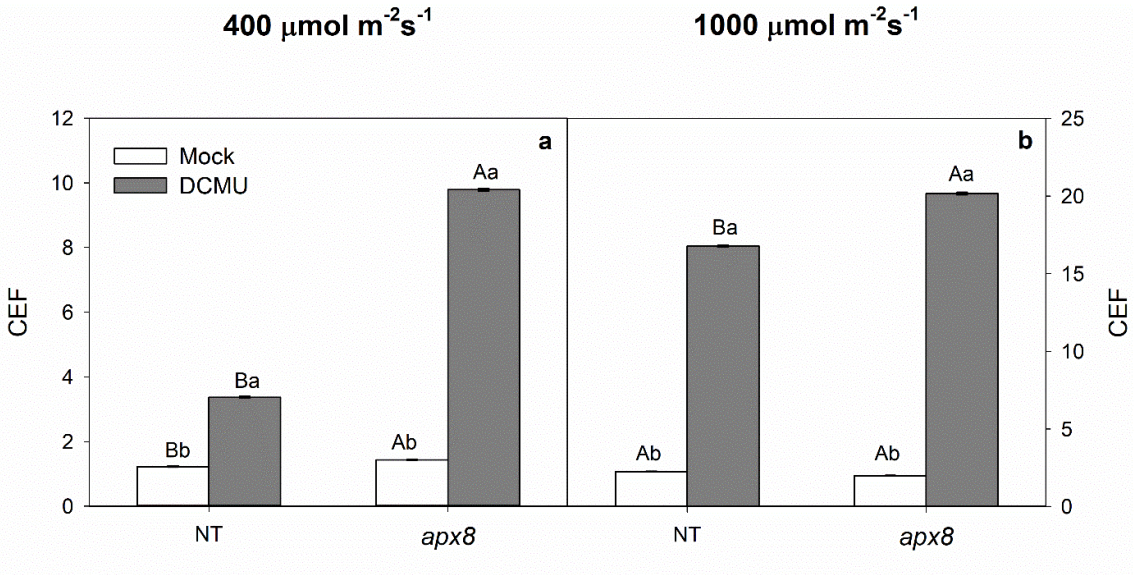


Figure 4. The estimation of the cyclic electron flow around PSI under moderate light (a) and high light (b) in NT and *apx8* rice plants. White bar represents the absence of DCMU and dark bars the vacuum infiltration of 100 μM DCMU in dark for 30 minutes. Data are the means of five replicates ± SD. Different capital letters represent significant differences between genotypes. Different lowercase letters represent significant differences between DCMU treatments for the same genotype by Tukey´s test ($P<0.05$).

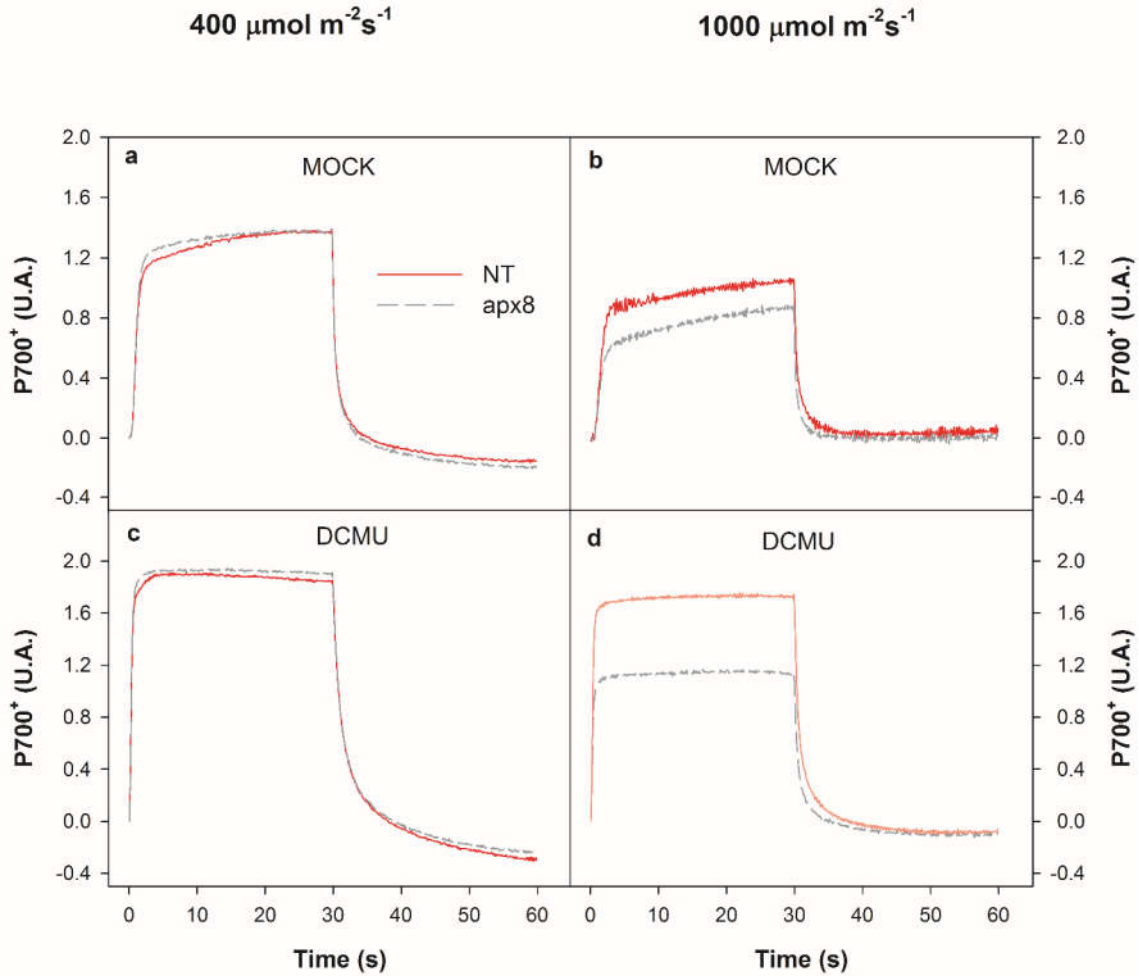


Figure 5. Normalized P700 kinetics at far-red light illumination recorded immediately after exposition of leaf in moderate light (left panel) or high light (right panel) in leaves of NT and *apx8* rice plants in absence (a,b) of presence of DCMU (c,d). Data are the means of thirty replicates. For more details see M&M section.

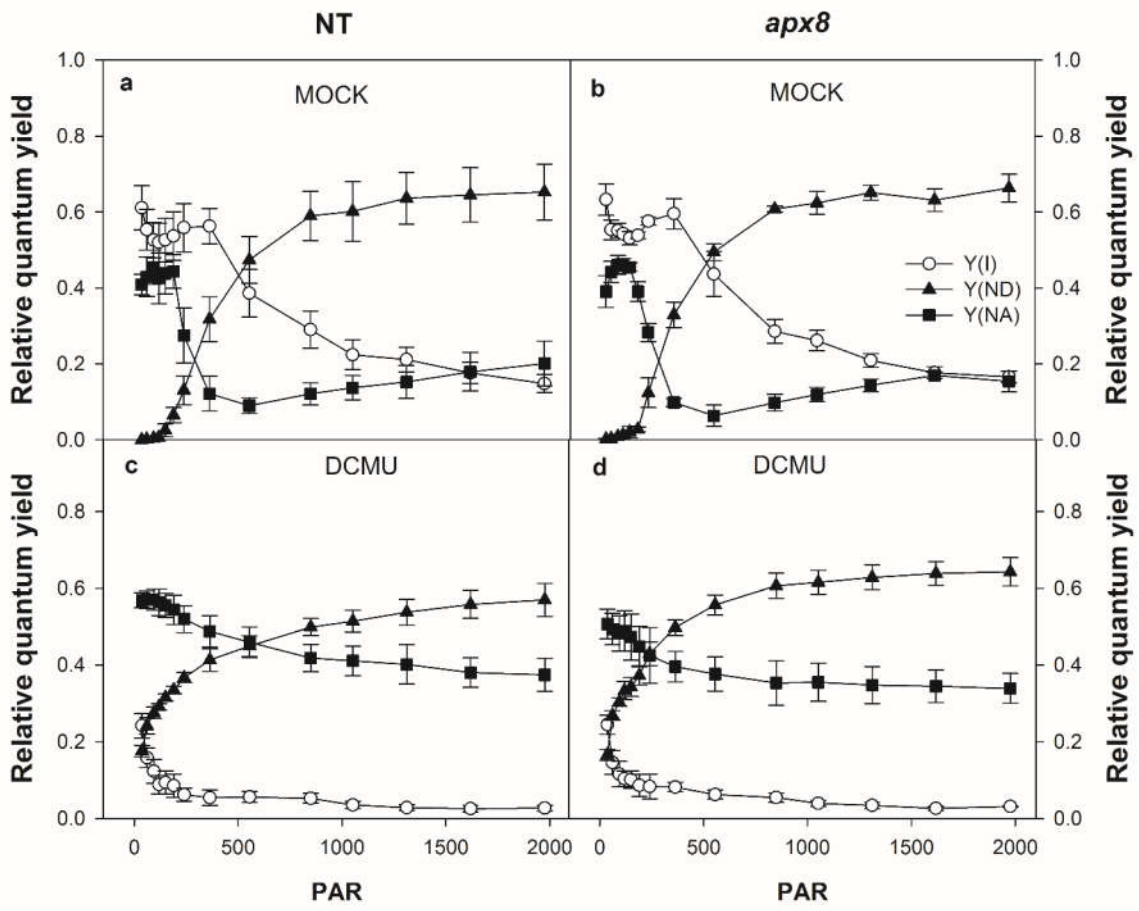


Figure 6. Rapid light responses of parameters related to PSI in NT (left panel) and *apx8* (right panel) in absence of presence of DCMU. Open circles represent the effective quantum yield of PSI, dark triangles the quantum yield of non-photochemical energy dissipation due to donor-side limitation of PSI and dark squares the quantum yield of non-photochemical energy dissipation due to acceptor-side limitation of PSI. Data are the means of five to ten replicates ± SD. The rapid light curves were obtained after previous induction at moderate light. The duration of each interval with a given light intensity was 30 s (for details see M&M).

