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PHOTOSYNTHETIC AND PHOTORESPIRATORY RESPONSES TO HIGH H_2O_2 ACCUMULATION TRIGGERED BY PEROXISOMAL ASCORBATE PEROXIDASE KNOCKDOWN AND CATALASE INHIBITION IN RICE PLANTS: PHYSIOLOGICAL AND PROTEOMIC APPROACHES

FORTALEZA
2018

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Tese apresentada ao programa de pós-graduação em Bioquímica da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Doutor em Bioquímica. Área de concentração: Bioquímica vegetal.

Orientador: Prof. Dr. Joaquim Albenísio Gomes da Silveira (UFC).

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Aprovada em 15/02/2018.

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"Há pessoas que desejam saber só por saber, e isso é curiosidade; outras, para alcançarem fama, e isso é vaidade; outras, para enriquecerem com a sua ciência, e isso é um negócio torpe; outras, para serem edificadas, e isso é prudência; outras, para edificarem os outros, e isso é caridade."

Santo Agostinho

RESUMO

A produção de espécies reativas de oxigênio (ERO) é um processo natural em celulas vegetais. A acumulação excessiva de ERO resulta em danos em processos celulares essenciais, como fotosíntese e síntese proteica. No entanto, várias evidências têm apontado para o envolvimento de ERO em processos de sinalização. Entre elas, o peróxido de hidrogênio (H_2O_2) é considerado a principal molécula sinalizadora, visto que ele possui longa meia vida e pode migrar para diferentes compartimentos celulares. Diversos trabalhos destacam o papel de sinalização do H_2O_2 na tolerância ao estresse oxidativo. Os peroxisomos são o principal local de produção de H_2O_2. Por isso, há muitos antioxidantes enzimáicos e não enzimáicos presentes nessa organela e, dentre eles, a catalase (CAT) é a enzima mais importante na remoção de H_2O_2. A ausência de CAT resulta em diversos danos para o metabolismo vegetal, principalmente redução no crescimento e na eficiêncica da fotossíntese. O ciclo ascorbato/glutationa (ASC/GSH) é outro sistema eficaz na remoção de H_2O_2, por meio da enzima ascorbato peroxidase (APX). No entanto, pouco se conhece acerca do papel da isoforma peroxisomal de APX (pAPX). Já é conhecido que o silenciamento da APX4, uma isoforma de pAPX, em plantas de arroz induz menor sensibilidade a inibição da CAT, mas não é completamente compreendido o papel de APX nos peroxissomos. Portanto, abordagens proteômicas e fisiológicas foram utilizadas neste trabalho para avaliar quais sistemas antioxidantes são acionados por plantas de arroz silenciadas em APX4 com atividade de CAT inibida e quais as consequências na eficiência fotossintética. Nesssas condições plantas APX4 apresentaram elevada resiliência da fotossíntese e acumulação de proteínas do metabolismo antioxidante, principalmente as do ciclo ASC/GSH. A maior integridade da maquinaria fotosintética em plantas APX4 sob alta fotorrespiração foi associada a um maior fluxo fotorrespiratório e acumulação de proteínas antioxidantes nos cloroplastos. Em conclusão, o silenciamento de APX4 aciona um proccesso de sinalização, induzindo uma eficiente resposta antioxidante à acumulação de H_2O_2 peroxissomal, através de diferentes mecanismos de proteção que protegem o aparato fotossintético dos efeitos tóxicos causados por altos níveis de H_2O_2. Os resultados obtidos neste trabalho auxiliam no entendimento do papel da isoforma peroxisomal de APX no processo de sinalização por H_2O_2 peroxisomal.

Palavras-chave: Peroxidase do ascorbato. Catalase. Estresse oxidativo.

ABSTRACT

Synthesis of reactive oxygen species (ROS) is a natural process in plant cells. The excessive ROS accumulation implies in damage for important cellular processes, such as photosynthesis and protein synthesis. However, several evidences have shown the role of ROS as signaling molecules. Among them, hydrogen peroxide (H_2O_2) is considered the main signaling molecule, since it has a relative high half-life and can migrate into different cellular compartments. Several works have reported H_2O_2 signaling inducing tolerance to oxidative stress. Peroxisome is the main site of H_2O_2 production. Many antioxidants act in the H_2O_2 detoxification in peroxisomes and catalase (CAT) is the most important of them. The absence of CAT results in several damages for plant metabolism by excessive H_2O_2 accumulation, such as strong impairment in growth and photosynthesis. The H_2O_2 detoxification by ascorbate/glutathione cycle, through the enzyme ascorbate peroxidase (APX), is also an important H_2O_2 scavenger. However, the role of peroxisomal ascorbate peroxidases (pAPX) is still unclear. It has shown that APX4 knockdown, a pAPX isoform, triggers less sensibility to CAT inhibition in rice plants. Nevertheless, it is not completely understood how pAPX knockout induces this favorable outcome. In the present study, proteomic and physiological approaches were utilized to evaluate which antioxidant systems are triggered by pAPX-knocked-down plants upon CAT inhibition and how this impacts the photosynthetic performance. High photosynthesis resilience and accumulation of protein from antioxidant metabolism, mainly the ascorbate/glutathione cycle, were observed in APX4-knocked-down plants (APX4) under inhibition of CAT. Additionally, the participation of photorespiration in photosynthesis resilience of APX4 plants was evaluated. The better photosynthesis performance of APX4 plants upon induced photorespiration was associated to enhanced photorespiratory flux and accumulation of chloroplastic antioxidant proteins. Therefore, the present thesis allows the conclusion that the deficiency of APX4 induces a previous signaling that triggers an efficient antioxidant response to peroxisomal H_2O_2 accumulation by different protective mechanisms. This response protects the photosynthetic apparatus against oxidative damage caused by high H_2O_2. The results obtained in this work expand the understanding of the role of pAPX and H_2O_2 signaling in peroxisomes.

Keywords: Ascorbate peroxidase. Catalase. Oxidative stress.

ABBREVIATION LIST

1O2: Singlet oxygen

2PG: 2-phosphoglycolate

3-AT: 3-amino-1,2,4-triazol

3PG: 3-phosphoglicerate

ABA: Abiscisic acid

Apot: Potential photosynthesis

APX: Ascorbate peroxidase

APX4: APX4-silenced rice plants

ASC: Ascorbate

Asn: Asparagine

Asp: Aspartate

CAT: Catalase

CBC: Calvin–Benson cycle

DHAR: Dehydroascorbate reductase

ETR: Electron transport rate of PSII

Fd-GOGAT: Ferredoxin-dependent glutamate synthase

FNR: ferredoxin-NADP reductase

Fv/Fm: Potential quantum yield of PSII

G6PDH: glucose-6-phosphate dehydrogenase

GDC: Glycine decarboxylase complex

GGAT: Glutamate:glyoxylate aminotransferase

Gln: Glutamine

Gln: Glutamine

Glu: Glutamate

GLYK: Glycerate-3-kinase

GPX: Glutathione peroxidase

GR: Glutathione reductase

GS2: Chloroplastic gluthamine synthetase

GSH: Glutathione

HPR: Hydroxypyruvate reductase

HSP: Heat shock protein

JC: Electron flow to carboxylation

JO: Electron flow to oxygenation

LHCB: Light-harvesting complex B

MAPK: Mitogen-activated protein kinases

MDHAR: Monodehydroascorbate reductase

NO: Nitric oxide

O2.-: Superoxide radical

pAPX: Peroxisomal ascorbate peroxidase

PCD: Plant cell death

PGLP: 2PG phosphatase

PN: Net photosynthesis

Pr: Photorespiration

Prx: Peroxiredoxins

PSBO: PSII subunit O protein

PSBP: PSII subunit O protein

PSBQ: PSII subunit Q protein

PSII: Photosystem II

rbcL: Rubisco large subunit

ROS: Reactive oxygen species

Rubisco: D-ribulose-1,5-bisphosphate carboxylase/oxygenase

SA: Salicylic acid

sAPX: Stromal ascorbate peroxidase

SGAT: Serine:glyoxylate aminotransferase

SHMT: Serine-hydroxymethyl transferase

SOD: Superoxide dismutase

tAPX: Thylakoidal ascorbate peroxidase

Vcmax: Maximum carboxylation velocity

ΦPSII: Yield of PSII

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OBJECTIVES

1. Understand the role of peroxisomal ascorbate peroxidase in the antioxidant metabolism.
2. Better understand the relation between photosynthesis regulation and photorespiration.
3. Indicate protection mechanisms triggered by H_2O_2 signaling.
4. Evaluate changes in the proteomic profile induced by high peroxisomal H_2O_2 in pAPX-knocked-down rice plants.

1 CHAPTER I: LITERATURE REVIEW

1.1 Introduction

Hydrogen peroxide is a reactive oxygen species (ROS) which in high concentration can damage biomolecules, such as DNA, lipids and proteins, inactivating key cellular functions (Gill and Tuteja, 2010; Del Río, 2015). However, H_2O_2 in subtle cellular levels can act as a signaling molecule for biological processes, such as development and stress perception, triggering plant tolerance to stress (Hossain et al., 2015; Corpas, 2015; Del Río, 2015). Taking into account the double role of H_2O_2, an efficient antioxidant system is crucial to control the H_2O_2 levels (Gill and Tuteja, 2010).

In C3 plants, peroxisome is the main site of H_2O_2 production, by the photorespiratory glycolate oxidase enzyme (GO) (Foyer and Noctor, 2003). Plants have a robust antioxidant system in peroxisomes composed by ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferase (GST) (Del Río et al., 2016). Among these enzymes, CAT is highlighted as the main H_2O_2 scavenger in the plant cell (Mhamdi et al., 2012). The function of the peroxisomal APX (pAPX) is controversial but this enzyme has been reported to act in the H_2O_2 signaling in pAPX knocked down rice, triggering less oxidative damage under CAT inhibition (Sousa et al., 2015).

It has been reported that *Arabidopsis* CAT knockout induces accumulation of H_2O_2 associated with strong impairment of growth and photosynthesis (Mhamdi et al., 2010). This outcome emphasizes the physiological importance of the regulation of peroxisomal H_2O_2 production by photorespiration and H_2O_2 scavenger by antioxidant

enzymes. However, despite the eventual damages in photosynthetic apparatus originated from photorespiratory H_2O_2 over-accumulation, the photorespiratory flux is essential for the photosynthesis performance. Several works have shown that disruption of photorespiratory flux by suppression of photorespiratory enzymes results in decreased photosynthetic activity (Timm et al., 2012a; Dellero et al., 2015; Lu et al., 2014). In this context, this review will discuss some important issues about the antioxidant metabolism in peroxisome, the importance of CAT and pAPX in redox regulation and, finally the synergism between photorespiration and photosynthesis.

1.2 Redox metabolism in peroxisomes

Peroxisomes are small organelles with diameter 0,1-1,7 μm, surrounded by a single membrane (Kaur et al., 2009). This organelle acts in important cellular processes, such as lipid metabolism (β-oxidation), hormone synthesis, glyoxylate cycle, ureides metabolism and photorespiration (Kaur et al., 2009; Hu et al., 2012). The most of these processes result in production of ROS, such as H_2O_2, superoxide radical ($O_2^{\cdot-}$), and singlet oxygen ($^1O_2$). The peroxisome homeostasis is highly controlled by the cellular redox status (Wang et al., 2015). For example, under oxidative stress, a high H_2O_2 accumulation affects peroxisome proliferation and induces peroxisome aggregation (Shibata et al., 2013).

The $O_2^{\cdot-}$ produced in peroxisomes takes place in a short electron chain associated with the NADH/NADPH-driven peroxisomal membrane and also in the peroxisomal matrix by xantine oxidoreductase and uricase (Sandalio et al. 2013). To scavenger $O_2^{\cdot-}$, the peroxisomes have the enzyme superoxide dismutase (SOD), which catalyzes

disproportionation of $O_2^{\cdot -}$ into H_2O_2 and O_2. The presence of Mn-SOD, CuZn-SOD, and Fe-SOD has been demonstrated in peroxisomes of different plant species (Corpas et al., 2017). It has already been reported the presence of $^1O_2$ in peroxisomes and, different than $^1O_2$ generated in chloroplasts, this ROS is produced in peroxisomes by a light-independent reaction, the Haber-Weiss mechanism (Mor, et al., 2014).

The peroxisomes are the main site of H_2O_2 production in plant cells. In C3 plants, the main process generating H_2O_2 in peroxisomes is the photorespiration, followed by the acyl-CoA oxidase in the fatty acid β-oxidation, the flavin oxidases and the spontaneous or enzymatic $O_2^{\cdot -}$ dismutation (Baker and Graham, 2013; DEL Río et al. 2002, Foyer et al. 2009) (Figure 1). The glycolate oxidase (GO) is a photorespiratory enzyme that catalyzes the oxidation from glycolate to glyoxylate with H_2O_2 production. This reaction is the main source of H_2O_2 in peroxisomes with about 2- and 50-fold higher rates of H_2O_2 production than that reported for chloroplasts and mitochondria, respectively (Foyer et al., 2009). Another enzyme involved in H_2O_2 production in peroxisomes is the acyl-CoA oxidase, the first enzyme of the β-oxidation which catalyzes the formation of 2-trans-enoyl-CoA using oxygen as the electron acceptor and generating H_2O_2 (Arent et al., 2008). Peroxisomes also contain many H_2O_2-producing flavin oxidases depending on the organism and tissue origins, such as enzymes of polyamine catabolism, xanthine oxidase, sulfite oxidase and sarcosine oxidase (Corpas et al., 2009; Sandalio and Romero-Puertas, 2015).