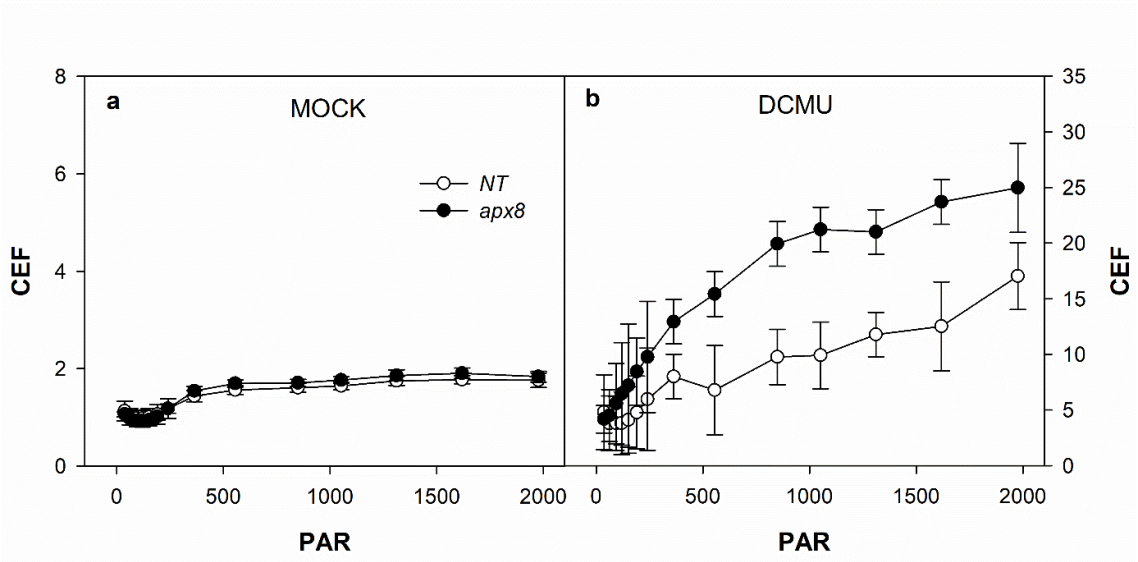


Figure 7. Rapid light responses of the estimated CEF in in NT (white circles) and *apx8* (dark circles) in absence (a) of presence of DCMU (b). Data are the means of five to ten replicates ± SD. The rapid light curves were obtained after previous induction at moderate light. The duration of each interval with a given light intensity was 30 s (for details see M&M).

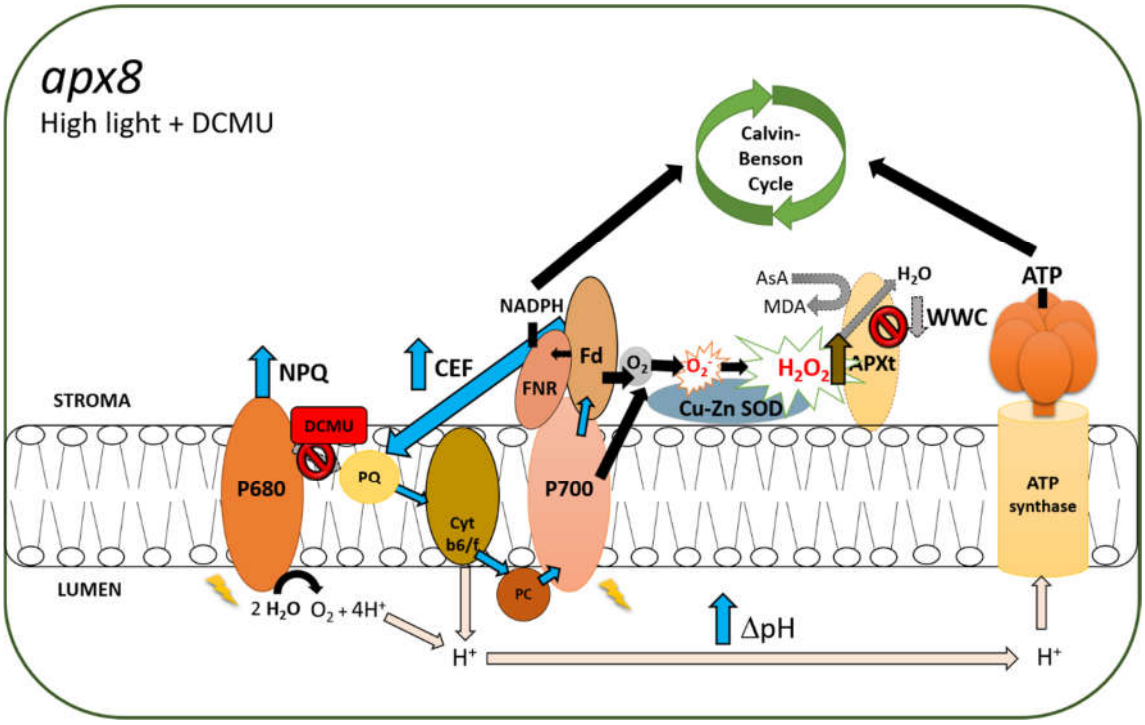
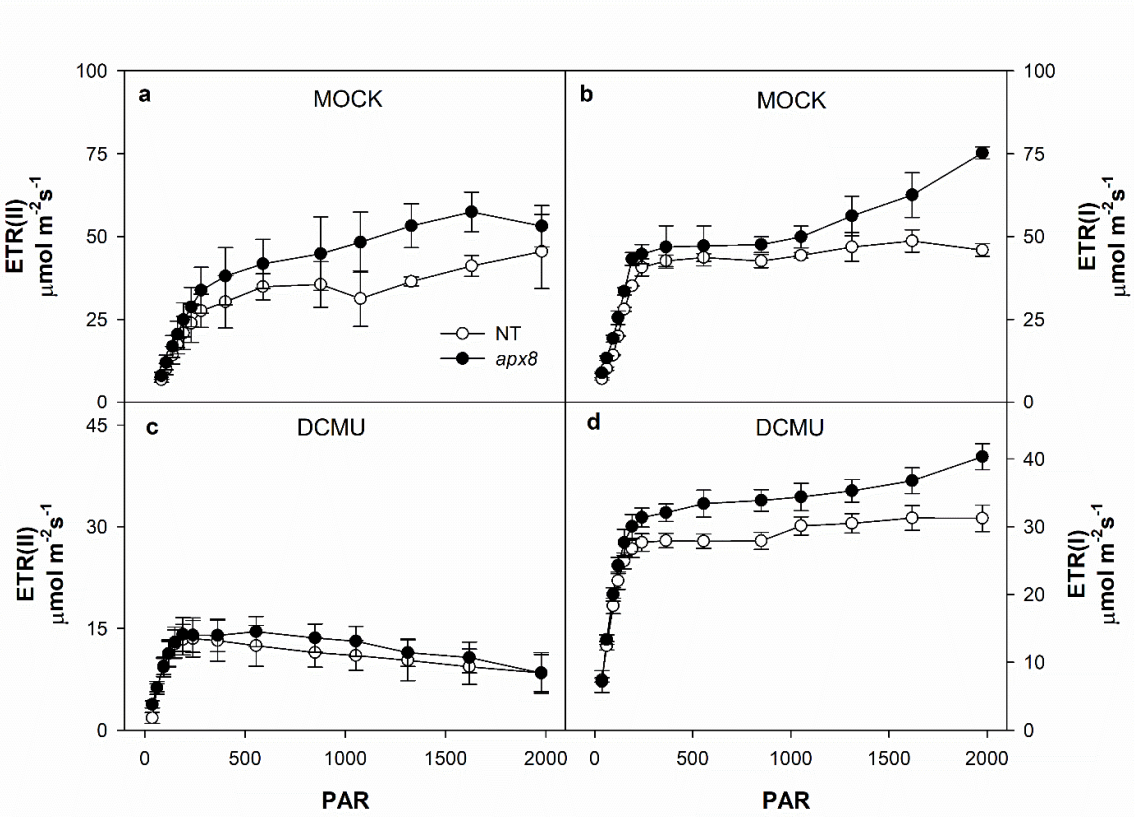


Figure 8. Schematic model summarizing the main mechanisms in *apx8* plants related to mitigation of PSI inhibition in presence of DCMU and high light. tAPX knockdown rice plants, with disturbing water-water cycle (WWC) and increased H_2O_2 content, triggers cyclic electron flow (CEF) as a compensatory mechanism to avoid PSI photoinhibition. As a consequence of DCMU exposure, *apx8* plants showed an increase in NPQ, which promote increases in ΔpH, dissipating excessive energy.



Supplementary figure 1. Rapid light responses of photosynthetic electron transport of PSII and PSI in NT (white circles) and *apx8* (dark circles) in absence of presence of DCMU. Data are the means of five to ten replicates ± SD. The rapid light curves were obtained after previous induction at moderate light. The duration of each interval with a given light intensity was 30 s (for details see M&M).

8 CONCLUSIONS

The results obtained in this work utilizing RNAi-silenced chlAPX plants showed that both chlAPXs isoforms has individual and specific role in chloroplast redox maintenance, however plant responses were depending on the intensity of silencing. Comparing different lines with different levels of transcripts may help to clarify this complex phenomenon. In the present study, differences in the transcript levels of the two isoforms were reflected in the activities of other isoforms in the APX family and in H_2O_2 accumulation levels. This change also leads to proteomic and physiological altered responses, especially in photosynthesis and mild drought-tolerance resistance. Possibly, such changes also affected other genetic and biochemical processes leading to impairment in plant growth for *apx8* plants. In a near future, gene silencing has the potential to become an effective biotechnological tool capable of modulate plant phenotype and induce plant resistance to different types of abiotic stresses.

Therefore, the data reported in the current doctoral thesis allow reaching the following main conclusions:

1. tAPX deficiency compromises the water-water cycle and reduces AsA-GSH cycle, triggering different ROS-scavenging compensatory mechanisms in *apx8* plants with only a small impairment of growth;
2. tAPX activity seems to be crucial to overcome oxidative stress induced by mild drought stress exposure. As already described by our group, tAPX activity is not essential when plants are exposed to intense drought stress. This seems to happen probably because of the high sensitivity of rice plants to drought stress;
3. Silencing of sAPX did not induced impairment in growth nor major alteration in proteomic profile of *apx7* plants when compared to NT plants;

4. sAPX silencing induced increased tolerance to drought stress trough maintenance of photosynthetic efficiency probably due to increasing drought-resistance pathways in rice plants;
5. tAPX RNAi-knockdown rice plants trigger photoprotective mechanisms (CEF and NPQ) to compensate water-water cycle impairment, especially under high light exposure to avoid PSI photoinhibition.

REFERENCES

AGRAWAL GK, RAKWAL R, JWA NS, AGRAWAL VP. Effects of signaling molecules, protein phosphatase inhibitors and blast pathogen (*Magnaporthe grisea*) on the mRNA level of a rice (*Oryza sativa* L.) phospholipid hydroperoxide glutathione peroxidase (*OsPHGPX*) gene in seedling leaves. **Gene** 283: 227–236, 2002.

ALRIC, J., JOHNSON, X. Alternative electron transport pathways in photosynthesis: a confluence of regulation. **Curr. Opin. Plant Biol.** 37, 78–86, 2017.

AMAKO K, CHEN G-X, ASADA K. Separate Assays Specific for Ascorbate Peroxidase and Guaiacol Peroxidase and for the Chloroplastic and Cytosolic Isozymes of Ascorbate Peroxidase in Plants. **Plant Cell Physiol** 35: 497–504, 1994.

APEL K, HIRT H. REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction. **Annu Rev Plant Biol** 55: 373–399, 2004.

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

ASADA, K. Production and scavenging of reactive oxygen species in chloroplasts and their functions. **Plant Physiol** 141: 391–396, 2006.

ASADA, K. The water-water cycle as alternative photon and electron sinks. **Philos. Trans. R. Soc. Lond. B. Biol. Sci.** 355, 1419–31, 2000.

AWAD J, STOTZ H, FEKETE A, KRISCHKE M, ENGERT C, HAVAUX M, BERGER S, MUELLER MJ. 2-Cys peroxiredoxins and thylakoid ascorbate peroxidase create a water-water cycle that is essential to protect the photosynthetic apparatus under high light stress conditions. **Plant Physiol** 167, 1592-603, 2015.

BAGARD, D. LE THIEC, E. DELACOTE, M.-P.P. HASENFRATZ-SAUDER, J. BANVOY, J. GÉRARD, P. DIZENGREMEL, Y. JOLIVET, Ozone-induced changes in photosynthesis and photorespiration of hybrid poplar in relation to the developmental stage of the leaves., **Physiol. Plant**. 134, 559–574, 2008.

BAKER, Chlorophyll fluorescence: A probe of photosynthesis *in vivo*, **Annu. Rev. Plant Biol.** 59, 89–113, 2008.

BENNETZEN J. THE RICE GENOME: Opening the Door to Comparative Plant Biology. **Science**, 296: 60–63, 2002.

BLUM A, EBERCON A. Cell Membrane Stability as a Measure of Drought and Heat Tolerance in Wheat. **Crop Sci** 21: 43–47, 1981.

BONDARAVA N, GROSS CM, MUBARAKSHINA M, GOLECKI JR, JOHNSON GN, KRIEGER-LISZKAY A. Putative function of cytochrome b559 as a plastoquinol oxidase. **Physiol Plant** 138: 463–473, 2010.

BONIFACIO, M.O. MARTINS, C.W. RIBEIRO, A. V. FONTENELE, F.E.L. CARVALHO, M. MARGIS-PINHEIRO, J.A.G. Silveira, Role of peroxidases in the compensation of

cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress, **Plant, Cell Environ**. 34 (2011).

BRADFORD MM. A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. **Analy** 254: 248–254, 1976.

BUCHANAN, BB. Regulation of CO_2 assimilation in oxygenic photosynthesis: The ferredoxin/thioredoxin system. **Arch Biochem Biophys** 288: 1–9, 1991.

BUCHANAN, BB. The carbon (formerly dark) reactions of photosynthesis. **Photosynth Res** 128: 215–217, 2016.

CAI, Y. F.F., Q.-Y.Y. YANG, S.-F.F. LI, J.-H.H. WANG, W. HUANG, The water-water cycle is a major electron sink in Camellia species when CO_2 assimilation is restricted, **J. Photochem. Photobiol. B Biol.** 168, 59–66, 2017.

CAKMAK, W.J. HORST, Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*), **Physiol. Plant**. 83, 463–468, 1991.

CAPELL T, BASSIE L, CHRISTOU P. Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. **Proc Natl Acad Sci U S A** 101: 9909–14, 2004.

CAPLAN JL, KUMAR AS, PARK E, PADMANABHAN MS, HOBAN K, MODLA S, CZYMMEK K, DINESH-KUMAR SP. Chloroplast stromules function during innate immunity. **Dev Cell** 34: 48–56, 2016.

CARVALHO FEL, RIBEIRO CW, MARTINS MO, BONIFACIO A, STAATS CC, ANDRADE CMB, CERQUEIRA J V., MARGIS-PINHEIRO M, SILVEIRA JAG. Cytosolic APX knockdown rice plants sustain photosynthesis by regulation of protein expression related to photochemistry, Calvin cycle and photorespiration. **Physiol Plant** 150: 632–645, 2014.

CASTEL SE, MARTIENSSEN RA. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. **Nat Rev Genet** 14: 100–112, 2013.

CAVERZAN A, BONIFACIO A, CARVALHO FEL, ANDRADE CMB, PASSAIA G, SCHÜNEMANN M, MARASCHIN F DOS S, MARTINS MO, TEIXEIRA FK, RAUBER R. The knockdown of chloroplastic ascorbate peroxidases reveals its regulatory role in the photosynthesis and protection under photo-oxidative stress in rice. **Plant Sci** 214: 74–87, 2014.

CHAE HZ, UHM TB, RHEE SG. Dimerization of thiol-specific antioxidant and the essential role of cysteine 47. **Proc Natl Acad Sci U S A,** 91: 7022–7026, 1994.

CHANG CCC, SLESAK I, JORDA L, SOTNIKOV A, MELZER M, MISZALSKI Z, MULLINEAUX PM, PARKER JE, KARPINSKA B, KARPINSKI S. Arabidopsis Chloroplastic Glutathione Peroxidases Play a Role in Cross Talk between Photooxidative Stress and Immune Responses. **Plant Physiol** 150: 670–683, 2009.

CHAUX, F., PELTIER, G., JOHNSON, X. A security network in PSI photoprotection: regulation of photosynthetic control, NPQ and O2 photoreduction by cyclic electron flow. **Front. Plant Sci.** 6, 1–7, 2015.

CHAVES MM, FLEXAS J, PINHEIRO C. Photosynthesis under drought and salt stress: Regulation mechanisms from whole plant to cell. **Ann Bot** 103: 551–560, 2009.

CHINTAKOVID N, MAIPOKA M, PHAONAKROP N, MICKELBART M V., ROYTRAKUL S, CHADCHAWAN S. Proteomic analysis of drought-responsive proteins in rice reveals photosynthesis-related adaptations to drought stress. **Acta Physiol Plant**. 2017.

CLEMENT M, LEONHARDT N, DROILLARD M-J, REITER I, MONTILLET J-L, GENTY B, LAURIERE C, NUSSAUME L, NOEL LD. The Cytosolic/Nuclear HSC70 and HSP90 Molecular Chaperones Are Important for Stomatal Closure and Modulate Abscisic Acid-Dependent Physiological Responses in Arabidopsis. **Plant Physiol** 156: 1481–1492, 2011.

D. TODAKA, K. SHINOZAKI, K. YAMAGUCHI-SHINOZAKI, Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants., **Front. Plant Sci.** 6, 84, 2015.

D'AUTRÉAUX B, TOLEDANO MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. **Nat Rev Mol Cell Biol** 8: 813–824, 2007.

DANNA, C.G. BARTOLI, F. SACCO, L.R. INGALA, G.E. SANTA-MARÍA, J.J. GUIAMET, R.A. UGALDE, Thylakoid-bound ascorbate peroxidase mutant exhibits impaired electron transport and photosynthetic activity, **Plant Physiol**. 132, 2116–2125, 2003.

DAT J, VANDENABEELE S, VRANOVÁ E, VAN MONTAGU M, INZÉ D, VAN BREUSEGEM F. Dual action of the active oxygen species during plant stress responses. **Cell Mol Life Sci** 57: 779–795, 2000.

DESIKAN R, A-H-MACKERNESS S, HANCOCK JT, NEILL SJ. Regulation of the Arabidopsis transcriptome by oxidative stress. **Plant Physiol** 127: 159–172, 2001.

DIETZ KJ, HELL R. Thiol switches in redox regulation of chloroplasts: Balancing redox state, metabolism and oxidative stress. **Biol Chem** 396: 483–494, 2015.

DIETZ KJ, JACOB S, OELZE ML, LAXA M, TOGNETTI V, DE MIRANDA SMN, BAIER M, FINKEMEIER I. The function of peroxiredoxins in plant organelle redox metabolism. **J Exp Bot** 57: 1697–1709, 2006.

DIETZ K-J, MITTLER R, NOCTOR G. Recent Progress in Understanding the Role of Reactive Oxygen Species in Plant Cell Signalling. **Plant Physiol** 171: 1535–1539, 2016.

DIETZ K-J, TURKAN I, KRIEGER-LISZKAY A. Redox- and reactive oxygen species-dependent signalling in and from the photosynthesizing chloroplast. **Plant Physiol** 171: 1541–1550, 2016.