To remove H_2O_2 in peroxisome, plants have an efficient antioxidant apparatus. The ascorbate-glutathione (ASC-GSH) cycle constitutes a system which enables plants to control H_2O_2 accumulation.

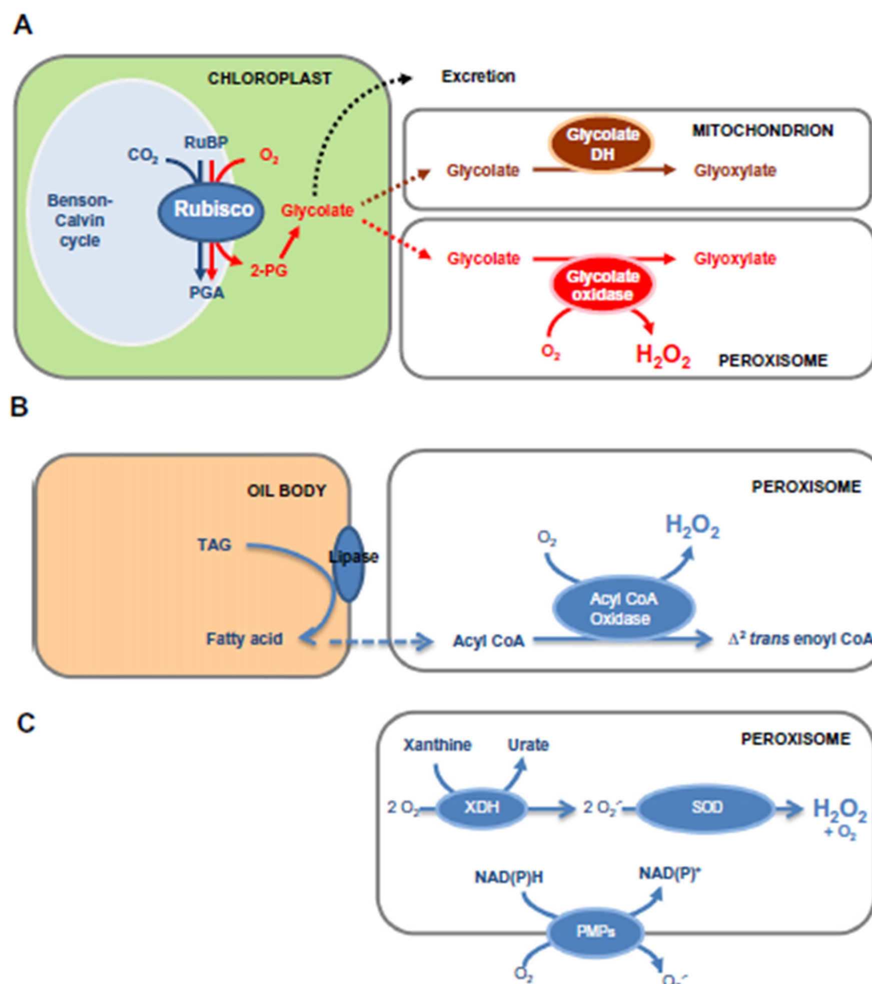

Figure 1. Some of the principal pathways of H_2O_2 production in plant peroxisomes. For discussion of other reactions, see text. (A) The photorespiratory pathway through peroxisomal glycolate oxidase. Other fates of glycolate, mainly described in unicellular algae, are shown on upper right. (B) b-Oxidation of fatty acids; (C) generation of H_2O_2 from superoxide. The xanthine dehydrogenase (XDH) reaction is shown as an example of a superoxide-generating enzyme. 2-PG, 2-phosphoglycolate. PGA, 3-phosphoglycerate. PMP, peroxisomal membrane polypeptide. RuBP, ribulose-1,5-bisphosphate. TAG, triacylglyceride. Reproduced from Mhamdi et al., (2012) - *Archives of Biochemistry and Biophysics*.

It has already been reported all components of the ASC/GSH cycle in peroxisomes, which are ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), ascorbate (ASC) and glutathione (GSH) (Jiménez et al., 1997; Romero-Puertas et al., 2006; Mhamdi et al., 2012). The presence of glutathione S-transferases (GST) in peroxisomes from *Arabidopsis* plants have also been reported (Dixon et al., 2009). The GST removes

H_2O_2 in addition to participating in the detoxification of xenobiotic compounds and lipid peroxide products (Dixon et al., 2009). It has been reported another peroxidase, glutathione peroxidase, in leaf peroxisome of tomato plants (Kużniak and Sklodowska 2005). The presence of a protein immunorelated to peroxiredoxins (Prx) in the matrix of pea leaf peroxisomes differently regulated by oxidative stress suggests that peroxiredoxin could also be involved in the mechanism of regulation of H_2O_2 in peroxisomes (Corpas et al., 2017). Although peroxisomes have several H_2O_2-scavengers, the catalase is highlighted as the main antioxidant involved in removing H_2O_2 (Mhamdi et al., 2012). The figure 2 shows the major H_2O_2 scavenger enzymes in peroxisomes from *Arabidopsis*.

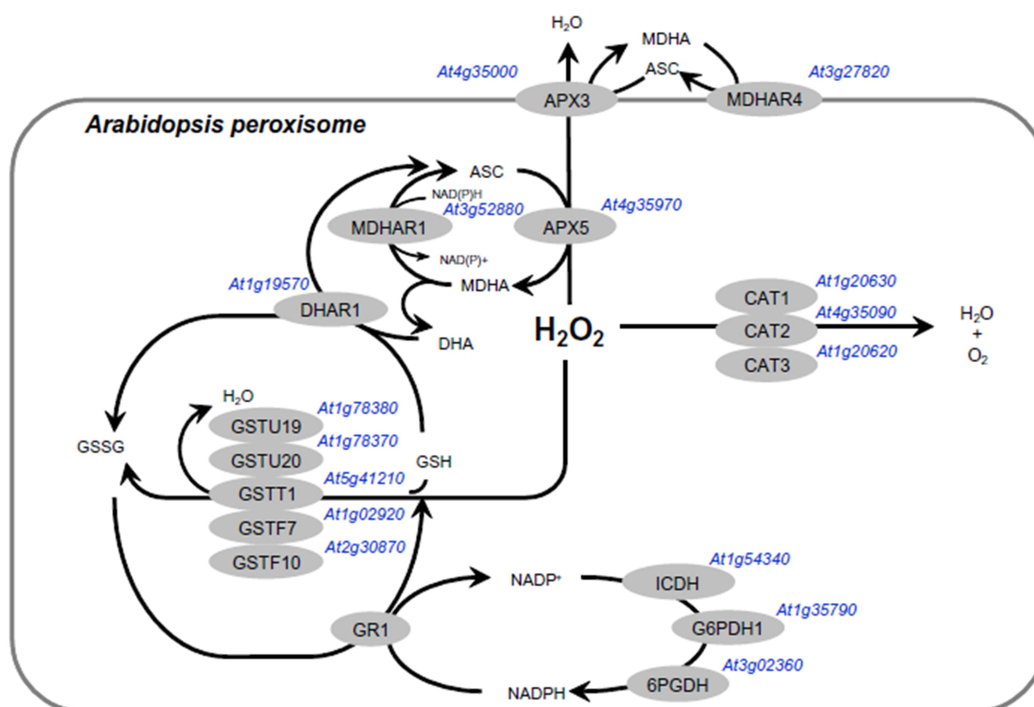

Figure 2. Known and possible major peroxisomal enzymes involved in H_2O_2 metabolism in plants. Gene annotation of enzymes predicted to be peroxisomal in *Arabidopsis* was performed according to the description and associated references on the TAIR website (http://www.*Arabidopsis*.org). Reactions are not necessarily showncstoichiometrically. *Arabidopsis* gene identifier codes are shown in blue type. NADPH generation is shown as coupled to GR activity but is also required for other enzymes (e.g., MDHAR). For simplicity, NAD(H) turnover is omitted. APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; G6PDH, glucose 6-phosphate dehydrogenase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide; GST, glutathione S-transferase. ICDH, isocitrate dehydrogenase; MDHA, monodehydroascorbate;

MDHAR, monodehydroascorbate reductase; 6PGDH, 6-phosphogluconate dehydrogenase. Reproduced from Mhamdi et al., (2012) - *Archives of Biochemistry and Biophysics*.

1.3 Physiological consequences of CAT deficiency

The presence of CAT in peroxisomes is known since the first characterization of these organelles by De Duve and Baudhuin, (1966). Different from peroxidases which require a small reducing molecule, CAT evolves directly O_2 from H_2O_2 (Loew, 1900). It is necessary two H_2O_2 molecules for the CAT reaction, where the first molecule is transformed to water and the second converted to O_2 (Zamocky et al., 2008). Apart from its function in the control of H_2O_2, CAT also has a key role in the maintenance of cellular redox homeostasis (Mhamdi et al., 2012, Sandalio and Romero-Puertas, 2015). CAT has low affinity for H_2O_2, with apparent Km value in the range 40–600 mM, and is susceptible to the inhibitor 3-amino-1,2,4-triazole (Chelikani and Fita, 2004; Regelsberger et al., 2002). The molecular mass of plant catalase monomers is approximately 55 kDa (Corpas et al., 1999).

Arabidopsis has three CAT isoforms (CAT1, CAT2, and CAT3), in which CAT2 expression is associated with the photorespiration pathway, CAT1 with fatty acid b-oxidation, and CAT3 is related to senescence processes (Mhamdi et al., 2010, Mhamdi et al. 2012). The specific function of CAT2 in leaves was confirmed by no phenotypic alteration in CAT1 and CAT3 knockouts plants (Hu et al., 2010). In Rice, the family of CATs comprises three isoforms, namely OsCATA, OsCATB, and OsCATC (Higo and Higo 1996; Morita et al., 1994; Agrawal et al. 2001). Rice CatA, CatB, and CatC are involved in environmental stress response, homeostasis related to root growth, and photorespiration, respectively (Joo et al., 2014).

The CAT activity can be reduced for some stresses as cold or salt under light condition, drought and high light (Streb and Feierabend, 1996; Nahar et al., 2015; Hertwig and Feierabend, 1992; Schmidt et al., 2002; Hasanuzzaman and Fujita, 2011). For example, in high light stress, CAT activity can be decreased through insufficient rates of resynthesis to replace photoinactivated enzyme (Streb and Feierabend, 1996). In general, decreases in CAT activity by stress are associated with similar effects to those described in deficient mutants in CAT2, such as reduction of the glutathione redox state (Streb and Feierabend, 1996; Volk and Feierabend, 1989).

Arabidopsis cat2 mutants developed lesions day length and light intensity dependent, associated with reduced glutathione redox status (Mhamdi et al., 2010). However, under high CO_2 growth, these responses are abolished, once photorespiration and hence H_2O_2 production are suppressed (Mhamdi et al., 2010). Deficient plants in CAT have always high levels of H_2O_2 (Bueso et al., 2007; Hu et al., 2010). CAT-lacking tobacco plants show bleaching and more susceptibility to diseases, while young leaves are less susceptible than the older leaves and show reduction in glutathione redox status, upon high light exposure (Willekens et al., 1997; Chamnongpol et al., 1998).

Accumulated glutathione in cat2 is mainly localized in the vacuole, followed by chloroplasts and cytosol (Queval et al., 2011). Perturbations of glutathione in catalase-deficient plants are presumably driven by engagement of GSH-dependent peroxidatic pathways, although the contribution of different subcellular compartments to these reactions is unclear (Mhamdi et al., 2012). It has also found a strong up-regulation of cytosolic components of the ascorbate–glutathione system, whereas peroxisomal APXs were not up-regulated, in a transcriptome analysis of *Arabidopsis* cat2 mutants (Mhamdi et al., 2010). The effects triggered by CAT pharmacological inhibition are

similar to those triggered by CAT knockdown. Rice plants CAT-inhibited by 3-AT showed reduced glutathione redox status, membrane damage and reduction in net photosynthesis (Sousa et al., 2015).

1.4 Role of ascorbate peroxidase isoforms

Ascorbate peroxidase (APX) is a member from the plant-type heme peroxidase superfamily that reduces hydrogen peroxidase to water, using ASC (Shigeoka et al., 2002). This enzyme contains a heme prosthetic group in which iron plays an important role for the enzymatic reaction and it has been shown that iron deficiency results in reduced APX activity even with high ASC concentration (Zaharieva and Abadia, 2003). This enzyme is involved in the fine regulation of H_2O_2 since APX has a high affinity for H_2O_2, with Km values below 100 µM (Mittler and Zilinskas, 1991). The APX activity is inhibited for Cyanide and azide, once APX is a heme peroxidase (Mittler and Zilinskas, 1991).

APX participates in the ascorbate-glutathione (ASC-GSH) cycle; the main H_2O_2 detoxification system; present in chloroplast, cytosol, mitochondria and peroxisomes (Asada, 1992; Mittler et al., 2004). In the ASC-GSH cycle, APX produces two molecules of monodehydroascorbate (MDHA) which are reduced to ASC by enzyme monodehydroascorbate reductase (MDHAR). MDHAR can produce spontaneously dehydroascorbate (DHA). Finally, DHA is reduced back to ASC by enzyme dehydroascorbate reductase (DHAR) which uses GSH as the reducing substrate and the oxidized glutathione (GSSG) produced is reduced back to GSH by glutathione reductase (GR) (Noctor and Foyer, 1998; Asada, 1999).