DIETZ K-J. Thiol-Based Peroxidases and Ascorbate Peroxidases: Why Plants Rely on Multiple Peroxidase Systems in the Photosynthesizing Chloroplast? **Mol Cells** 39: 20–5, 2016.

DING X, RICHTER T, CHEN M, FUJII H, SEO YS, XIE M, ZHENG X, KANRAR S, STEVENSON RA, DARDICK C. A Rice Kinase-Protein Interaction Map. **Plant Physiol** 149: 1478–1492, 2009.

DRIEVER SM, BAKER NR. The water-water cycle in leaves is not a major alternative electron sink for dissipation of excess excitation energy when CO2 assimilation is restricted. **Plant, Cell Environ** 34: 837–846, 2011.

EXPOSITO-RODRIGUEZ M, LAISSUE PP, YVON-DUROCHER G, SMIRNOFF N, MULLINEAUX PM. Photosynthesis-dependent H_2O_2 transfer from chloroplasts to nuclei provides a high-light signalling mechanism. **Nat Commun** 8: 49, 2017.

FARMER EE, MUELLER MJ. ROS-Mediated Lipid Peroxidation and RES-Activated Signalling. **Annu Rev Plant Biol** 429–450, 2013.

FENG, Y. HAN, G. LIU, B. AN, J. YANG, G. YANG, Y. LI, Y. ZHU, Overexpression of sedoheptulose-1,7-bisphosphatase enhances photosynthesis and growth under salt stress in transgenic rice plants, **Funct. Plant Biol.** 34, 822–834, 2007.

FINAZZI, G., JOHNSON, G.N. Cyclic electron flow : facts and hypotheses. **Photosynth. Res.** 129, 227–230, 2016.

FISCHER BB, DAYER R, SCHWARZENBACH Y, LEMAIRE SD, BEHRA R, LIEDTKE A, EGGEN RIL. Function and regulation of the glutathione peroxidase homologous gene GPXH/GPX5 in Chlamydomonas reinhardtii. **Plant Mol Biol** 71: 569–583, 2009.

FISCHER BB, KRIEGER-LISZKAY A. Production, Detection, and Signalling of Singlet Oxygen. **Antioxid Redox Signal** 18: 2145–2162, 2013.

FLEXAS J, MEDRANO H. Drought-inhibition of photosynthesis in C3 plants: Stomatal and non-stomatal limitations revisited. **Ann Bot** 89: 183–189, 2002.

FLEXAS J, ORTUÑO MF, RIBAS-CARBO M, DIAZ-ESPEJO A, FLÓREZ-SARASA ID, MEDRANO H. Mesophyll conductance to CO2 in Arabidopsis thaliana. **New Phytol** 175: 501–511, 2007.

FLEXAS, J. BOTA, F. LORETO, G. CORNIC, T.D. SHARKEY, Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants, **Plant Biol.** 6, 269–279, 2004.

FLEXAS, M.F. ORTUÑO, M. RIBAS-CARBO, A. DIAZ-ESPEJO, I.D. FLÓREZ-SARASA, H. MEDRANO, Mesophyll conductance to CO_2 in *Arabidopsis thaliana*, **New Phytol.** 175, 501–511, 2007.

Food and Agriculture Organization of the United Nations - FAO. Available in < http://www.fao.org/faostat/en/#rankings/commodities_by_country>. Acession in 07.06.2018.

FOREMAN J, DEMIDCHIK V, BOTHWELL JHF, MYLONA P, MIEDEMA H, TORRES MA, LINSTEAD P, COSTA S, BROWNLEE C, JONES JDG. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. **Nature** 442–446, 2003.

FOYER CH, NOCTOR G. Oxidant and antioxidant signalling in plants: A re-evaluation of the concept of oxidative stress in a physiological context. **Plant, Cell Environ** 28: 1056–1071, 2005.

FOYER CH, NOCTOR G. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. **Physiol Plantarum**, 355–364, 2003.

FOYER CH, NOCTOR G. Stress-triggered redox signalling : what ' s in pROSpect ? **Plant, Cell Environ** 951–964, 2016.

FOYER CH, RUBAN A V, NOCTOR G. Viewing oxidative stress through the lens of oxidative signalling rather than damage. **Biochem J,** 877–883, 2017.

FOYER CH, SHIGEOKA S. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. **Plant Physiol** 155: 93–100, 2011.

FOYER, C.H., HALLIWELL, B. The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. **Planta** 133, 21–25, 1976.

FUCCI L, OLIVER CN, COON MJ, STADTMAN ER. Inactivation of key metabolic enzymes by mixed-function oxidation reactions: Possible implication in protein turnover and ageing. **Proc Natl Acad Sci** 80: 1521–1525, 1983.

GABER A, OGATA T, MARUTA T, YOSHIMURA K, TAMOI M, SHIGEOKA S. The involvement of *Arabidopsis* glutathione peroxidase 8 in the suppression of oxidative damage in the nucleus and cytosol. **Plant Cell Physiol** 53: 1596–1606, 2012.

GADJEV I, GADJEV I, VANDERAUWERA S, VANDERAUWERA S, GECHEV TS, GECHEV TS, LALOI C, LALOI C, MINKOV IN, MINKOV IN. Transcriptomic Footprints Disclose Specificity of Reactive Oxygen Species Signalling in *Arabidopsis*. **Society** 141: 436–445, 2006.

GALVEZ-VALDIVIESO G, MULLINEAUX PM. The role of reactive oxygen species in signalling from chloroplasts to the nucleus. **Physiol Plant** 138: 430–439, 2010.

GIACOMELLI L, MASI A, RIPOLL DR, LEE MJ, VAN WIJK KJ. *Arabidopsis thaliana* deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low. **Plant Mol Biol** 65: 627–644, 2007.

GILL SS, TUTEJA N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. **Plant Physiol Biochem** 48: 909–930, 2010.

GOH, C.-H., KO, S.-M., KOH, S., KIM, Y.-J., BAE, H.-J. Photosynthesis and Environments: Photoinhibition and Repair Mechanisms in Plants. **J. Plant Biol.** 93–101, 2011.

GUO Z, WANG F, XIANG X, AHAMMED GJ, WANG M, ONAC E, ZHOU J, XIA X, SHI K, YIN X. Systemic induction of photosynthesis via illumination of the shoot apex is mediated by phytochrome B. **Plant Physiol** 172, 1259-1272, 2016.

HALD, S., NANDHA, B., GALLOIS, P., JOHNSON, G.N. Feedback regulation of photosynthetic electron transport by NADP(H) redox poise. **Biochim. Biophys. Acta** 1777, 433–40, 2008.

HEBER, R. BLIGNY, P. STREB, R. DOUCE, Photorespiration is essential for the protection of the photosynthetic apparatus of C3 plants against photoinactivation under sunlight, **Bot. Acta**. 109, 307–315, 1996.

HENZLER T, STEUDLE E. Transport and metabolic degradation of hydrogen peroxide in Chara corallina: model calculations and measurements with the pressure probe suggest transport of H_2O_2 across water channels. **J Exp Bot** 51: 2053–2066, 2000.

HERBETTE S, ROECKEL-DREVET P, DREVET JR. Seleno-independent glutathione peroxidases: More than simple antioxidant scavengers. **FEBS J** 274: 2163–2180, 2007.

HERNANDEZ M, FERNANDEZ-GARCIA N, DIAZ-VIVANCOS P, OLMOS E. A different role for hydrogen peroxide and the antioxidative system under short and long salt stress in Brassica oleracea roots**. J Exp Bot** 61: 521–535, 2010.

HIGA T, SUETSUGU N, KONG S-G, WADA M. Actin-dependent plastid movement is required for motive force generation in directional nuclear movement in plants. **Proc Natl Acad Sci** 111: 4327–4331, 2014.

HOAGLAND, D.I. ARNON, The water-culture method for growing plants without soil, **Calif. Agric. Exp. Stn. Circ.** 347, 1–32, 1950.

HOSSAIN MA, BHATTACHARJEE S, ARMIN S-M, QIAN P, XIN W, LI H-Y, BURRITT DJ, FUJITA M, TRAN L-SP. Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging. **Front Plant Sci** 6: 1–19, 2015.

HUANG, W., TIKKANEN, M., ZHANG, S.-B. Photoinhibition of photosystem I in Nephrolepis falciformis depends on reactive oxygen species generated in the chloroplast stroma. **Photosynth. Res.** 137, 129–140, 2018.

ISHIDA H, NISHIMORI Y, SUGISAWA M, MAKINO A, MAE T. The Large Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase is Fragmented into 37-kDa and 16-kDa Polypeptides by Active Oxygen in the Lysates of Chloroplasts from Primary Leaves of Wheat. **Plant Cell Physiol** 38: 471–479, 1997.

ISLAM T, MANNA M, KAUL T, PANDEY S, REDDY CS, REDDY MK. Genome-Wide Dissection of Arabidopsis and Rice for the Identification and Expression Analysis of Glutathione Peroxidases Reveals Their Stress-Specific and Overlapping Response Patterns. **Plant Mol Biol Report** 33: 1413–1427, 2015.

JACOB P, HIRT H, BENDAHMANE A. The heat-shock protein/chaperone network and multiple stress resistance. **Plant Biotechnol J** 15: 405–414, 2017.

JARDIM-MESSEDER D, CAVERZAN A, RAUBER R, CUNHA JR, CARVALHO FEL, GAETA ML, DA FONSECA GC, COSTA JM, FREI M, SILVEIRA JAG, MARGIS-PINHEIRO, M. Thylakoidal APX modulates hydrogen peroxide content and stomatal closure in rice (*Oryza sativa* L.). **Environ Exp Bot** 150: 46–56, 2018.

JENSEN ON. Interpreting the protein language using proteomics. **Nat Rev Mol Cell Biol** 7: 391–403, 2006.

JI K, WANG Y, SUN W, LOU Q, MEI H, SHEN S, CHEN H. Drought-responsive mechanisms in rice genotypes with contrasting drought tolerance during reproductive stage. **J Plant Physiol** 169: 336–344, 2012.