In higher plants, APX is encoded by multigenic families. *Arabidopsis* contains nine APX genes, three were found to be encoded in cytosol whereas the other six were distributed in chloroplast and peroxisome (Chew et al., 2003; Mittler et al., 2004). The presence of eight isoenzymes has been confirmed in *Arabidopsis*, with the following localization: two in chloroplast (sAPX and tAPX), three in cytosol (APX1, APX2, APX6) and three in microsome (APX3, APX4, APX5) (Jespersen et al., 1997; Panchuk et al., 2002). Rice has eight APX genes: two cytosolic (OsAPx1 and OsAPx2), two peroxisomal (OsAPx3 and OsAPx4), three chloroplastic (OsAPx5, OsAPX7 and OsAPx8) and one mitochondrial (OsApx6) (Teixeira et al., 2004; Teixeira et al., 2006). The existence of multiple isoforms of APX within different cellular compartments indicates the important and specific role played by APXs in antioxidant defense (Ishikawa et al., 1998; Shigeoka et al., 2002).

In *Arabidopsis*, cytosolic APXs play an important role in the oxidative protection of chloroplasts under abiotic and biotic stresses (Pnueli et al., 2003; Koussevitzky et al., 2008; Wu et al., 2014). APX1 is important for plant growth and development since *Arabidopsis* APX1-knockout has shown a reduction in plant growth and photosynthesis, delay of flowering, and enhanced protein oxidation under high light (Pnueli et al., 2003). APX2 expression is induced by stress, such as high light, heat stresses and wounding conditions (Panchuk et al., 2005; Mullineaux et al., 2006). In rice, the expressions of OsAPX1 and OsAPX2 are developmentally regulated and the silencing of OsAPX1 or OsAPX2 genes resulted in strong impairment on plant development (Agrawal et al., 2003; Rosa et al., 2010). Whereas overexpression of OsAPX1 enhances tolerance to chilling at the booting stage and overexpression of OsAPX2 improves salt tolerance in transgenic *Arabidopsis* (Lu et al., 2007).

In chloroplast, APX integrates the water-water cycle, involved in ROS production and scavenging (Asada, 1999; Shigeoka et al., 2002, Foyer and Shigeoka, 2011). In the water–water cycle, electrons excised from water at PSII are transferred to oxygen by PSI, resulting in the formation of $O_2^{\cdot-}$ which is subsequently converted into H_2O_2 by SOD and, APX, in turn, reduces H_2O_2 back into water (Asada, 1999). Chloroplast APXs are indispensable for photosynthetic activity and photoprotection under photooxidative stress (Kangasjärvi et al., 2008). In *Arabidopsis*, chloroplastic APXs have contributed for photoprotection regulating H_2O_2-responsive genes during photooxidative stress (Maruta et al., 2009).

The importance of APX in peroxisomes is not fully clear, whereas this organelle has CAT, an efficienty H_2O_2-scavenger (Mhamdi et al., 2010). However, several works have reported protective role of pAPX against stress. Transgenic plants overexpressing pAPX were significantly more tolerant to heat stress (Shi et al., 2001; Wei-Feng et al., 2008). Enhanced protection against oxidative stress was reported in tobacco plants overexpressing peroxisomal APX (Wang et al., 1999). Tabacco plants overexpressing pAPX from *Halophyte salicornia* have shown salt and drought stress tolerance (Singh et al., 2014).

Overexpression of a Populus peroxisomal ascorbate peroxidase (PpAPX) gene in transgenic tobacco has been reported for enhanced cellular protection against drought and salinity (Li et al., 2009). However, Narendra et al., (2006) working with *Arabidopsis* knocked out in APX3, concluded that this isoform may not be an important antioxidant enzyme in *Arabidopsis*. Similarly, rice plants knocked down in APX4 have not shown impairment phenotypic and have shown enhanced tolerance to CAT

inhibition (Sousa et al., 2015). Therefore, there is no a consensus in concern the importance of peroxisomal APX isoform.

1.5 H_2O_2 as a signaling molecule

The Hydrogen peroxide (H_2O_2) molecule is the result of two reductions via $O^{\cdot-}_2$, and can be produced in different cellular sites by several processes. The major players in H_2O_2 generation in plants are the photorespiratory GO, the photosynthetic electron transport chain, the respiratory electron transport chain, and NADPH oxidases located at the plasmalemma (Noctor et al., 2017). Initially, H_2O_2 had been considered a toxic molecule without any function in the cellular metabolism. However, in the last decades H_2O_2 has been receiving highlight as an important signaling molecule (Neil et al., 2002; Maruta et al., 2012; Noctor et al., 2014).

The highest half-life (1 ms) of H_2O_2 among the other ROS and its small size, associated to selective reactive, ability to cross membrane through aquaporins and stability make H_2O_2 an ideal signaling molecule (García-Mata and Lamattina, 2013; Noctor et al., 2014). Thus, as consequence of these early mentioned properties, H_2O_2 can diffuse to different cellular compartments and affect protein redox status, alter its function and structure, activate or inactivate target proteins (Suzuki et al., 2012; Maruta et al., 2012; Jacques et al., 2013).

A study with *Arabidopsis* has shown that H_2O_2 originated in peroxisomes and chloroplasts induces different signals that regulate the plant transcriptome differentially (Sewelam et al., 2014). Recently, Exposito-Rodriguez et al., (2017) attested that

chloroplast-derived H_2O_2 can be detected in the nucleus, suggesting that a nuclear gene expression respond to changes in photosynthetic activity, via H_2O_2 signaling. The figure 3 shows schematically the different types of H_2O_2 signaling in the plant cell, highlighting that the signaling can be in the site of H_2O_2 production or the H_2O_2 molecule can migrate to trigger a signaling response in another compartment.

The H_2O_2 signaling cross talk with many different signaling molecules, such as calcium, abiscisic acid (ABA), nitric oxide, salicylic acid (SA), ascorbic acid, jasmonate and ethylene, protein kinase and many other small signaling molecules; and, consequently, regulates important metabolic pathways (González et al., 2012; Shi. et al., 2015; Saxena et al., 2016). For example, hormones like ABA, jasmonate and SA mediated the activation of the mitogen-activated protein kinases (MAPK) cascade by H_2O_2 (Rodríguez et al., 2010). In addition, H_2O_2 is known as a signaling molecule in response to multiple defenses to environmental stresses (Petrov and Vanbreusegem, 2012). Changes of H_2O_2 level may impact metabolic and antioxidant enzyme activity in favor of plant growth and development (Barba-Espín et al., 2011). Several studies have reported that pre-treatment with low concentrations of H_2O_2 can enhance abiotic stress tolerance through modulation of physiological processes, such as photosynthesis and activating different stress-responsive pathways (Chao et al.,2009; Xu et al., 2011; Wang et al., 2014).

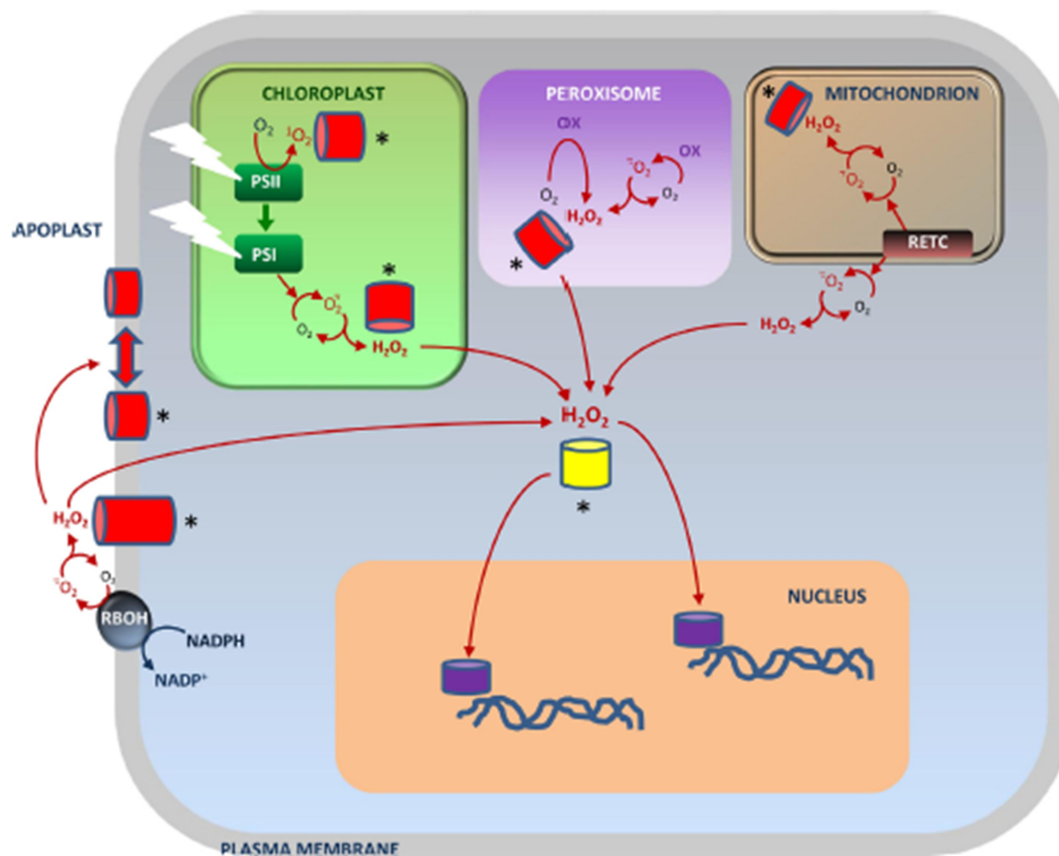

Figure 3. Integration of multiple pathways of ROS signaling in plant cells. The most stable ROS, H_2O_2, can move from the compartments in which it is mainly produced to alter cytosolic and nuclear redox states, which can be perceived by receptor proteins (yellow barrel). In addition, site-specific receptor systems (red barrels) may perceive singlet oxygen- or H_2O_2-driven redox changes more locally, leading to (in)activation of signaling networks (*). This might involve redox modifications of protein–protein interactions or second messengers. Ultimately, gene expression will be modified by altered activity of transcription factors (purple) that may not themselves be redox-modified. This extensive vocabulary of generic and site-specific signaling confers both specificity and flexibility in the redox regulation of gene expression. Reproduced from Noctor et al., (2017) - *Seminars in Cell & Developmental Biology*.

Although many works show the process of signaling by H_2O_2, the most of them do not provide a clear picture of signaling pathways during stress response in various conditions and studies about how the signaling process occurs in plants are still scarce. Oxidation of cysteine residues from redox-sensitive proteins is the best described mechanism through which H_2O_2 act as signaling molecules. The conformation as a thiolate anion (S _) and the low pKa at physiological pH, make the susceptible cysteine

residues more reactive than the protonated cysteine thiol group (SH) and provide selectivity and specificity (Finkel, 2012). Thus, H_2O_2 oxidization of the thiolate anion to the sulfenic form (SO _) by H_2O_2 results on cellular signaling by changing protein conformation and activity. Recently, König et al. (2018) working with glutathione and ascorbate deficient mutants *pad2* and *vtc1*, concluded that the light-triggered rapid gene regulation is a glutathione-dependent processes, independent on cellular H_2O_2.

There are two main mechanisms proposed to explain the H_2O_2 signaling by oxidation of cysteine thiol groups. The first mechanism, thiol peroxidase-based redox relays, is considered the major mechanism for the specific and efficient transmission of oxidative signals under conditions of nanomolar-range H_2O_2 signaling in animals (STÖKER et al., 2017). In the redox relay, H_2O_2 oxidizes scavenger enzymes that transfer the oxidation to the target protein. The second mechanism is called floodgate and is a direct signaling. In this mechanism, the scavenger enzymes are inactivated by H_2O_2 that floods the area with and oxidizes specific thiol within the target protein. Aditionaly, other two mechanisms, one involving oxidation of thioredoxins (Trx) and GSH as intermediate step and the other involving a dissociation of target protein from scavenger enzyme through oxidation of scavenger by H_2O_2. The figure 4 shows the four mechanisms.