JIMBO, R. YUTTHANASIRIKUL, T. NAGANO, T. HISABORI, Y. HIHARA, Y. NISHIYAMA, Oxidation of translation factor EF-Tu inhibits the repair of photosystem II, Plant Physiol. 176, 2018.

JOHNSON, G.N. Cyclic electron transport in C3 plants: fact or artefact? **J. Exp. Bot.** 56, 407–16, 2005.

JOHNSON, G.N., STEPIEN, P. Plastid Terminal Oxidase as a route to improving plant stress tolerance - Known Knowns and Known Unknowns. **Plant Cell Physiol.** 57, 1387–1396, 2016.

JOLIOT, P., JOLIOT, A. Cyclic electron flow in C3 plants. **Biochim. Biophys. Acta** 1757, 362–8, 2006.

JOLIOT, P., JOLIOT, A. Quantification of cyclic and linear flows in plants. **Proc. Natl. Acad. Sci. U. S. A.** 102, 4913–8, 2005.

K.J. LIVAK, T.D. SCHMITTGEN, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods. 25, 402–408, 2001.

KANG S-G, JEONG HK, SUH HS. Characterization of a New Member of the Glutathione Peroxidase Gene Family in Oryza sativa. **Mol Cells** 17: 23–28, 2003.

KANGASJÄRVI S, LEPISTÖ A, HÄNNIKÄINEN K, PIIPPO M, LUOMALA E-MM, ARO E-MM, RINTAMÄKI E, KANGASJARVI S, LEPISTO A, HANNIKAINEN K. Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses. **Biochem J** 412: 275–85, 2008.

KARPINSKI S. Systemic Signalling and Acclimation in Response to Excess Excitation Energy in Arabidopsis. **Science** 284: 654–657, 1999.

KATO Y, KAMOSHITA A, YAMAGISHI J. Evaluating the resistance of six rice cultivars to drought: Restriction of deep rooting and the use of raised beds. **Plant Soil** 300: 149–161, 2007.

KIM Y-J, JANG M-G, NOH H-Y, LEE H-J, SUKWEENADHI J, KIM J-H, KIM S-Y, KWON W-S, YANG D-C. Molecular characterization of two glutathione peroxidase genes of Panax ginseng and their expression analysis against environmental stresses. **Gene** 535: 33–41, 2014.

KITAJIMA, Hydrogen peroxide-mediated inactivation of two chloroplastic peroxidases, ascorbate peroxidase and 2-Cys peroxiredoxin, **Photochem. Photobiol**. 84, 1404–1409, 2008.

KLUGHAMMER C, SCHREIBER U. An improved method, using saturating light pulses, for the determination of photosystem I quantum yield via P700+-absorbance changes at 830 nm. **Planta.** 1994.

KOJIMA K, OSHITA M, NANJO Y, KASAI K, TOZAWA Y, HAYASHI H, NISHIYAMA Y. Oxidation of elongation factor G inhibits the synthesis of the D1 protein of photosystem II. **Mol Microbiol** 65: 936–947, 2007.

KOMATSU S, NANJOA Y, NISHIMURAB M. Proteomic analysis of the flooding tolerance mechanism in mutant soybean. J Proteomics 79: 231–250, 2013.

KÖNIG J, GALLIARDT H, JÜTTE P, SCHÄPER S, DITTMANN L, DIETZ KJ. The conformational bases for the two functionalities of 2-cysteine peroxiredoxins as peroxidase and chaperone. **J Exp Bot** 64: 3483–3497, 2013.

KONO M, NOGUCHI K, TERASHIMA I. Roles of the cyclic electron flow around PSI (CEF-PSI) and O_2-dependent alternative pathways in regulation of the photosynthetic electron

flow in short-term fluctuating light in *Arabidopsis thaliana*. **Plant Cell Physiol** 55: 990–1004, 2014.

KONO, M., TERASHIMA, I. Elucidation of Photoprotective Mechanisms of PSI Against Fluctuating Light photoinhibition. **Plant Cell Physiol**. 57, 2016.

KONO, M., TERASHIMA, I. Long-term and short-term responses of the photosynthetic electron transport to fluctuating light. **J. Photochem. Photobiol. B.** 137, 89–99, 2014.

LABS, M., RUHLE, T., LEISTER, D. The antimycin A-sensitive pathway of cyclic electron flow: from 1963 to 2015. **Photosynth. Res.** 1–8, 2016.

LAD L, MEWIES M, BASRAN J, SCRUTTON NS, RAVEN EL. Role of histidine 42 in ascorbate peroxidase: Kinetic analysis of the H42A and H42E variants. **Eur J Biochem** 269: 3182–3192, 2002.

LALOI C, HAVAUX M. Key players of singlet oxygen-induced cell death in plants. **Front Plant Sci** 6: 1–9, 2015.

LENKA SK, KATIYAR A, CHINNUSAMY V, BANSAL KC. Comparative analysis of drought-responsive transcriptome in Indica rice genotypes with contrasting drought tolerance**.** **Plant Biotechnol** J 9: 315–327, 2011.

LIEBTHAL M, MAYNARD D, DIETZ K-J. Peroxiredoxins and Redox Signalling in Plants. **Antioxid Redox Signal** 28, 2017.

LIMA NETO, M.C., A.K.M. LOBO, M.O. MARTINS, A.V. FONTENELE, J.A.G. SILVEIRA, Dissipation of excess photosynthetic energy contributes to salinity tolerance: A comparative study of salt-tolerant *Ricinus communis* and salt-sensitive *Jatropha curcas*, **J. Plant Physiol.** 171, 23–30, 2014.

LIMA NETO, M.C., CERQUEIRA, J.V.A., CUNHA, J.R., RIBEIRO, R.V., SILVEIRA, J.A.G. Cyclic electron flow, NPQ and photorespiration are crucial for the establishment of young plants of Ricinus communis and Jatropha curcas exposed to drought. **Plant Biol.** 19, 650–659, 2017.

LÓPEZ-CALCAGNO, T.P. HOWARD, C.A. RAINES, The CP12 protein family: a thioredoxin-mediated metabolic switch? **Front. Plant Sci.** 5, 9, 2014.

M. BAGARD, D. LE THIEC, E. DELACOTE, M.P. HASENFRATZ-SAUDER, J. BANVOY, J. GÉRARD, P. DIZENGREMEL, Y. JOLIVET (2008) Ozone-induced changes in photosynthesis and photorespiration of hybrid poplar in relation to the developmental stage of the leaves, Physiol. Plant. 134: 559–574, 2008.

M.J. GARCÍA-MURRIA, H.P.K. SUDHANI, J. MARÍN-NAVARRO, M.M. SÁNCHEZ DEL PINO, J. MORENO, Dissecting the individual contribution of conserved cysteines to the redox regulation of RubisCO, **Photosynth. Res.** 137, 251–262, 2018.

MAKINO, A., MIYAKE, C., YOKOTA, A. Physiological Functions of the Water–Water Cycle (Mehler Reaction) and the Cyclic Electron Flow around PSI in Rice Leaves. **Plant Cell Physiol.** 43, 1017–1026, 2002.

MANO, J., USHIMARU, T., ASADA, K. Ascorbate in thylakoid lumen as an endogenous electron donor to Photosystem II : Protection of thylakoids from photoinhibition and

regeneration of ascorbate in stroma by dehydroascorbate reductase. **Photosynthesis Research**, 197–204, 1997.

MARGIS R, DUNAND C, TEIXEIRA FK, MARGIS-PINHEIRO M. Glutathione peroxidase family - An evolutionary overview. **FEBS J** 275: 3959–3970, 2008.

MARUTA T, NOSHI M, TANOUCHI A, TAMOI M, YABUTA Y, YOSHIMURA K, ISHIKAWA T, SHIGEOKA S. H_2O_2-triggered Retrograde Signaling from Chloroplasts to Nucleus Plays Specific Role in Response to Stress. **J Biol Chem** 287: 11717–11729, 2012.

MARUTA T, SAWA Y, SHIGEOKA S, ISHIKAWA T. Diversity and evolution of ascorbate peroxidase functions in chloroplasts: More than just a classical antioxidant enzyme? **Plant Cell Physiol** 57: 1377–1386, 2016.

MARUTA T, TANOUCHI A, TAMOI M, YABUTA Y, YOSHIMURA K, ISHIKAWA T, SHIGEOKA S. *Arabidopsis* chloroplastic ascorbate peroxidase isoenzymes play a dual role in photoprotection and gene regulation under photooxidative stress. **Plant Cell Physiol** 51: 190–200, 2010.

MARUTA, Y. SAWA, S. SHIGEOKA, T. ISHIKAWA, Diversity and evolution of ascorbate peroxidase functions in chloroplasts: More than just a classical antioxidant enzyme? **Plant Cell Physiol**. 57, 1377–1386, 2016.

MAXWELL, K., JOHNSON, G.N. Chlorophyll fluorescence--a practical guide. **J. Exp. Bot.** 51, 659–68, 2000.

MIAO Y, LV D, WANG P, WANG X-C, CHEN J, MIAO C, SONG C-P. An *Arabidopsis* Glutathione Peroxidase Functions as Both a Redox Transducer and a Scavenger in Abscisic Acid and Drought Stress Responses. **Plant Cell Online** 18: 2749–2766, 2006.

MICHELET L, ZAFFAGNINI M, MORISSE S, SPARLA F, PÉREZ-PÉREZ ME, FRANCIA F, DANON A, MARCHAND CH, FERMANI S, TROST P. Redox regulation of the Calvin-Benson cycle: something old, something new. **Front Plant Sci** 4: 470, 2013.

MIGNOLET-SPRUYT L, XU E, IDÄNHEIMO N, HOEBERICHTS FA, MÜHLENBOCK P. Spreading the news: subcellular and organellar reactive oxygen species production and signalling. **J Exp Bot** 67: 3831–3844, 2017.

MIKI D, SHIMAMOTO K. Simple RNAi vectors for stable and transient suppression of gene function in rice. **Plant Cell Physiol** 45: 490–495, 2004.

MIKI, K. SHIMAMOTO, Simple RNAi vectors for stable and transient suppression of gene function in rice, **Plant Cell Physiol**. 45, 490–495, 2004.

MILLER G, SUZUKI N, CIFTCI-YILMAZ S, MITTLER R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. **Plant, Cell Environ** 33: 453–467, 2010.

MILLER G, SUZUKI N, RIZHSKY L, HEGIE A, KOUSSEVITZKY S, MITTLER R. Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. **Plant Physiol** 144: 1777–1785, 2007.

MITTLER R, VANDERAUWERA S, GOLLERY M, VAN BREUSEGEM F. Reactive oxygen gene network of plants. **Trends Plant Sci** 9: 490–498, 2004.

MITTLER R, VANDERAUWERA S, SUZUKI N, MILLER G, TOGNETTI VB, VANDEPOELE K, GOLLERY M, SHULAEV V, VAN BREUSEGEM F. ROS signaling: The new wave? **Trends Plant Sci** 16: 300–309, 2011.

MITTLER R. Oxidative stress, antioxidants and stress tolerance. **Trends Plant Sci** 7: 405–410, 2002.

MITTLER R. ROS Are Good. **Trends Plant Sci** 22: 11–19, 2016.

MIYAKE C, ASADA K. Inactivation Mechanism of Ascorbate Peroxidase at Low Concentrations of Ascorbate; Hydrogen Peroxide Decomposes Compound I of Ascorbate Peroxidase. **Plant Cell Physiol** 37: 423–430, 1996.

MIYAKE C, ASADA K. Thylakoid-Bound Ascorbate Peroxidase in Spinach Chloroplasts and Photoreduction of Its Primary Oxidation Product Monodehydroascorbate Radicals in Thylakoids. **Measurement** 33: 541–553, 1992.

MIYAKE C. Alternative electron flows (water-water cycle and cyclic electron flow around PSI) in photosynthesis: Molecular mechanisms and physiological functions. **Plant Cell Physiol** 51: 1951–63, 2010.

MIYAKE, K. ASADA, Inactivation mechanism of ascorbate peroxidase at low concentrations of ascorbate; hydrogen peroxide decomposes compound I of ascorbate peroxidase, **Plant Cell Physiol**. 37, 423–430, 1996.

MØLLER IM, JENSEN PE, HANSSON A. Oxidative Modifications to Cellular Components in Plants. **Annu Rev Plant Biol**. 459–483, 2007.

MØLLER IM, SWEETLOVE LJ. ROS signalling - specificity is required**. Trends Plant Sci** 15: 370–374, 2010.

MOUSTAFA K, ABUQAMAR S, JARRAR M, AL-RAJAB AJ, TRÉMOUILLAUX-GUILLER J. MAPK cascades and major abiotic stresses. **Plant Cell Rep** 33: 1217–1225, 2014.

MUBARAKSHINA MM, IVANOV BN, NAYDOV IA, HILLIER W, BADGER MR, KRIEGER-LISZKAY A. Production and diffusion of chloroplastic H_2O_2 and its implication to signalling. **J Exp Bot** 61: 3577–3587, 2010.

MULLER K, LINKIES A, VREEBURG RAM, FRY SC, KRIEGER-LISZKAY A, LEUBNER-METZGER G. In Vivo Cell Wall Loosening by Hydroxyl Radicals during Cress Seed Germination and Elongation Growth. **Plant Physiol** 150: 1855–1865, 2009.

MULLINEAUX PM, BAKER NR. Oxidative stress: antagonistic signaling for acclimation or cell death? **Plant Physiol** 154: 521–525, 2010.

MULLINEAUX, Spatial dependence for hydrogen peroxide-directed signaling in light-stressed plants, **Plant Physiol**. 141, 346–350, 2006.

MUNEKAGE, Y., HOJO, M., MEURER, J., ENDO, T., TASAKA, M., SHIKANAI, T., PGR5 Is Involved in Cyclic Electron Flow around Photosystem I and Is Essential for

Photoprotection in Arabidopsis. **Cell** 110, 361–371, 2002.

MUNNÉ-BOSCH, S.; QUEVAL, G.; FOYER CH, MUNNE-BOSCH S, QUEVAL G, FOYER CH. The Impact of Global Change Factors on Redox Signaling Underpinning Stress Tolerance. **Plant Physiol** 161: 5–19, 2013.

MURGIA, D. TARANTINO, C. VANNINI, M. BRACALE, S. CARRAVIERI, C. SOAVE, *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death, **Plant J**. 38, 940–953, 2004.

NAKANO, Y., ASADA, K. Hydrogen peroxide is scavenged by ascorbato specific peroxidase in spinach chloroplasts. **Plant Cell Physiol** 22, 867–880, 1981.

NARANJO, A. DIAZ-ESPEJO, M. LINDAHL, F.J. CEJUDO, Type-f thioredoxins have a role in the short-term activation of carbon metabolism and their loss affects growth under short-day conditions in *Arabidopsis thaliana*, **J. Exp. Bot**. 67, 1951–1964, 2016.

NAVROT N, COLLIN V, GUALBERTO J, GELHAYE E, HIRASAWA M, REY P, KNAFF DB, ISSAKIDIS E, JACQUOT J-P, ROUHIER N. Plant Glutathione Peroxidases Are Functional Peroxiredoxins Distributed in Several Subcellular Compartments and Regulated during Biotic and Abiotic Stresses**. Plant Physiol** 142: 1364–1379, 2006.

NEILL S, DESIKAN R, HANCOCK J. Hydrogen peroxide signalling. **Curr Opin Plant Biol** 5: 388–395, 2002.

NISHIKAWA, Y., YAMAMOTO, H., OKEGAWA, Y., WADA, S., SATO, N., TAIRA, Y., SUGIMOTO, K., MAKINO, A., SHIKANAI, T. PGR5-dependent cyclic electron transport around PSI contributes to the redox homeostasis in chloroplasts rather than CO_2 fixation and biomass production in rice. **Plant Cell Physiol**. 53, 2117–2126, 2012.

NISHIYAMA Y, ALLAKHVERDIEV SI, MURATA N. Protein synthesis is the primary target of reactive oxygen species in the photoinhibition of photosystem II. **Physiol Plant** 142: 35–46, 2011.

NISHIYAMA, Y, ALLAKHVERDIEV, SI, MURATA, N. A new paradigm for the action of reactive oxygen species in the photoinhibition of photosystem II. **Biochimica et Biophysica Acta - Bioenergetics** 7: 742-749, 2006.

NISHIYAMA, Y., ALLAKHVERDIEV, S.I., MURATA, N. Protein synthesis is the primary target of reactive oxygen species in the photoinhibition of photosystem II. **Physiol. Plant.** 142, 35–46, 2011.

NOCTOR G, FOYER CH. Intracellular Redox Compartmentation and ROS-Related Communication in Regulation and Signalling. **Plant Physiol.** 171: 1581–1592, 2016.

NOCTOR G, REICHHELD J-P, FOYER CH. ROS-related redox regulation and signalling in plants. **Semin Cell Dev Biol**, 80: 3-12, 2017.

PANDEY S, FARTYAL D, AGARWAL A, SHUKLA T, JAMES D, KAUL T, NEGI YK, ARORA S, REDDY MK. Abiotic Stress Tolerance in Plants: Myriad Roles of Ascorbate Peroxidase. **Front Plant Sci** 8: 1–13, 2017.

PASSAIA G, CAVERZAN A, FONINI LS, CARVALHO FEL, SILVEIRA JAG, MARGIS-PINHEIRO M. Chloroplastic and mitochondrial GPX genes play a critical role in rice development. **Biol Plant** 58: 375–378, 2014.

PASSAIA G, MARGIS-PINHEIRO M. Glutathione peroxidases as redox sensor proteins in plant cells**. Plant Sci** 234: 22–26, 2015.

PASSAIA G, SPAGNOLO FONINI L, CAVERZAN A, JARDIM-MESSEDER D, CHRISTOFF AP, GAETA ML, DE ARAUJO MARIATH JE, MARGIS R, MARGIS-PINHEIRO M. The mitochondrial glutathione peroxidase GPX3 is essential for H_2O_2 homeostasis and root and shoot development in rice. **Plant Sci** 208: 93–101, 2013.

PÉREZ-RUIZ JM, CEJUDO FJ. A proposed reaction mechanism for rice NADPH thioredoxin reductase C, an enzyme with protein disulfide reductase activity. **FEBS Lett** 583: 1399–1402, 2009.

PEREZ-RUIZ JM, SPÍNOLA MC, KIRCHSTEIGER K, MORENO J, SAHRAWY M, CEJUDO. Rice NTRC Is a High-Efficiency Redox System for Chloroplast Protection against Oxidative Damage. **Plant Cell Online** 18: 2356–2368, 2006.

PINHEIRO, M.M. CHAVES, Photosynthesis and drought: Can we make metabolic connections from available data?, **J. Exp. Bot.** 62, 869–882, 2011.

POPESCU S, POPESCU G, BACHAN S, ZHANG Z, GERSTEIN M, SNYDER M, DINESH-KUMAR SP. MAPK target networks in Arabidopsis thaliana revealed using functional protein microarrays. **Genes Dev** 23: 80–92, 2009.

PULIDO, E. LLAMAS, M. RODRIGUEZ-CONCEPCION, Both Hsp70 chaperone and Clp protease plastidial systems are required for protection against oxidative stress, Plant Signal. Behav. 12, 1–4, 2017.

QUAN LJ, ZHANG B, SHI WW, LI HY. Hydrogen peroxide in plants: A versatile molecule of the reactive oxygen species network. **J Integr Plant Biol** 50: 2–18, 2008.

RAO KUPR, RICHA TA, KUMAR KU, RAGHURAM BA, SINHA ALOKKR. In Silico Analysis Reveals 75 Members of Mitogen-Activated Protein Kinase Kinase Kinase Gene Family in Rice. **DNA Res.** 139–153, 2010.