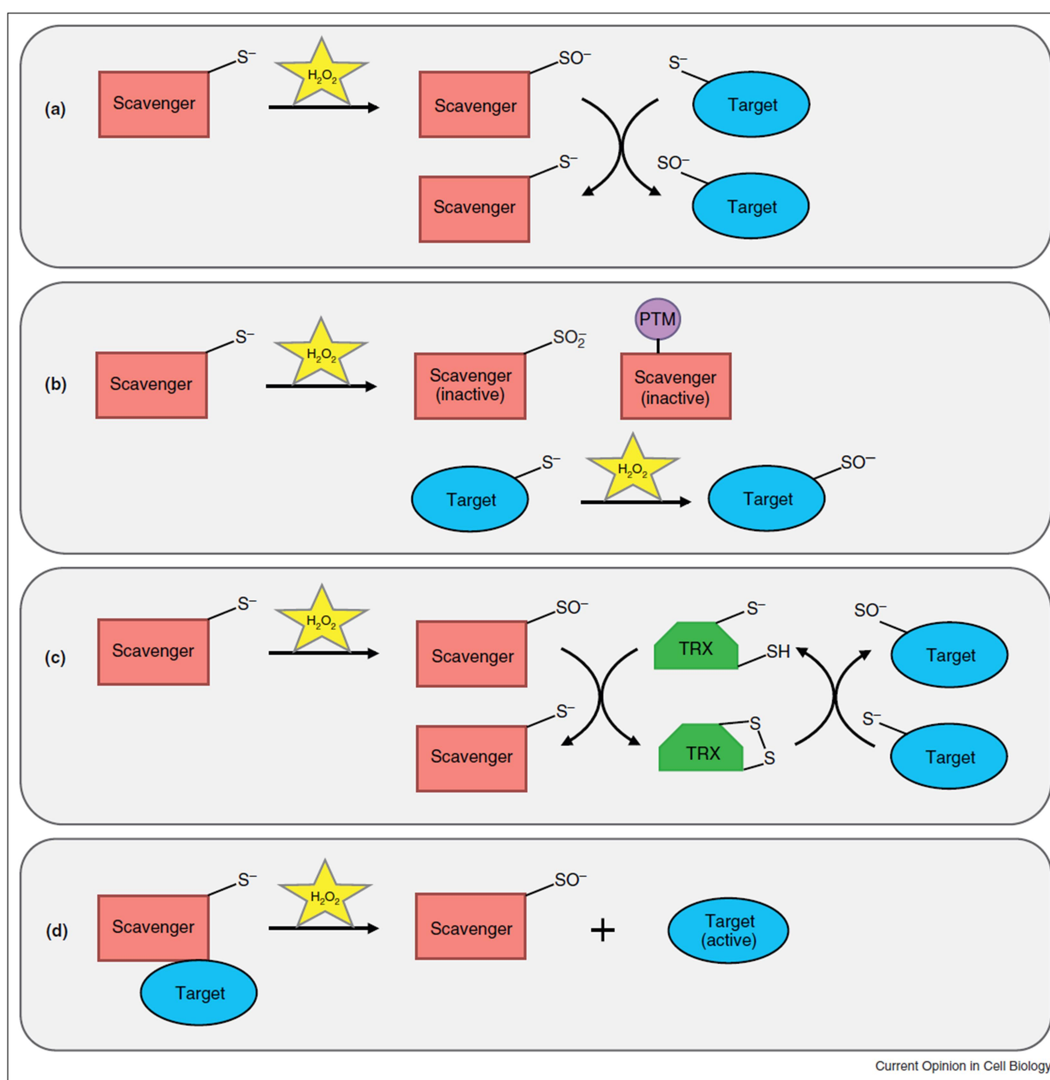

Figure 4. Possible mechanisms for H_2O_2-dependent signal transduction. (a) The redox relay mechanism uses a scavenging enzyme such as glutathione peroxidase (GPX) or peroxiredoxin (PRX) to transduce the H_2O_2 signal and oxidize the target protein. (b) With the floodgate model, H_2O_2 inactivates the scavenger, perhaps through hyperoxidation to sulfinic (SO2 _) acid or through a posttranslational modification (PTM), to allow for H_2O_2-mediated oxidation of the target protein. (c) The scavenging enzyme accepts H_2O_2 oxidation and transfers the oxidation to an intermediate redox protein such as thioredoxin (TRX), which subsequently oxidizes the target protein. (d) Dissociation of the target protein from the oxidized scavenging enzyme results in target protein activation. *Reproduced from Reczek & Chandel, (2015) – Current Opinion in Cell Biology.*

To transduce signals, H_2O_2-induced thiol oxidation must be target selective (affecting specific cysteines on specific proteins), fast (acting on time scales from seconds to several minutes) and efficient (acting on low abundance target proteins). The specificity and efficiency of H_2O_2 as a signaling molecule are the most difficult issues

to explain (STÖKER et al., 2017). The signaling process displayed for ROS to regulate and maintain normal physiological functions mainly via interacting with cysteine (Cys) residues of proteins is called redox biology (SCHIEBER and CHANDEL, 2014; RECZEK and CHANDEL, 2015; TRUONG and CARROLL, 2013). The figure 5 shows an example of H_2O_2 altering the structure and function of a target protein through oxidation of Cys thiolate anions (Cys–S _) to sulfenic form (Cys–SOH).

This change in function of target proteins can trigger several consequences for cellular metabolism, as instance affect transcription, phosphorylation, and other important signaling events, and/or alter metabolic fluxes and reactions in the cell by altering enzymatic properties (TRUONG and CARROLL, 2013; RECZEK and CHANDEL, 2015) In addition, many redox relays exist in cells, and these can transduce and/or amplify an initial ROS-derived redox event (RECZEK and CHANDEL, 2015).

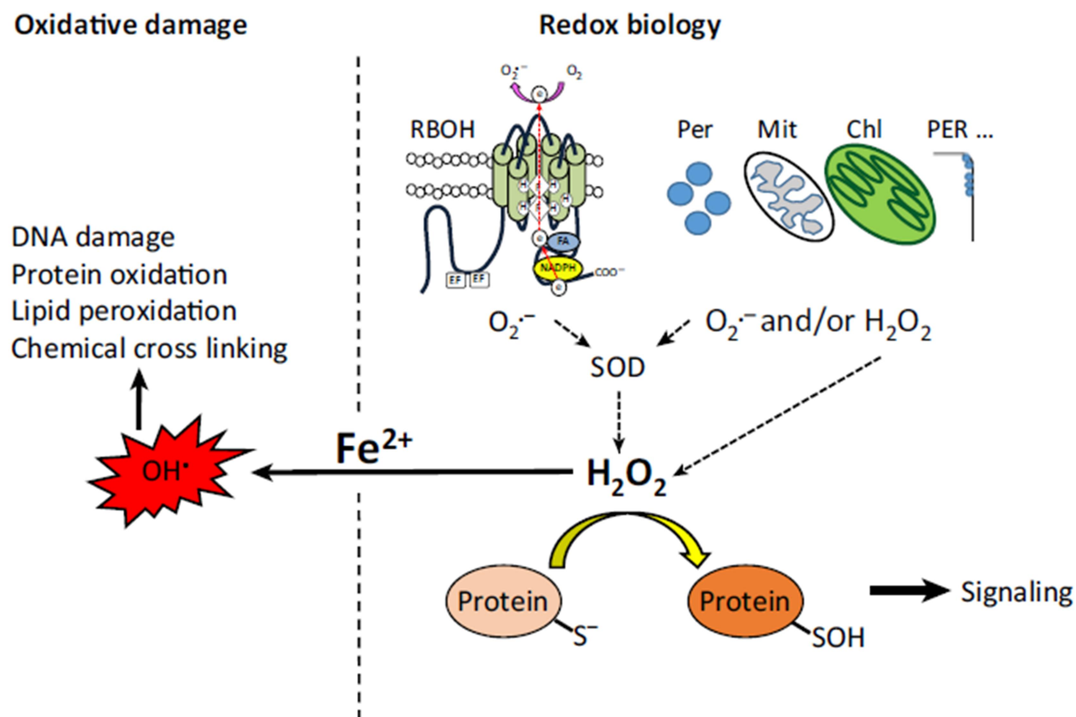

Figure 5. ROS and Redox Biology. ROS produced by respiratory burst oxidase homologs (RBOHs), peroxisomes (Per), mitochondria (Mit), chloroplasts (Chl), and cell wall bound peroxidases (PER) result in the accumulation of H2O2 that mediates the oxidation of cysteine residues on proteins, affecting their structure and function and triggering/regulating cellular signaling pathways (Signaling). However, the presence of labile iron in the form of Fe2+ can tip the cellular balance of ROS/redox reactions and cause oxidative stress via the formation of hydroxyl radicals. Maintaining the cellular pool of labile iron as low as possible is therefore crucial for redox biology and for the regulation of metabolism and other cellular functions by ROS. Copied from Mittler, (2017) – *Trends in Plant Science*.

1.6 Photorespiration and its impact in photosynthesis

The incorporation of CO_2 into biological compounds is played by D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the major enzyme assimilating atmospheric CO_2 in the biosphere (ANDERSSON and BACKLUND, 2008). For each CO_2 carboxylated by Rubisco, two molecules of 3-phosphoglicerate (3PG) are produced and metabolized in the Calvin-Benson (CB) cycle for carbohydrate synthesis (LEEGOOD et al., 1995). However, the oxygenase capacity of Rubisco results in the synthesis of one molecule of 3-PG and one molecule of 2-phosphoglycolate (2PG) for

each O_2 metabolized (OGREN and BOWES, 1971). The 2PG accumulation can result in photosynthesis collapse, once this molecule is a potent inhibitor of, at least, two CB cycle enzymes: triosephosphate isomerase and phosphofructokinase (ANDERSON, 1971; KELLY and LATZKO, 1976). To salvage the 3PG from 2PG, takes place a complex process that involves more than 20 enzymes in three organelles, the photorespiration (HAGEMANN and BAUWE, 2016).

The photorespiratory pathway initiates in chloroplast where 2PG is dephosphorylated to glycolate, by 2PG phosphatase (PGLP) reaction. Glycolate is then exported to cytosol through a glycolate/glycerate antiporter (PICK et al., 2013) and diffuses to peroxisome. In peroxisomes, GO oxidizes glycolate to glyoxylate with production of H_2O_2. Subsequently, glutamate:glyoxylate aminotransferase (GGAT) and serine:glyoxylate aminotransferase (SGAT) catalyze the transamination of glyoxylate to glycine. The produced glycine migrates to mitochondria and is converted in serine by glycine decarboxylase complex (GDC) acoupled to serine-hydroxymethyl transferase with release of ammonium and CO_2. Serine is then exported to peroxisome and its amino group is moved to glyoxylate in the SGAT reaction, producing hydroxypyruvate (HP). Still in peroxisomes, the enzyme hydroxypyruvate reductase (HPR) reduces pyruvate to glycerate. Finally, glycerate returns into to chloroplast through glycerate/glycolate antiporter and is phosphorylated to 3PG by glycerate-3-kinase (GLYK) reaction (BAUWE et al., 2012; HAGEMANN and BAUWE, 2016). The whole photorespiratory cycle is shown at Figure 6.

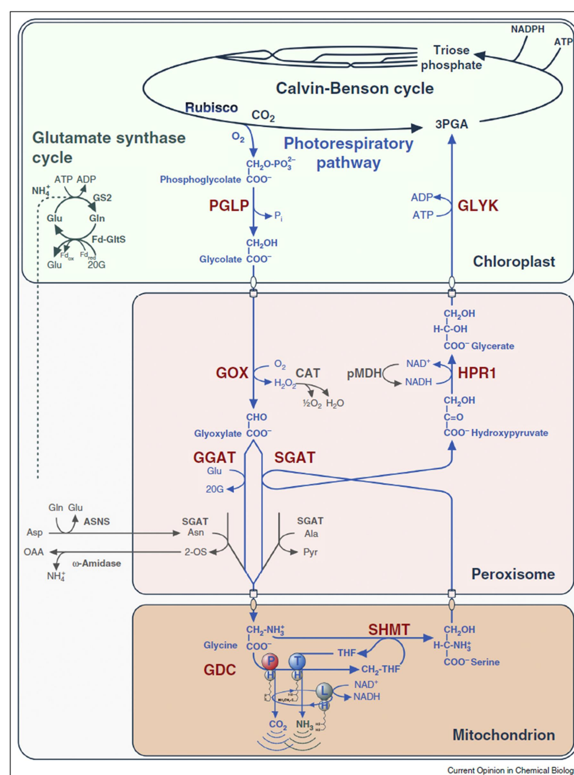

Figure 6. The photorespiratory pathway and its interconnection with photosynthetic Calvin–Benson cycle and NH3 assimilation in higher plants. (2OG, 2- oxoglutarate; 2-OS, 2-oxosuccinamate; 3PGA, 3-phosphoglycerate; Ala, alanine; Asn, asparagine; ASNS, asparagine synthetase; Asp, aspartate; CAT, catalase; FD-GltS, ferredoxin-dependent glutamate synthase; GDC, glycine decarboxylase complex; GGAT, glutamate:glyoxylate aminotransferase; Gln, glutamine; Glu, glutamate; GLYK, glycerate 3-kinase; GOX, glycolate oxidase; GS2, glutamine synthetase; HPR1, hydroxypyruvate reductase; OAA, oxaloacetate; PGLP, phosphoglycolate phosphatase; Pyr, pyruvate; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; SGAT, serine:glyoxylate aminotransferase; SHMT, serine hydroxymethyltransferase.). Reproduced from Hagemann and Bauwe, (2016) – *Current Opinion in Chemical Biology.*

Photorespiration cycle results in loss of previously fixed CO_2, release of NH3 and cost of energy, in addition to reduce 23% of the photosynthesis efficiency in C3 plants (SAGE et al., 2012). Approximately 50% of the photorespiratory CO_2 is lost in ambient. Taking into account that photorespiration results in a reduction in photosynthesis efficiency several works have been developed with the aim to reduce photorespiratory rate. However, instead improvement of photosynthesis, plants with deficiency in photorespiratory enzymes have been displayed photosynthesis impairment and could not grow in ambient air but can be rescued in air with a 20-fold to 30-fold

higher CO2 concentration than normal, where RuBP oxygenation becomes inhibited (Xu et al., 2009; Timm et al., 2012a; Schjoerring et al., 2006).

For example, a study with *Arabidopsis* knocked down in the major photorespiratory isoform of glutamate:glyoxylate aminotransferase 1 (GGT1) has shown reduced photosynthesis in consequence of glyoxylate accumulation (Dellero et al., 2015). *Arabidopsis* knocked out in PGLP1 displayed a strong reduction of net photosynthesis and growth even under 1% CO_2, suggesting that a significant amount of 2PG is produced even in high CO_2 concentration and this compound could be affecting harmfully CB cycle enzymes (Timm et al., 2012a). Glycerate is other photorespiratory metabolite with potential impact on the CB cycle since this compound inhibits sedoheptulose 1,7-bisphosphatase and fructose 1,6-bisphosphatase (Schimkat *et al.*, 1990). Glycine chelates magnesium ions and, thus could potentially affect Calvin–Benson cycle activity (Eisenhut *et al.*, 2007).

Timm et al., (2016) propose two hypotheses for the fact that blocked photorespiration cycle implies in photosynthesis impairment. Firstly, some photorespiratory metabolites could inhibit Calvin–Benson cycle enzymes that could inhibit RuBP regeneration and CO_2 fixation and, in a feedback loop, cause a reduction of photorespiration by lower RuBP supply (Timm et al., 2012a). Secondly, with a slower response time, some photorespiratory metabolites could be involved in transcriptional and translational regulation, as was experimentally shown at least for serine and glycine (Timm et al., 2013).

Additionally, some works have been shown that overexpression of photorespiratory enzymes implies in improvement photosynthesis. *Arabidopsis* overexpressing H-protein (Timm et al., 2012b) and L-protein (Timm et al., 2015) from

GDC and rice overexpressing SHMT1 (Zhang et al., 2015) displayed improved grown higher, net photosynthesis and lowered CO_2 compensation point. These results highlight the metabolic importance and the complexity of the photorespiration pathway.

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Peroxisomal APX knockdown and CAT inhibition enhance synthesis of antioxidant proteins mitigating photosynthesis impairment in rice

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Abstract:	Signaling pathways triggered by excess of peroxisomal H2O2 and downstream oxidative changes are still not completely understood. Here we employed an integrated approach involving peroxisomal ascorbate peroxidase knockdown (RNAiOsAPX4) and CAT inhibition by 3-AT in rice plants, and performed proteomics and physiological analyses. The aim was to elucidate why RNAiOsAPX4 plants under CAT inhibition display protective mechanisms more effective to mitigate photosynthesis impairment. APX4 deficiency differently regulated several proteins, inducing a metabolic rearrangement, possibly trigged by increased peroxisomal H2O2 or other oxidative signal. Higher photosynthetic efficiency displayed by RNAiOsAPX4 plants under CAT inhibition was related to higher amount and activity of Rubisco and photochemical efficiency of PSII, which was associated with alterations in the protein profile. The remarkable increase in proteins belonging to the ascorbate-glutathione cycle and other antioxidant pathways were probably crucial for photosynthesis protection against excess ROS. Besides, these plants also accumulated other important

	proteins related to sugar metabolism, photorespiration, protein translation and heat shock. We propose that RNAiOsAPX4 plants exhibit a metabolic reprogramming in response to CAT inhibition, possibly induced by signaling involving H2O2 or other oxidative signals. This biochemical rearrangement triggers a more effective antioxidant system to mitigate photosynthesis impairment under CAT deficiency.

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2 CHAPTER II: PEROXISOMAL APX KNOCKDOWN AND CAT INHIBITION ENHANCE SYNTHESIS OF ANTIOXIDANT PROTEINS MITIGATING PHOTOSYNTHESIS IMPAIRMENT IN RICE

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Running title: Antioxidant protection and photosynthesis in rice.

Abstract – Signaling pathways triggered by excess of peroxisomal H_2O_2 and downstream oxidative changes are still not completely understood. Here we employed an integrated approach involving peroxisomal ascorbate peroxidase knockdown (RNAiOsAPX4) and CAT inhibition by 3-AT in rice plants, and performed proteomics and physiological analyses. The aim was to elucidate why RNAiOsAPX4 plants under CAT inhibition display protective mechanisms more effective to mitigate photosynthesis impairment. APX4 deficiency differently regulated several proteins, inducing a metabolic rearrangement. Higher photosynthetic efficiency displayed by RNAiOsAPX4 plants under CAT inhibition was related to higher amount and activity of Rubisco and photochemical efficiency of PSII, which was associated with alterations in the protein profile. The remarkable increase in proteins belonging to the ascorbate-glutathione cycle and other antioxidant pathways were probably crucial for photosynthesis protection against excess ROS. Besides, these plants also accumulated other important proteins related to sugar metabolism, photorespiration, protein translation and heat shock. We propose that RNAiOsAPX4 plants exhibit a metabolic reprogramming in response to CAT inhibition, possibly induced by signaling involving H_2O_2 or other oxidative signals. This biochemical rearrangement triggers a more effective antioxidant system to mitigate photosynthesis impairment under CAT deficiency.

Keywords: H_2O_2 signaling; oxidative stress; photosynthetic efficiency; proteome; redox metabolism.

2.1 Introduction

Plant peroxisomes are the most important cellular site for H_2O_2 production in C3 plant species exposed to light. Several studies have revealed that redox changes in this organelle are able to affect the metabolic regulation in other cellular compartments by cross-talking mechanisms (Nyathi & Baker, 2006; Sewelam *et al.* 2014; Corpas, 2015). The majority of these finds have been achieved by using different CAT-deficient plants, evidencing that such responses are associated with photorespiratory H_2O_2 accumulation and downstream oxidative signaling events (Willekens *et al.* 1997; Rizhsky *et al.* 2002; Vandenabeele *et al.* 2004; Vanderauwera *et al.* 2011; Han *et al.* 2013; Rahantaniaina *et al.* 2017). Transcriptomic and proteomics analyses have revealed that these alterations in peroxisome metabolism are able to trigger several transcriptional and translational modifications, affecting some metabolic pathways including changes in various ribosomal and heat shock proteins (Vandenabeele *et al.* 2004; Vanderauwera, 2005; Vanderauwera *et al.* 2011).

The comprehension of how the deficiency in CAT activity and the H_2O_2 signaling can affect such processes is still plenty of gaps (Sousa *et al.* 2015). This problem has a practical importance because the majority of the common abiotic stresses can enhance the photorespiratory H_2O_2 production and some of them are capable of inducing CAT degradation and consequently decreasing its activity (Hertwig *et al.* 1992; Polidoros *et al.* 1997; Voss *et al.* 2013). In addition, much less is known about which other peroxisomal peroxidases are capable of overlapping CAT activity under such stressful conditions. For many years, some authors have suggested that peroxisomal APX (pAPX) isoforms are the most important plant peroxidases related to compensation and/or to supplementation of CAT activity (Yamaguchi *et al.* 1995; Mullen, 1999; Wang *et al.* 1999; Kavitha *et al.* 2008; Xu *et al.* 2008). However, there is no consensus on this issue, since some studies employing

Arabidopsis and rice plants deficient in peroxisomal APXs have suggested minor importance for these proteins as H_2O_2 scavengers (Narendra *et al.* 2006; Sousa *et al.* 2015).

CAT and pAPX present very contrasting K_M values for H_2O_2 and this fact has suggested that these peroxidases could display complementary or different metabolic roles in the peroxisomes (Mhamdi *et al.* 2012). In this context, pAPX could be important to keep fine-tuning and low concentrations of H_2O_2, allowing cellular signaling (Corpas, 2015). It has been proposed that these enzymes are externally bound to peroxisome membranes, with the catalytic site facing the cytosol (Yamaguchi *et al.* 1995; Kavitha *et al.* 2008; Ribeiro *et al.* 2017). This fact could be favorable for establishment of a signaling interface between peroxisomes and cytoplasm since pAPX activity could interfere in the H_2O_2 flux intensity from peroxisome to cytosol. Indeed, experimental evidences have demonstrated that peroxisomal H_2O_2 might migrate to cytosol and other neighbor organelles throughout membranes and triggers specific signals for gene expression (Mubarakshina *et al.* 2010; Corpas, 2015; Exposito-Rodriguez *et al.* 2017).

Some reports have proposed that H_2O_2 signaling in plant cell is very complex and apparently it involves cross-compartment relationships (Sewelam *et al.* 2014). Deficiency in cytosolic APX1 might induce increase in the chloroplast H_2O_2 levels associated with down-regulation in both chloroplastic APX isoforms, evidencing a cross-talking mechanism between chloroplast and cytosol (Davletova *et al.* 2005). These authors have suggested that the cytosolic ascorbate-glutathione (ASC-GSH) cycle is able to scavenge the excess H_2O_2 produced in other organelles such as peroxisomes, chloroplasts, and mitochondria, acting as an antioxidant buffer. Hydrogen peroxide produced in peroxisomes and chloroplasts is able to trigger differential gene expression (Sewelam *et al.* 2014), evidencing that H_2O_2 cellular localization associated with effectiveness of local antioxidant systems are important for induction of specific signaling responses.

The complexity of H_2O_2 signaling might explain some unexpected results reported in literature. For instance, tobacco and Arabidopsis double-mutant lines deficient in CAT2 and APX1 were more resistant to paraquat-induced oxidative stress than single mutants (Rizhsky *et al.* 2002; Vanderauwera *et al.* 2011). The authors have proposed that specific ROS signaling due to CAT and APX deficiency contributed for a more effective DNA repair system associated with cellular cycle control and suppression of the plant cell death (PCD) pathway, which resulted in higher resistance to tobacco double mutants (Vanderauwera *et al.* 2011). Our group has reported similar results in rice plants deficient in peroxisomal or cytosolic APX followed by pharmacological CAT inhibition (Sousa *et al.* 2015; Bonifacio *et al.* 2016). These results reinforce that physiological responses generated by signaling involving H_2O_2 and other ROS are very intricate since they are dependent on multifaceted metabolic and gene networks.

Cytosolic or peroxisomal APX-silenced rice plants exposed to CAT inhibition displayed lower ROS accumulation and higher photosynthesis resilience in comparison to CAT-inhibited NT plants (Sousa *et al.* 2015; Bonifacio *et al.* 2016). The mechanisms underlying such responses, especially a more effective antioxidant protection associated to a relative better photosynthetic performance, are still unknown to date. We have postulated that the increased constitutive-peroxisomal H_2O_2 due to APX deficiency is able to trigger over-expression of some antioxidant genes favoring the oxidative defense for a further oxidative stress. We have hypothesized that this mechanism is able to mitigate oxidative dangerous effects generated by CAT inhibition on the photosynthetic apparatus.

To test this hypothesis, we have performed an integrated approach employing reverse genetics by silencing of peroxisomal APX4 (RNAi-knockdown) combined with CAT pharmacological inhibition by 3-AT associated with high-throughput proteomic analysis and *in vivo* photosynthetic analyses. Our results corroborate the hypothesis evidencing that

peroxisomal APX deficiency followed by CAT pharmacological inhibition triggered a strong increase in several important proteins involved with crucial metabolic processes. Especially, accumulation of proteins belonging to the ASC-GSH cycle addressed to cytosol and chloroplasts, additionally to other antioxidants and protective proteins, were important to improve photosynthetic capacity. The physiological significance of these results for photosynthesis resilience under oxidative stress is discussed.

2.2 Material and methods

Growth and treatment conditions

Knockdown rice (*Oryza sativa japonica* CV. Niponbare) plants deficient in peroxisomal ascorbate peroxidase 4 (RNAiOsAPX4) were obtained as previously described (Ribeiro *et al*. 2017). Non-transformed (NT) and RNAiOsAPX4 seedlings were transferred to 2-L pots containing Hoagland-Arnon's nutritive solution (Hoagland & Arnon 1950) in a controlled growth chamber (day/night mean temperature of 29/24 °C, mean relative humidity of 68%, photoperiod of 12 h and 400 µmol photons m^{-2} s^{-1} PPFD) for 40 days.

For CAT inhibition treatment, 40-day-old NT and RNAiOsAPX4 plants were sprayed with 10 mM 3-amino-1,2,4-triazole (3-AT) dissolved in 10 mM N-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid (HEPES) buffer at pH 6.5 containing 1.5 mM $CaCl_2$ and 0.1% Triton X-100. Shoots were sprayed once with 50 mL of 3-AT solution at 6:00 pm and plants were kept in the dark during 12 hours. Afterwards, the plants were maintained for 12 h under light condition (400 µmol photons m^{-2} s^{-1}) and, at the end of the photoperiod, the gas exchange determinations and leaf sampling for proteomic analysis were performed. The

control plants were sprayed with a similar 3-AT-free solution. For the short-term CAT inhibition and H_2O_2 accumulation experiment, 40-day-old NT and RNAiOsAPX4 leaves were sprayed once with 50 mL AT and control solutions similarly to described above and leaf sampling was performed at 0, 0.5, 1.5 and 2.5 hours after 3-AT exposure. During this time course experiment, plants were kept under moderate light regime (400 µmol photons m^{-2} s^{-1}). Aiming to investigate the consequences of CAT and APX4 deficiencies on PSII activity, 40-day-old NT and RNAiOsAPX4 plants were sprayed with 10 mM 3-AT solution and kept in moderate light regime (400 µmol photons m^{-2} s^{-1}) during 1 hour. Subsequently, plants were exposed to high light (1000 µmol photons m^{-2} s^{-1}) for one hour and *in vivo* chlorophyll *a* fluorescence measures were performed. Controls were sprayed with a similar 3-AT-free solution.