RECZEK CR, CHANDEL NS. ROS-dependent signal transduction. **Curr Opin Cell Biol** 33: 8–13, 2015.

RODRIGUEZ MILLA MA, MAURER A, HUETE AR, GUSTAFSON JP. Glutathione peroxidase genes in *Arabidopsis* are ubiquitous and regulated by abiotic stresses through diverse signalling pathways. **Plant J** 36: 602–615, 2003.

ROSA SB, CAVERZAN A, TEIXEIRA FK, LAZZAROTTO F, SILVEIRA JAG, FERREIRA-SILVA SL, ABREU-NETO J, MARGIS R, MARGIS-PINHEIRO M. Cytosolic APx knockdown indicates an ambiguous redox responses in rice. **Phytochemistry** 71: 548–558, 2010.

ROSENZWEIG C, ELLIOTT J, DERYNG D, RUANE AC, MÜLLER C, ARNETH A, BOOTE KJ, FOLBERTH C, GLOTTER M, KHABAROV N. Assessing agricultural risks of climate change in the 21st century in a global gridded crop model intercomparison. **Proc Natl Acad Sci** 111: 3268–3273, 2014.

ROUHIER N, JACQUOT J. The plant multigenic family of thiol peroxidases. **Free Radic Med** 1413–1421, 2005.

ROUHIER N, JACQUOT JP. Plant peroxiredoxins: Alternative hydroperoxide scavenging enzymes. **Photosynth Res** 74: 259–268, 2002.

RUBAN A V, JOHNSON MP, DUFFY CDP. The photoprotective molecular switch in the photosystem II antenna. **BBA - Bioenerg** 1817: 167–181, 2012.

RUBAN, A. V. Quantifying the efficiency of photoprotection. **Philos. Trans. R. Soc. Lond. B. Biol.** Sci. 372, 201, 2017.

SÁ, JCM, LAL, R, CERRI, CC, LORENTZ, K, HUNGRIA, M, CARVALHO, PCF. Low-carbon agriculture in South America to mitigate global climate change and advance food security. **Environmental International**, 90: 102-112, 2017.

SAKSCHEWSKI B, VON BLOH W, HUBER V, MÜLLER C, BONDEAU A. Feeding 10 billion people under climate change: How large is the production gap of current agricultural systems? **Ecol Modell** 288: 103–111, 2014.

SATO Y, YOKOYA S. Enhanced tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, sHSP17.7. **Plant Cell Rep** 27: 329–334, 2008.

SAXENA I, SRIKANTH S, CHEN Z. Cross Talk between H_2O_2 and Interacting Signal Molecules under Plant Stress Response. **Front Plant Sci** 7: 1–16, 2016.

SCHEIBE, R., DIETZ, K.J. Reduction-oxidation network for flexible adjustment of cellular metabolism in photoautotrophic cells. **Plant. Cell Environ**. 35, 202–216, 2012.

SCHMITTGEN, K.J. LIVAK, Analyzing real-time PCR data by the comparative CT method, **Nat. Protoc**. 3, 1101–1108, 2008.

SEJIMA, T., TAKAGI, D., FUKAYAMA, H., MAKINO, A., MIYAKE, C. Repetitive short-pulse light mainly inactivates photosystem i in sunflower leaves. **Plant Cell Physiol.** 55, 1184–1193, 2014.

SELGA T, SELGA M, GOBINS V, OZOLINA A. Plastid-nuclear complexes: permanent structures in photosynthesizing of vascular plants. **Environ Exp Biol** 8: 85–92, 2010.

SELVARAJ MG, ISHIZAKI T, VALENCIA M, OGAWA S, DEDICOVA B, OGATA T, YOSHIWARA K, MARUYAMA K, KUSANO M, SAITO K. Overexpression of an Arabidopsis thaliana galactinol synthase gene improves drought tolerance in transgenic rice and increased grain yield in the field. **Plant Biotechnol J** 15: 1465–1477, 2017.

SERRATO AJ, PÉREZ-RUIZ JM, SPÍNOLA MC, CEJUDO FJ. A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in Arabidopsis thaliana. **J Biol Chem** 279: 43821–43827, 2004.

SHAN, S. ZHANG, X. OU, The roles of H_2S and H_2O_2 in regulating AsA-GSH cycle in the leaves of wheat seedlings under drought stress, **Protoplasma**, 2018.

SHARKEY TD, BERNACCHI CJ, FARQUHAR GD, SINGSAAS EL. Fitting photosynthetic carbon dioxide response curves for C3 leaves. **Plant, Cell Environ** 30: 1035–1040, 2007.

SHI LX, THEG SM. The chloroplast protein import system: From algae to trees. **Biochim Biophys Acta - Mol Cell Res** 1833: 314–331, 2013.

SHIGEOKA S, ISHIKAWA T, TAMOI M, MIYAGAWA Y, TAKEDA T, YABUTA Y, YOSHIMURA K. Regulation and function of ascorbate peroxidase isoenzymes. **J Exp Bot** 53: 1305–1319, 2002.

SHIGEOKA S, MARUTA T. Cellular redox regulation, signaling, and stress response in plants. **Biosci Biotechnol Biochem** 78: 1457–1470, 2014.

SHIGEOKA, T. ISHIKAWA, M. TAMOI, Y. MIYAGAWA, T. TAKEDA, Y. YABUTA, K. YOSHIMURA, Regulation and function of ascorbate peroxidase isoenzymes., **J. Exp. Bot.** 53. 1305–1319, 2002.

SHU L, LOU Q, MA C, DING W, ZHOU J, WU J, FENG F, LU X, LUO L, XU G. Genetic, proteomic and metabolic analysis of the regulation of energy storage in rice seedlings in response to drought. **Proteomics** 11: 4122–4138, 2012.

SIES H, BERNDT C, JONES DP. Oxidative Stress. **Annu Rev Biochem** 715–48, 2017.

SILVEIRA JAG, ARAÚJO SAM, LIMA JPMS, VIÉGAS RA. Roots and leaves display contrasting osmotic adjustment mechanisms in response to NaCl-salinity in Atriplex nummularia. **Environ Exp Bot** 66: 1–8, 2009.

SILVEIRA JAG, CARVALHO FEL. Proteomics, photosynthesis and salt resistance in crops: An integrative view. **J Proteomics** 143: 24–35, 2016.

SILVEIRA, S.A.M. ARAÚJO, J.P.M.S. LIMA, R.A. VIÉGAS, Roots and leaves display contrasting osmotic adjustment mechanisms in response to NaCl-salinity in *Atriplex nummularia*, **Environ. Exp. Bot**. 66, 1–8, 2009.

SINGH R, JWA NS. The rice MAPKK-MAPK interactome: The biological significance of MAPK components in hormone signal transduction. **Plant Cell Rep** 32: 923–931, 2013.

SINGH R, LEE M-O, LEE J-E, CHOI J, PARK JH, KIM EH, YOO RH, CHO J-I, JEON J-S, RAKWAL R. Rice Mitogen-Activated Protein Kinase Interactome Analysis Using the Yeast Two-Hybrid System. **Plant Physiol** 160: 477–487, 2012.

SLESAK I, LIBIK M, KARPINSKA B, KARPINSKI S, MISZALSKI Z. The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. **Acta Biochim Pol** 54: 39–50, 2007.

SMIRNOFF N. The Function and Metabolism of Ascorbic Acid in Plants. **Ann Bot** 78: 661–669, 1996,

SONOIKE K. Photoinhibition of photosystem I: Its physiological significance in the chilling sensitivity of plants. **Plant Cell Physiol** 37: 239–247, 1996.

SOUSA RH V, CARVALHO FEL, RIBEIRO CW, PASSAIA G, CUNHA JR, LIMA-MELO Y, MARGIS-PINHEIRO MM, SILVEIRA JAG. Peroxisomal APX knockdown triggers antioxidant mechanisms favourable for coping with high photorespiratory H2O2 induced by CAT deficiency in rice. **Plant, Cell Environ** 38: 499–513, 2015.

SPÍNOLA MC, PÉREZ-RUIZ JM, PULIDO P, KIRCHSTEIGER K, GUINEA M, GONZÁLEZ M, CEJUDO FJ. NTRC new ways of using NADPH in the chloroplast. **Physiol Plant** 133: 516–524, 2008.

STRAND, D.D., LIVINGSTON, A.K., SATOH-CRUZ, M., FROEHLICH, J.E., MAURINO, V.G., KRAMER, D.M. Activation of cyclic electron flow by hydrogen peroxide in vivo. **Proc. Natl. Acad. Sci.** 112, 5539–5544, 2015.

SUETSUGU N, HIGA T, GOTOH E, WADA M. Light-Induced Movements of Chloroplasts and Nuclei Are Regulated in Both Cp-Actin-Filament-Dependent and -Independent Manners in *Arabidopsis thaliana*. **PLoS One** 11, 2016.

SUGANAMI M, SUZUKI Y, SATO T, MAKINO A. Relationship between Rubisco activase and Rubisco contents in transgenic rice plants with overproduced or decreased Rubisco content. **Soil Sci Plant Nutr** 1–8, 2018.

SUORSA, M., JÄRVI, S., GRIECO, M., NURMI, M., PIETRZYKOWSKA, M., RANTALA, M., KANGASJÄRVI, S., PAAKKARINEN, V., TIKKANEN, M., JANSSON, S., ARO, E.-M. PROTON GRADIENT REGULATION5 is essential for proper acclimation of Arabidopsis photosystem I to naturally and artificially fluctuating light conditions. **Plant Cell** 24, 2934–48, 2012.

SWATUK L, MCMORRIS M, LEUNG C, ZU Y. Seeing “invisible water”: Challenging conceptions of water for agriculture, food and human security. **Can J Dev Stud** 36: 24–37, 2015.

TAKAGI D, TAKUMI S, HASHIGUCHI M, SEJIMA T, MIYAKE C. Superoxide and singlet oxygen produced within the thylakoid membranes both cause photosystem I photoinhibition. **Plant Physiol**. vol. 171 no. 3 1626-1634, 2016.