Gas exchange measurements

The intercellular CO_2 partial pressure (C_I)-dependent photosynthesis (A) curve was measured using a portable infrared gas analyzer system, equipped with an LED source and a leaf chamber (IRGA LI-6400XT, LI-COR, Lincoln, NE, USA). For the A/C_I curve, the C_I was varied between 50 and 1,800 ppm CO_2 and the photosyntheticaly active radiation (PAR) was set as 1,000 µmol photons m^{-2} s^{-1}. The maximum Rubisco carboxylation rate (V_{cmax}) and maximum photosynthetic electron transport rate (J_{max}) were calculated from the A/C_I curve (Sharkey *et al*. 2007).

Chlorophyll a fluorescence

The saturation pulse method (Schreiber *et al.* 1995) was used in order to evaluate the *in vivo* photosystem II activity. To perform the measurements, a Dual-PAM-100 fluorometer (Walz, Germany) was employed. Initially, detached rice leaves were dark-acclimated during 30 minutes in order to assess the maximum quantum efficiency of PSII estimated as [Fv/Fm = (Fm-Fo)/Fm], where Fo and Fm represent the minimal and the maximum fluorescence after a saturation pulse in the dark, respectively. The saturation pulse intensity and duration were 8,000 µmol photons m^{-2} s^{-1} and 0.6 s, respectively. Next, the actinic light (500 µmol photons $m^{-2}.s^{-1}$) was turned on and a second saturation pulse was triggered after 3 min under this light condition, in order to assess the actual quantum efficiency estimated as [YII = (Fm'-Fs)/Fm'], where Fm' and Fs consisted in the maximum and steady state fluorescence in the light, respectively. The electron transport ratio from PSII was estimated as [ETRII = YII×500×0.5×0.8], assuming that 80 percent of incident light reached the PSII antennas and an equal energy distribution between PSII and PSI had occurred.

Western blot

The Rubisco large subunit (rbcL) was identified in total soluble extract by immunoblotting. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10 µg proteins from total soluble extract. After electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose membrane (Towbin *et al.* 1979), and polypeptide detection was performed using specific polyclonal antibodies against the rbcL (Agrisera©, Vännäs, Sweden). The tagged protein was detected by luminescence,

through the reaction between the second antibody bound to the target protein and the ECL reagent (GE ref. RPN2106).

Hydrogen peroxide concentration

The H_2O_2 concentration was assayed by the resorufin production method (Zhou *et al.* 1997), using the Amplex-red^{TM} kit. Leaf extracts were incubated with a reaction buffer containing Amplex Red (Invitrogen) and horseradish peroxidase (Sigma). After 30 min of reaction, the resorufin was quantified in spectrophotometer set 560 nm and the H_2O_2 concentration was estimated by a standard curve.

Catalase activity

Catalase activity was assayed from total soluble protein extract. The protein extraction was performed utilizing N_2 liquid in the presence of 100 mM phosphate buffer, pH 7.5, containing 2 mM EDTA and 1 mM ASC at 4 °C. The activity was measured by following the oxidation of H_2O_2 at 240 nm over a 300 s interval at 25 °C in the presence of 50 mM potassium phosphate buffer pH 7.0, containing 20 mM H_2O_2 (Havir & McHale 1987). The CAT activity was calculated according to the H_2O_2 molar extinction coefficient = 36 M^{-1} cm^{-1} and the results were expressed as µmol H_2O_2 mg $protein^{-1}$ min^{-1}. The total protein concentration was performed according to Bradford (1976).

Protein extraction, digestion and desalt for proteomic analysis

Approximately 700 mg of leaves fresh matter from each plant were ground to a powder, frozen and dried by lyophilization. The extracted proteins were precipitated in 10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone, and resuspended in a lysis buffer containing 8 M urea, 2 M thiourea, 5% CHAPS and 2 mM tributylphosphine. The protein concentrations were determined using the Bradford method (Bradford, 1976) with bovine serum albumin as standard. The samples were purified with methanol and chloroform to remove detergent from samples and centrifuged at 20,000 × *g* for 10 min to achieve phase separation. The upper phase was discarded and methanol was added to the lower phase. The solutions were again centrifuged at 20,000 × *g* for 10 min and the resulting pellets were dried. Dried samples were reduced with 25 mM dithiothreitol and alkylated with 30 mM iodoacetamide. 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 the dark. Peptides were acidified with 20% formic acid (pH < 3) and desalted with a C18-pipette tip (Nikkyo Technos, Tokyo, Japan). The samples were analyzed by nano-liquid chromatography with *in tandem* mass spectrometry (LC-MS/MS).

Protein identification using nano-liquid chromatography mass spectrometry

The peptide samples were separated using an Ultimate 3000 nano-LC system (Dionex, Germering, Germany), and the peptide ions were detected using a LTQ Orbitrap Discovery MS nanospray (Thermo Fisher Scientific, San Jose, CA, USA) with data-dependent

acquisition mode with Xcalibur software (version 2.1, Thermo Fisher Scientific). The peptide samples were loaded onto a C18 PepMap trap column (300 µm I.D. × 5 mm, 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^{-1}. The eluted peptides were loaded and separated on a C18 capillary tip column (75 µm I.D. × 120 mm, NikkyoTechnos) 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 90 sec to prevent the repetitive selection of peptides (Zhang *et al.* 2009).

Data acquisition by mass spectrometry analysis

Identification of proteins was performed using Mascot search engines (version 2.4.1., Matrix Science, London, UK) with a rice protein database (50,253 sequences and 15,266,515 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_ 2013-4-24) and protein sequences predicted computationally (IRGSP-1.0_ predicted protein_2013-3-9). The Proteome Discoverer software (version 1.4, Thermo Fisher Scientific) was used to process the acquired raw data files. For the Mascot searches, the carbamido methylation 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 at 0.5 Da, and the peptide charge 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

The commercial label-free quantification package SIEVE was used for the differential analysis of relative abundances of peptides and proteins between samples. 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 bin codes (http://mapman.gabipd.org/) (Usadel *et al.* 2005).

Assay of pAPX activity

Activitiy of pAPX in the leaf extract were separately determined as previously reported by Amako, Chen and Asada, 1994 and Miyake and Asada, 1996, utilizing the different sensitivities of pAPXto a low-AsA condition. Rice leaves were ground to a fine powder in liquid N2 and the homogenated 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 CaCl2 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 120 000 × g membrane fraction was washed and suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ascorbate. The membrane fraction (10µl) was added to 5.0 ml of N2-bubbling 50 mM potassium phosphate buffer (pH 7.0) containing 10 µM H2O2. At 1.5, 2, 2.5, and 3 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 H2O2. The oxidation of ascorbate was followed by a decrease in the A290n, and the results are plotted on the graph. pAPX activity was calculated from the inactivation curve of each isoenzyme.

Statistical Analyses

The experiments were carried out in a completely randomized design with four replicates, each one represented by an individual pot containing two plants. Data were analyzed by ANOVA and averages were compared by Tukey's test at the 0.05 confidence level. The standard deviation is plotted in all graphics. The proteomics analyses were performed as previously described ($p \leq 0.05$).

2.3 Results

RNAi-silencing targeted to rice peroxisomal OsAPX4 strongly down-regulated both OsApx3 and OsApx4 gene expression and AT supplying rapidly inhibited CAT activity

As previously reported (Ribeiro *et al*. 2017), RNAi-silenced rice lines do not display phenotypic changes at morphological and physiological levels during the vegetative phase despite both peroxisomal Os*APX3* and *OsAPX4* transcript amounts have been strongly decreased after knockdown (Ribeiro *et al.* 2017). This intense silencing was related to remarkable decrease in APX activity in the microbody fraction (peroxisome membranes), which reached non-detectable levels when compared to NT plants (Figure S1). The silenced line displayed slight increase in the constitutive H_2O_2 level in leaves compared to NT plants. NT and RNAiOsAPX4 rice were supplied with 10 mM 3-AT to promote a rapid CAT inhibition (an inhibition of almost 100% occurred after 90 min – Fig. 1A). However, after a

fast 3-AT exposure (30 min), the H_2O_2 levels prominently increased in both treated plants (85% in NT versus 63% in RNAiOsAPX4, respectively) and abruptly decreased to initial levels after 150 min, also in both plants (Fig. 1B). This strategy allowed the simultaneous deficiencies of pAPX and CAT in the transgenic line and the single CAT deficiency in NT leaves for about 24 h, which was enough to affect the translation and proteomics profile (Bonifacio *et al.* 2016). After 3-AT supplying for 24 h, both NT and transformed plants did not exhibit any visual toxicity symptoms (Figure S2), evidencing that the employed 3-AT dose (on 40-day-old plants) was not enough to cause generalized side effects as previously reported (Bonifacio *et al.* 2016). In order to verify if the effects induced by 3-AT on photosynthesis were exclusively due to CAT inhibition or by indirect mechanisms, an experiment was carried out under non-photorespiratory conditions (3% CO_2). Under absence of photorespiration, both NT and RNAiOsAPX4 plants displayed similar photosynthesis in presence of 3-AT, compared to their respective controls (data not shown), indicating that the effects were essentially related to CAT inhibition and downstream oxidative effects.

The RNAiOsAPX4 line displayed higher photosynthetic capacity than NT plants after CAT inhibition

To verify if rice plants deficient in peroxisomal APX followed by CAT inhibition display lower photosynthetic capacity than 3-AT-treated NT plants, A-Ci curves were performed (Figure S3). Both genotypes showed similar photosynthesis (P_N) values under normal growth conditions. Catalase inhibition induced strong decline in P_N of both plant types, but, unexpectedly, the decrease was more prominent in NT than in the RNAiOsAPX4 line (63% and 37%, respectively compared to their respective controls in ambient CO_2 –

Figure 2A). The higher decrease in P_N was accompanied by a strong reduction in V_{cmax} and J_{max}, which decreased 66% and 57% in NT, and 14% and 17% in RNAiOsAPX4, respectively (Figures 2B and 2C). The higher Rubisco carboxylation rate and P_N exhibited by RNAiOsAPX4 plants was strongly related to the increased Rubisco abundance, which was significantly increased in RNAiOsAPX4 and decreased in NT leaves, both in comparison to their respective controls (Figure 3). In order to verify if the effects caused by 3-AT on gas exchange parameters were related to photochemical disturbances, some PSII activity indicators were determined. The potential quantum yield of PSII (Fv/Fm) in both NT and RNAiOsAPX4 plants under normal growth conditions were similar. However, after a short time exposure to 3-AT, the Fv/Fm significantly decreased only in the non-transformed genotype (Figure 4A). These photochemical alterations were paralleled by changes in actual quantum yield of PSII (ΦPSII) and electron transport rate of PSII (ETR) in both genotypes (Figures 4B and 4C).

CAT inhibition in RNAiOsAPX4 plants induced strong increase in abundance of proteins involved in several metabolic pathways

Silenced rice plants displayed increase of 211 different proteins involved in several biochemical pathways, in comparison to NT, under normal growth conditions (Figure 5). Remarkably, RNAiOsAPX4 plants displayed increase in 649 proteins after CAT inhibition, whereas NT plants showed much lower increment (only 252 proteins). Quantitatively, the increase in abundance of proteins involved especially metabolic processes related to photosynthesis (59), TCA cycle (27), amino acids (37), biotic/abiotic stresses (33), redox pathways (32), and DNA/RNA (38) and protein metabolism (147) (Figure 5). Interestingly,

the amount of decreased proteins was much lower than those previously mentioned to increased proteins for all the treatments. Differently of increased proteins, NT and RNAiOsAPX4 plants displayed similar results for the amount of decreased proteins under CAT inhibition (70 and 74, respectively). Additionally, these proteins were related to a reduced number of metabolic classes than those with increased amount, especially in RNAiOsAPX4 plants.

The Venn diagrams summarize the global numbers of protein abundances relative to the effects of APX4-knockdown, CAT inhibition, and their intersections (Figure 7). Silenced plants grown under normal conditions displayed 70 proteins that were also increased only under CAT inhibition. Among the 636 proteins that had their amount increased in RNAiOsAPX4 in presence of CAT inhibition, only 121 (19%) were also increased in NT plants under the same experimental condition (Figure 7). Thus, these results indicate that the majority of the increased proteins in the RNAiOsAPX4 line, by effect of CAT inhibition, were not increased in NT plants.

RNAiOsAPX4 plants exhibited amount increase of several important proteins involved with photosynthesis, redox metabolism, abiotic stress and photorespiration in response to CAT inhibition

RNAiOsAPX4 plants under normal growth conditions displayed significant increase in abundance of the following photosynthetic proteins: fructose-diphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, triose-phosphate isomerase and ferredoxin-NADP reductase. Besides these proteins, silenced plants, in presence of CAT inhibition, exhibited intense increase in abundances of other important photosynthetic proteins,

especially those belonging to the Calvin-Benson cycle, such as fructose-diphosphate aldolase isoforms, fructose 1-6-diphosphatase, glyceraldehyde 3-phosphate dehydrogenase, Rubisco Large Subunit and triose-phosphate isomerase (Figure 8). Also, other proteins belonging to the photochemical phase were increased: putative ATPE, E subunit of chloroplastic ATPase, ferredoxin-NADP reductase (FNR), LHCB2 and LHCB4. All these proteins, except fructose-diphosphate aldolase 1, LHCB2 and LHCB4 were also increased in NT plants under CAT inhibition, but in a lesser extent compared to RNAiOsAPX4 (Figure 8). On the other hand, CAT inhibition induced decreased amount of some proteins involved in the photochemical phase, in both NT and RNAiOsAPX4 plants, such as ferredoxin 1, photosystem I reaction center subunit II, chlorophyll a-b binding protein, PSBO2, PSBP1 and PSBQ3, whereas CAT-inhibited RNAiOsAPX4 line displayed decreased also in LHCB1 amount. RNAiOsAPX4 displayed increase in some photorespiratory proteins including glycine decarboxylase P (GDC), serine hydroxy-methyl transferase (SHMT), chloroplastic gluthamine synthetase (GS2), and ferredoxin-dependent glutamate synthase (Fd-GOGAT). In presence of CAT inhibition, these silenced plants showed significant increase in the amounts of GDC, SHMT and GS2, whereas NT plants under CAT deficiency displayed lower accumulation of these proteins (Figure 8).

The figure 9 shows the responses of proteins involved with redox metabolism and stress defence. RNAiOsAPX4 plants showed increase in some proteins in this group, especially chloroplastic GR, cytosolic SOD Cu-Zn, 2-Cys peroxiredoxin, chaperone ClpC2, HSP 24-1 and HSP 90. Remarkably, the CAT inhibition in the RNAiOsAPX4 line induced a strong increase in most of the proteins belonging to redox defense, in comparison to 3-AT-treated NT plants. Some of these proteins are involved in the ASC-GSH cycle (MDHR, DHR, GR, APX and GPX) and ascorbate and glutathione synthesis pathways (GDP-mannose epimerases and glutamate-cysteine ligases, respectively), while others are classified as thiol

proteins (glutaredoxin, peroxiredoxins and thioredoxin H), chaperone isoforms, and heat shock proteins (HSP 24-1, HSP 17-4, HSP 70, HSP 81 and HSP 90). Interestingly, most of those redox proteins are addressed to cytosol and chloroplasts while none of them are targeted to peroxisomes (Figure 9).

CAT inhibition in RNAiOsAPX4 plants positively regulated proteins related to signaling pathways and metabolism of nucleic acids, proteins, sugars, and amino acids

RNAiOsAPX4 plants displayed increase in abundance of several proteins involved in polypeptide synthesis (Figure 10). Among these, some were strongly increased, such as puromycin-sensitive aminopepidase, aspartic proteinase, 26S proteasome regulatory ATPase subunit 4 and 6, and 40S, 50S and 60S ribosomal proteins. After CAT inhibition, the RNAiOsAPX4 line presented much higher increase in the amount of several proteins compared to all the other treatments. Among these positively regulated proteins are aminopetidase M1, proteasomes, chaperonins, elongations factors, 40S, 50S and 60S ribosomal proteins, and importin subunits. RNAiOsAPX4 plants also displayed increase in some proteins related to nucleus metabolism, such as histones, putative polyribonucleotide-nucleotidyltransferase, eukaryotic initiation factors, adenylsuccinate synthetase 2, and DEAD-box ATP-dependent RNA helicase 3 and 37 (Figure S4).

Although the RNAiOsAPX4 line grown under normal condition did not display any change in the amount of signaling proteins, they were strongly increased in silenced line under CAT inhibition, in comparison to NT (Figure S5). These proteins include 14-3-3-like proteins, calmodulin-like protein 1, calreticulin, ethylene-responsive small GTP-binding protein, a guanine nucleotide-binding protein subunit, and Obg-like ATPase 1. The APX4

deficiency virtually did not affect the amount of the identified proteins involved in amino acid and sugar metabolism (Figures S6). However, several of these proteins were strongly increased in these plants after CAT inhibition, including some important enzymes such as tryptophan synthase, aspartate aminotransferase, glutamate decarboxylase, glutamine synthetase, 4-alpha-glucanotransferase, fructokinase, sucrose synthases and sucrose phosphatase. In contrast, less than half of these proteins, which had slight amount increase, were identified in NT plants exposed to CAT inhibition (Figures S6).

2.4 Discussion

In the current study we have postulated the hypothesis that an increased constitutive H_2O_2 level in leaves of peroxisomal APX silenced rice could be associated with changes in synthesis of several important proteins involved in photosynthesis protection. We previously verified that rice RNAiOsAPX4 plants display early leaf senescence involving ROS signaling (Ribeiro *et al.* 2017). However, in that study no change in the extractable leaf H_2O_2 level was detected, suggesting that pAPX deficiency could affect the concentration of this ROS in a local manner, possibly in the cytosolic region adjacent to peroxisomes, since this enzyme is bound externally to membranes of this organelle (Sousa *et al*. 2015). Indeed, H_2O_2 detection in leaf tissues is very complex, as this ROS is produced in several cellular compartments and because it displays a very short lifetime. Previous study based on transcriptomic analyses in Arabidopsis plants in response to H_2O_2 derived from different organelles, has evidenced that this ROS displays complex signaling pathways, which is dependent on the cellular production site (Sewelam *et al.* 2014).

In this current study, total H_2O_2 level was slightly increased in RNAiOsAPX4 plants, suggesting that the observed changes in the protein profile could have been related to this accumulation. We have assumed that these alterations in proteomics data were possibly a direct or indirect consequence of a metabolic reprogramming signalized by H_2O_2 and/or other redox-signaling molecules. Similar responses have already been observed in rice plants deficient in both cytosolic APX (Ribeiro *et al.* 2012) and the authors have suggested that this priming effect induced by increased H_2O_2 favored the phenotypic plasticity. Indeed, the differential regulation of proteins exhibited by RNAiOsAPX4 in our study suggests their metabolic networks were modified. This changed biochemical network could specifically interact with peroxisomal H_2O_2/ROS or other derived redox signals produced from CAT inhibition, generating a new metabolic arrangement. We have argued that the metabolic network changed by pAPX deficiency is more responsive to high levels of peroxisomal H_2O_2 or other redox signals, displaying deeper physiological changes, which mitigate photosynthesis impairment. Indeed, all photosynthetic indicators studied here clearly revealed that, under CAT inhibition, RNAiOsAPX4 plants displayed higher photosynthesis efficiency than NT plants. Why RNAiOsAPX4 silenced plants showed a better photosynthetic performance under CAT inhibition?

The various proteins accumulated in transformed plants could have contributed direct or indirectly for mitigation of photosynthesis impairment under CAT deficiency. However, our main hypothesis is that accumulation of antioxidant proteins, especially the components of the ASC-GSH cycle, was the most important players responsible for generating a more effective protection in silenced plants. Indeed, it is widely known that the presence of an efficient antioxidant system in chloroplasts and cytosol is crucial for photosynthetic protection (Davletova *et al.* 2005; Maruta *et al.* 2009; Maruta *et al.* 2016; Naranjo *et al.* 2016). In this study, pAPX plants accumulated important antioxidant proteins localized in

cytosol and chloroplast, especially those belonging to the ASC-GSH cycle, some important peroxidases, glutaredoxins, thioredoxins, GDP-mannose epimerases (involved in the ASC synthesis pathway) and glutamate-cysteine ligases (the most important enzyme in the GSH synthesis pathway). It has been widely reported that a coordinate action of these enzymes and the reducing ASC and GSH agents contributes to maintain the H_2O_2 homeostasis, avoiding oxidative stress and contributing to mitigate photosynthesis impairment in presence of excess ROS condition (Foyer & Noctor, 2011; Rahantaniaina *et al.* 2017).

Interestingly, NT and RNAiOsAPX4 plants under CAT inhibition did not change the amounts of any antioxidant proteins addressed to peroxisomes. These findings suggest that antioxidant systems localized in cytosol and chloroplasts, especially the ASC-GSH cycle, are important to cope with excess peroxisomal H_2O_2. Some experimental evidences have suggested that cytosolic APX and ASC-GSH cycle display a central role in the control of excessive H_2O_2 produced in other organelles such as peroxisomes, chloroplasts and mitochondria (Davletova *et al.* 2005). In addition, these authors verified that excess cytosolic H_2O_2 generated by APX deficiency might inhibit photosynthesis by inducing oxidative stress in chloroplasts. In the current study, the higher accumulation of those antioxidant proteins in RNAiOsAPX4 plants exposed to CAT inhibition was closely related to a mitigation of photosynthesis impairment. Indeed, excess ROS can cause denaturation of crucial Calvin cycle proteins by carbonylation, including Rubisco (Davletova *et al.* 2005), and delays in the PSII repair process (Nishiyama *et al.* 2011). These dangerous processes induce strong restriction in CO_2 assimilation and photoinhibition, contributing to a generalized impairment in photosynthesis (Foyer *et al.* 2012).

RNAiOsAPX4 plants under CAT-inhibited condition also displayed intense accumulation of some important proteins related to photosynthesis efficiency. In accordance, the amounts of some photorespiration proteins such as GOX, SHMT, GDC subunits, GS2 and

Fd-GOGAT were enhanced suggesting that this process could have contributed as an alternative sink for excess photosynthetic energy (Voss *et al.* 2013). In addition, these plants also displayed up-regulation of proteins involved in C and N metabolism (SuSy, GS1, GDH, aspartate aminotransferase among others). The activities of these enzymes might favor the photosynthetic energy consumption and carbohydrate transport. These processes might contribute to reduce the sugar levels in chloroplast, which might attenuate a negative feedback on expression of photosynthesis-related genes (Lobo *et al.* 2015).

Other protective important proteins not directly related to photosynthesis also accumulated in RNAiOsAPX4 plants in the CAT inhibition treatment. The amounts of several heat shock proteins (HSP) were strongly increased, including the HSP 24-1 and HSP 17-4 that are responsive to osmotic stress (Zou *et al.* 2009) and increased H_2O_2 levels (Vandenabeele *et al.* 2004). Other accumulated heat shock proteins include HSP90, HSP81, HSP70 and three chaperone isoforms, which are involved in general responses to abiotic stress and protein structure protection (Timperio *et al.* 2008; Hoang *et al.* 2015; Wang *et al.* 2016). Besides these protective proteins, the RNAiOsAPX4 plants also displayed amount increase of several other processes involved especially with protein metabolism. Collectively, the expression of these non-photosynthetic proteins could have contributed to a better physiological performance and indirectly collaborating for a better photosynthetic efficiency.

The obtained results involving the mitigation of photosynthetic disturbance under conditions of peroxisomal APX knockdown followed by CAT deficiency are complex. We have attributed such responses to the complexity of the H_2O_2/ROS signaling as have been widely reported. Double mutants deficient in CAT2 and cytosolic APX1 exposed to oxidative stress have also displayed intriguing responses (Rizhsky *et al.* 2002; Vanderauwera *et al.* 2011). In these reports, double mutant tobacco and Arabidopsis lines were more resistant to paraquat-induced oxidative stress than single mutants. The authors have proposed that a

specific ROS signaling due to CAT and APX deficiency contributed for expression of a more effective DNA repair system associated with the control of cellular cycle and suppression of the PCD pathway, which conferred higher resistance for tobacco double mutants (Vanderauwera *et al.* 2011). Other works employing CAT deficient mutants have revealed deep changes in expression of genes involved in various cellular processes (Rizhsky *et al.* 2002; Vandenabeele *et al.* 2004).

The question concerning the signaling involving CAT deficiency and peroxisomal H_2O_2 accumulation has devoted several years of studies (Willekens *et al.* 1997; Rizhsky *et al.* 2002; Vandenabeele *et al.* 2004; Vanderauwera *et al.* 2011; Han *et al.* 2013; Rahantaniaina *et al.* 2017) but this problem has not been solved to date (König *et al.* 2017; Rahantaniaina *et al.* 2017). Several reports in various plant species have demonstrated that under these conditions a rapid and intense GSH oxidation occur accompanied by increase in the total glutathione pool (Willekens *et al.* 1997; Han *et al.* 2013; König *et al.* 2017). Some authors have questioned if the signaling agent is H_2O_2 or changes in the GSH redox state since that several experimental evidences have confirmed that this antioxidant might act as an important signaling molecule (König *et al.* 2017; Rahantaniaina *et al.* 2017). Interestingly, in the current study, two glutamate-cysteine ligases, the most important enzyme of GSH synthesis pathway, were strongly accumulated in RNAiOsAPX4 plants under CAT inhibition. We previously demonstrated that rice plants display strong increase in both GSH oxidation and total glutathione pool in this condition, suggesting that these redox changes could be involved in signaling alone or additionally to peroxisomal H_2O_2 (Sousa *et al.* 2015; Bonifacio *et al.* 2016).

Previously, our group has reported other unexpected physiological responses in rice plants deficient in peroxisomal APX followed by pharmacological CAT inhibition (Sousa *et al.* 2015). These plants displayed several phenotypic changes, exhibiting a more effective

antioxidant protection under high photorespiration conditions and a better photosynthetic performance compared to plants deficient only in CAT activity. However, the underlying mechanisms involved in these distinct responses triggered by peroxisomal H_2O_2 or other derivative ROS, or even by changes in the GSH redox state, are unknown. In the current study, we elucidated some of the reasons by which rice plants deficient in peroxisomal APX followed by CAT inhibition have higher photosynthetic resilience compared to NT plants exposed to CAT deficiency only. We also revealed the antioxidant players and the involved mechanisms displayed RNAiOsAPX4 plants that might confer protection for photosynthesis under an oxidative condition. The figure 11 summarizes the presented hypothesis.