TAKAGI, D., ISHIZAKI, K., HANAWA, H., MABUCHI, T., YAMAMOTO, H., MIYAKE, C. Diversity of strategies for escaping reactive oxygen species production within photosystem I among land plants: P700 oxidation system is prerequisite for alleviating photoinhibition in photosystem I. **Physiol Plant.** 161(1):56-74, 2017.

TEIXEIRA FK, MENEZES-BENAVENTE L, GALVÃO VC, MARGIS R, MARGIS-PINHEIRO M. Rice ascorbate peroxidase gene family encodes functionally diverse isoforms localized in different subcellular compartments. **Planta** 224: 300–314, 2006.

TEIXEIRA FK, MENEZES-BENAVENTE L, MARGIS R, MARGIS-PINHEIRO M. Analysis of the molecular evolutionary history of the ascorbate peroxidase gene family: Inferences from the rice genome. **J Mol Evol** 59: 761–770, 2004.

TIKKANEN, M., MEKALA, N.R., ARO, E.-M. Photosystem II photoinhibition-repair cycle protects Photosystem I from irreversible damage. **Biochim. Biophys. Acta** 1837, 210–5, 2014.

TODAKA D, SHINOZAKI K, YAMAGUCHI-SHINOZAKI K. Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. **Front Plant Sci** 6: 84, 2015.

TOPPO S, VANIN S, BOSELLO V, TOSATTO SCE. Evolutionary and Structural Insights Into the Multifaceted Glutathione Peroxidase (Gpx) Superfamily. **Antioxid Redox Signal** 10: 1501–1514, 2008.

TRUBITSIN, B. V, MAMEDOV, M.D., SEMENOV, A.Y., TIKHONOV, A.N. Interaction of ascorbate with photosystem I. **Photosynth. Res.** 122, 215–231, 2014.

UPADHYAYA NM, SURIN B, RAMM K, GAUDRON J, SCHÜNMANN PHD, TAYLOR W, WATERHOUSE PM, WANG M-B. Agrobacterium-mediated transformation of Australian rice cultivars Jarrah and Amaroo using modified promoters and selectable markers. **Funct Plant Biol** 27: 201, 2000.

USADEL B, NAGEL A, THIMM O, REDESTIG H, BLAESING OE, STEINHAUSER D, GIBON Y, MORCUENDE R, WEICHT D, MEYER S. Extension of the Visualization Tool MapMan to Allow Statistical Analysis of Arrays, Display of Coresponding Genes , and Comparison with Known Responses 1. **Plant Physiol** 138: 1195–1204, 2005.

VAAHTERA L, BROSCHÉ M, WRZACZEK M, KANGASJÄRVI J. Specificity in ROS signalling and transcript signatures. **Antioxid Redox Signal** 21: 1422–1441, 2014.

VOSS I, SUNIL B, SCHEIBE R, RAGHAVENDRA A. Emerging concept for the role of photorespiration as an important part of abiotic stress response. **Plant Biol (Stuttg)** 15: 713–722, 2013.

WANG W, VINOCUR B, SHOSEYOV O, ALTMAN A. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. **Trends Plant Sci** 9: 244–252, 2004.

WANG X, HOU C, LIU J, HE W, NAN W, GONG H, BI Y. Hydrogen peroxide is involved in the regulation of rice (*Oryza sativa* L.) tolerance to salt stress. **Acta Physiol Plant** 35: 891–900, 2013.

WOOD ZA, POOLE LB, KARPLUS PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signalling. **Science** 300: 650–653, 2003.

WOOD, W.H.J., MACGREGOR-CHATWIN, C., BARNETT, S.F.H., MAYNEORD, G.E., HUANG, X., HOBBS, J.K., HUNTER, C.N., MATTHEW, P. Dynamic thylakoid stacking regulates the balance between linear and cyclic photosynthetic electron transfer. **Nat. Plants** 4, 2018.

WU Y, MIRZAEI M, PASCOVICI D, CHICK JM, ATWELL BJ, HAYNES PA. Quantitative proteomic analysis of two different rice varieties reveals that drought tolerance is correlated with reduced abundance of photosynthetic machinery and increased abundance of ClpD1 protease. **J Proteomics** 143: 73–82, 2016.

YABUTA Y, MOTOKI T, YOSHIMURA K, TAKEDA T, ISHIKAWA T, SHIGEOKA S. Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. **Plant J** 32: 915–925, 2002.

YAMORI, W., MAKINO, A., SHIKANAI, T. A physiological role of cyclic electron transport around photosystem I in sustaining photosynthesis under fluctuating light in rice. **Sci. Rep.** 6, 1–12, 2016.

YAMORI, W., SAKATA, N., SUZUKI, Y., SHIKANAI, T., MAKINO, A. Cyclic electron flow around photosystem I via chloroplast NAD(P)H dehydrogenase (NDH) complex performs a significant physiological role during photosynthesis and plant growth at low temperature in rice. **Plant J.** 68, 966–76, 2011.

YAMORI, W., SHIKANAI, T. Physiological Functions of Cyclic Electron Transport Around

Photosystem I in Sustaining Photosynthesis and Plant Growth. **Annu. Rev. Plant Biol.** 67, 81–106, 2016.

YOSHIDA K, HISABORI T. Two distinct redox cascades cooperatively regulate chloroplast functions and sustain plant viability. **PNAS** 3967–3976, 2016.

ZHAI CZ, ZHAO L, YIN LJ, CHEN M, WANG QY, LI LC, XU ZS, MA YZ. Two Wheat Glutathione Peroxidase Genes Whose Products Are Located in Chloroplasts Improve Salt and H_2O_2 Tolerances in *Arabidopsis*. PLoS One, 8: 10-15 2013.

ZHANG Y, WEN Z, WASHBURN MP, FLORENS L. Effect of dynamic exclusion duration on spectral count based quantitative proteomics. **Anal Chem** 81: 6317–6326, 2009.

ZHOU J, WANG J, SHI K, XIA XJ, ZHOU YH, YU JQ. Hydrogen peroxide is involved in the cold acclimation-induced chilling tolerance of tomato plants. **Plant Physiol Biochem** 60: 141–149, 2012.

ZHOU M, DIWU Z, PANCHUK-VOLOSHINA N, HAUGLAND RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. **Anal Biochem** 253: 162–168, 1997.

ZIVCAK, M., BRESTIC, M., KUNDERLIKOVA, K., SYTAR, O. Repetitive light pulse-induced photoinhibition of photosystem I severely affects CO2 assimilation and photoprotection in wheat leaves. **Photosynth. Res.** 449–463, 2015.

ZIVCAK, M., KALAJI, H.M., SHAO, H.-B., OLSOVSKA, K., BRESTIC, M. Photosynthetic proton and electron transport in wheat leaves under prolonged moderate drought stress. **J. Photochem. Photobiol. B.** 137, 107–15, 2014.

APPENDIX A – ARTICLES PUBLISHED DURING DOCTORAL COURSE

The following articles were published during the period of doctoral course (2014-2018) and are very relevant for scientific formation of the candidate:

1. **CUNHA, J.R.**; LIMA NETO, M.C.; CARVALHO, F.E.L.; MARTINS, M.O.; JARDIM-MESSEDER, D.; MARGIS-PINHEIRO, M.; SILVEIRA, J.A.G. Salinity and osmotic stress trigger different antioxidant responses related to cytosolic ascorbate peroxidase knockdown in rice roots. **Environmental and Experimental Botany**, v. 131, p. 58-67, 2016. doi: 10.1016/j.envexpbot.2018.02.012.
2. BONIFACIO, A.; CARVALHO, F.E.L.; MARTINS, M.O.; LIMA NETO, M.C.; **CUNHA, J.R**.; RIBEIRO, C.W.; MARGIS-PINHEIRO, M.; SILVEIRA, J.A.G. Silenced rice in both cytosolic ascorbate peroxidases displays pre-acclimation to cope with oxidative stress induced by 3-aminotriazole-inhibited catalase. **Journal of Plant Physiology**, v. 201, p. 17-27, 2016. doi: 10.1016/j.jplph.2016.06.015.
3. LIMA NETO, M.C.; CERQUEIRA, J.V.A.; **CUNHA, J.R.**; RIBEIRO, R.V.; SILVEIRA, J.A.G. Cyclic electron flow, NPQ and photorespiration are crucial for the establishment of young plants of *Ricinus communis* and *Jatropha curcas* exposed to drought. **Plant Biology**, v. 19, p. 650-659, 2017. doi: 10.1111/plb.12573.
4. LIMA NETO, M.C.; SILVEIRA, J.A.G.; CERQUEIRA, J.V.A.; **CUNHA, J.R**. Regulation of the photosynthetic electron transport and specific photoprotective mechanisms in Ricinus communis under drought and recovery. **Acta Physiologiae Plantarum**, v. 39, p. 183, 2017. doi: 10.1007/s11738-017-2483-9.
5. JARDIM-MESSEDER, D.; CAVERZAN, A.; RAUBER, R.; **CUNHA, J.R.**; CARVALHO, F.E.L.; GAETA, M.L.; DA FONSECA, G.C.; COSTA, J.M.; FREI, M.; SILVEIRA, J.A.G.; MARGIS, R.; SAIBO, N.J.M.; MARGIS-PINHEIRO, M. Thylakoidal APX modulates hydrogen peroxide content and stomatal closure in rice (*Oryza sativa* L.). **Environmental and Experimental Botany**, v. 150, p. 46-56, 2018. doi: 10.1016/j.envexpbot.2018.02.012.
6. **CUNHA, J.R.**; CARVALHO, F.E.L.; LIMA-NETO, M.C.; JARDIM-MESSEDER, D.; CERQUEIRA, J.V.A.; MARTINS, M.O.; FONTENELE, A.V.; MÁRGIS-PINHEIRO, M.; KOMATSU, S.; SILVEIRA, J.A.G. Proteomic and physiological approaches reveal new insights for uncover the role of rice thylakoidal APX in response to drought stress. **Journal of Proteomics**, 2018. doi.org/10.1016/j.jprot.2018.08.014.