In summary, pAPX-deficient rice plants display several protein profile changes leading to a metabolic rearrangement, which is possibly trigged by increased H_2O_2/ROS or GSH oxidation. This could interact specifically with high peroxisomal H_2O_2 concentration or other derivative-signaling molecules generated by a transient CAT inhibition, generating a second phenotype different from rice plants only deficient in CAT. These phenotypic differences involved essentially the increased amount of several proteins related to various metabolic processes, in special antioxidant proteins localized in cytosol and chloroplasts which include components of the ASC-GSH cycle, other peroxidases such as GPX and peroxiredoxins, and other thiol-proteins such as GRX and TRX. Consequently, such changes could contribute to contrasting physiological responses, particularly those related to mitigation in photosynthesis impairment under oxidative stress condition.

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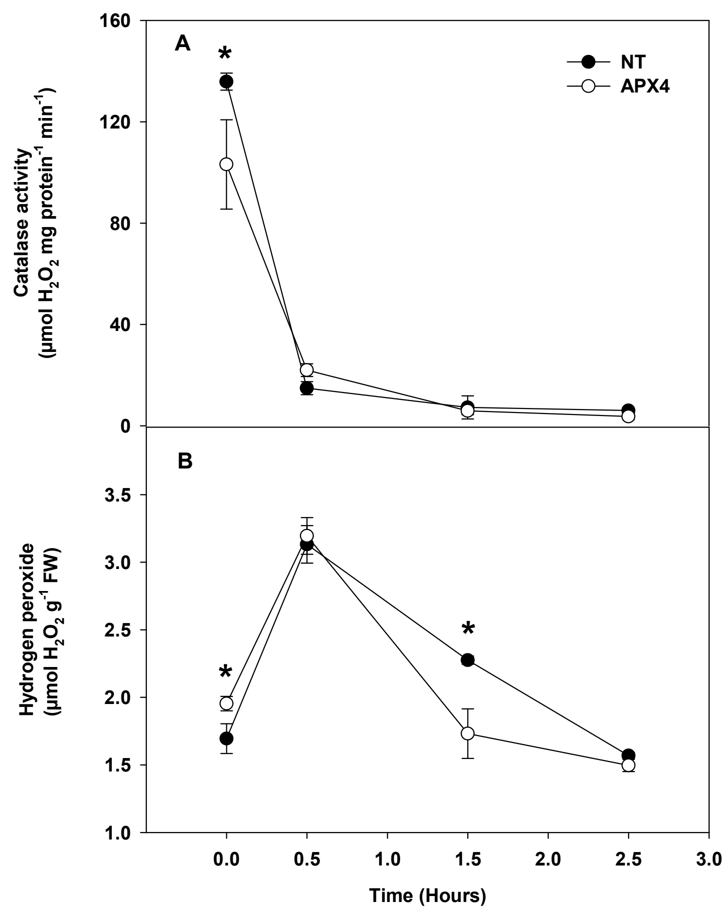


Figure 1. (A) CAT activity and (B) Hydrogen peroxide content in leaves of non-transformed (NT) and RNAiOsAPX4 rice plants exposed to different times of CAT inhibition with 10 mM AT (0; 30; 90 and 150 minutes). Points represent average and vertical bars the standard deviation (n=4).

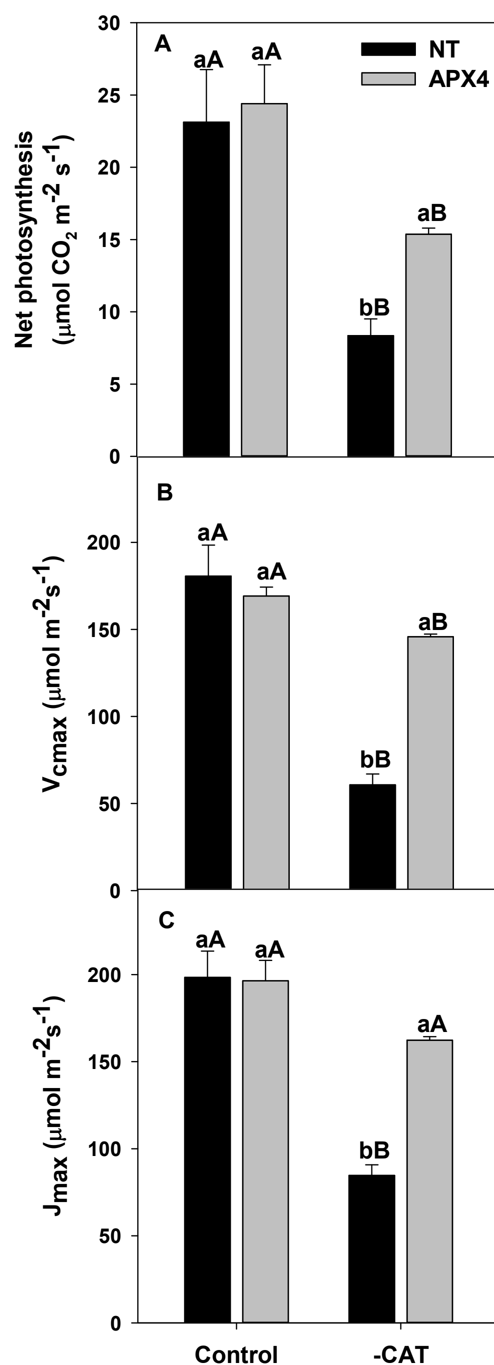


Figure 2. (A) Net photosynthesis (P_N), (B) Maximum carboxylation velocity (V_{cmax}), (C) Maximum electron flow (Jmax) in leaves non-transformed (NT) and RNAiOsAPX4 rice plants exposed 10 mM AT (-CAT) and control. The values represent average of four replicates and vertical bars represent standard deviation. Capital letters indicate significant differences between treatments (control and -CAT) within the same genotype and lower cases represent significant differences between genotypes (NT and APX4) within each treatment.

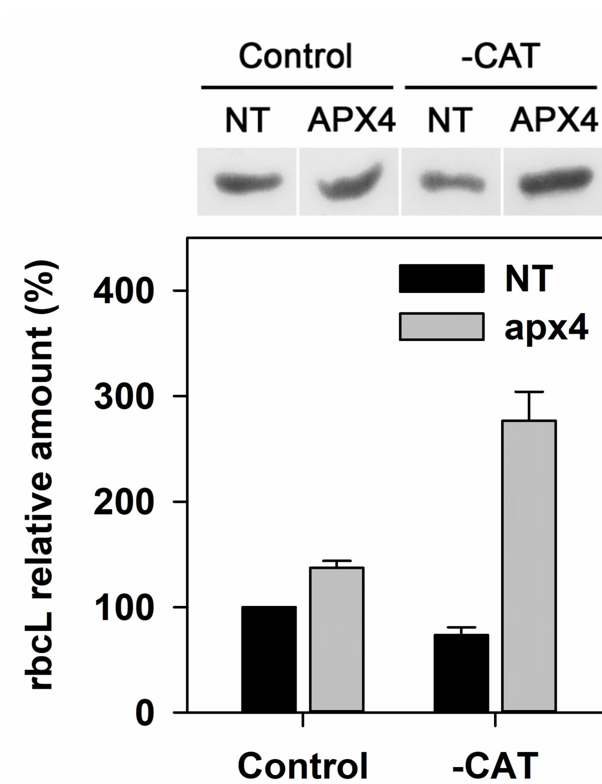


Figure 3. Western blot of Rubisco Large Subunit (RcbL) in leaves non-transformed (NT) and RNAiOsAPX4 rice plants exposed 10 mM AT (-CAT) and control. For SDS-page, 10 µg of soluble proteins was loaded in each lane. The blot image is representative of three independent runs.

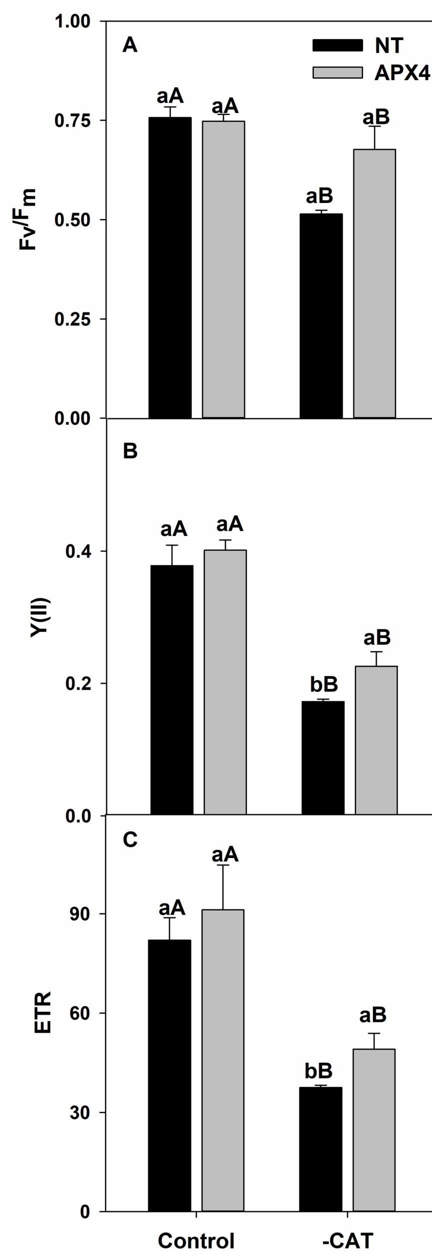


Figure 4. Changes in (A) Electron transport rate (ETR), (B) maximum efficiency of PSII and (C) maximum efficiency of PSII (F_v/F_m) in leaves non-transformed (NT) and RNAiOsAPX4 rice plants exposed 10 mM AT (-CAT) and control. Bars represent means of four replicates ±SD. Capital letters indicate significant differences between treatments (control and -CAT) within the same genotype and lower cases represent significant differences between genotypes (NT and APX4) within each treatment.

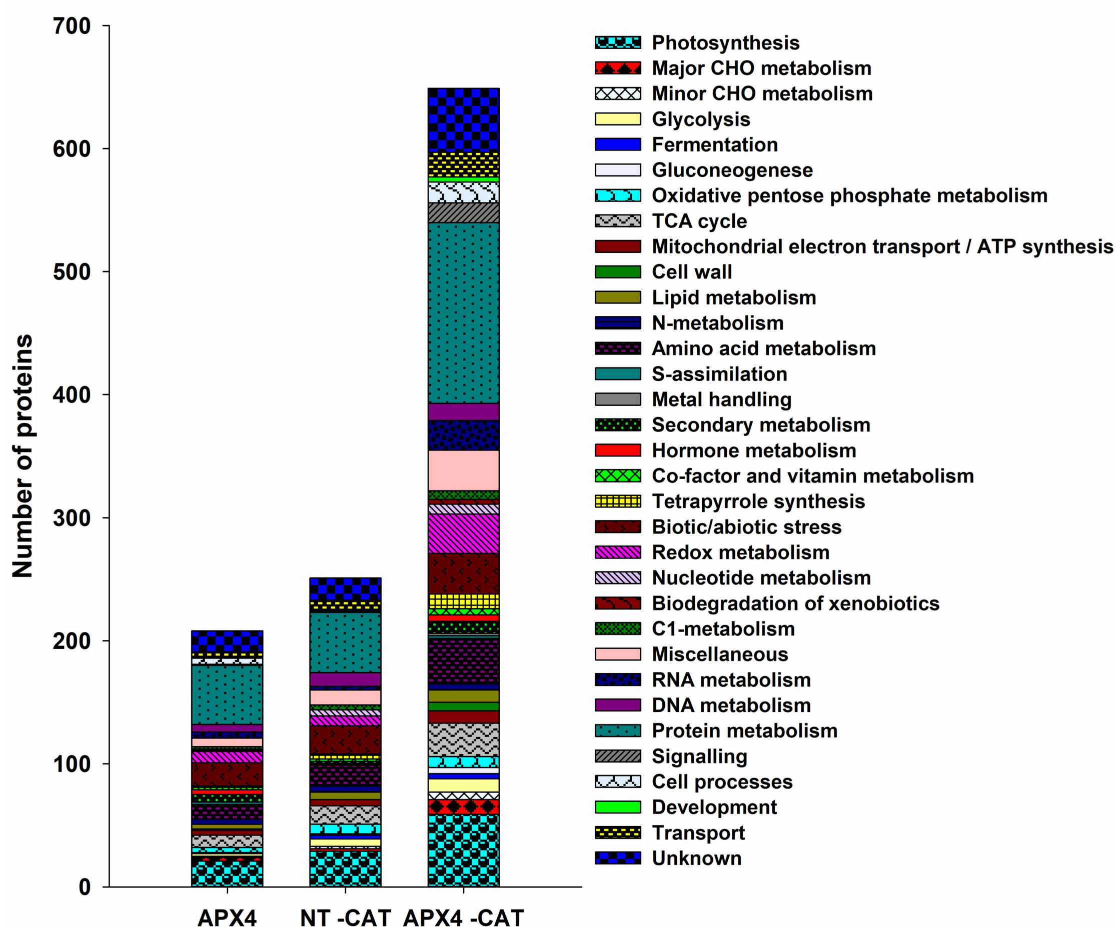


Figure 5. Functional classification of proteins with increased abundance. The amount of each protein in leaves of RNAiOsAPX4 under normal growth condition and in NT and RNAiOsAPX4 plants exposed to 10 mM AT (-CAT) were all compared to the respective protein amount found in NT control plants. Each protein content was considered increased if the $\log_2$ was higher than 0.5. The proteins were classified by the software (MapMan) according to their involvement in each metabolic process.

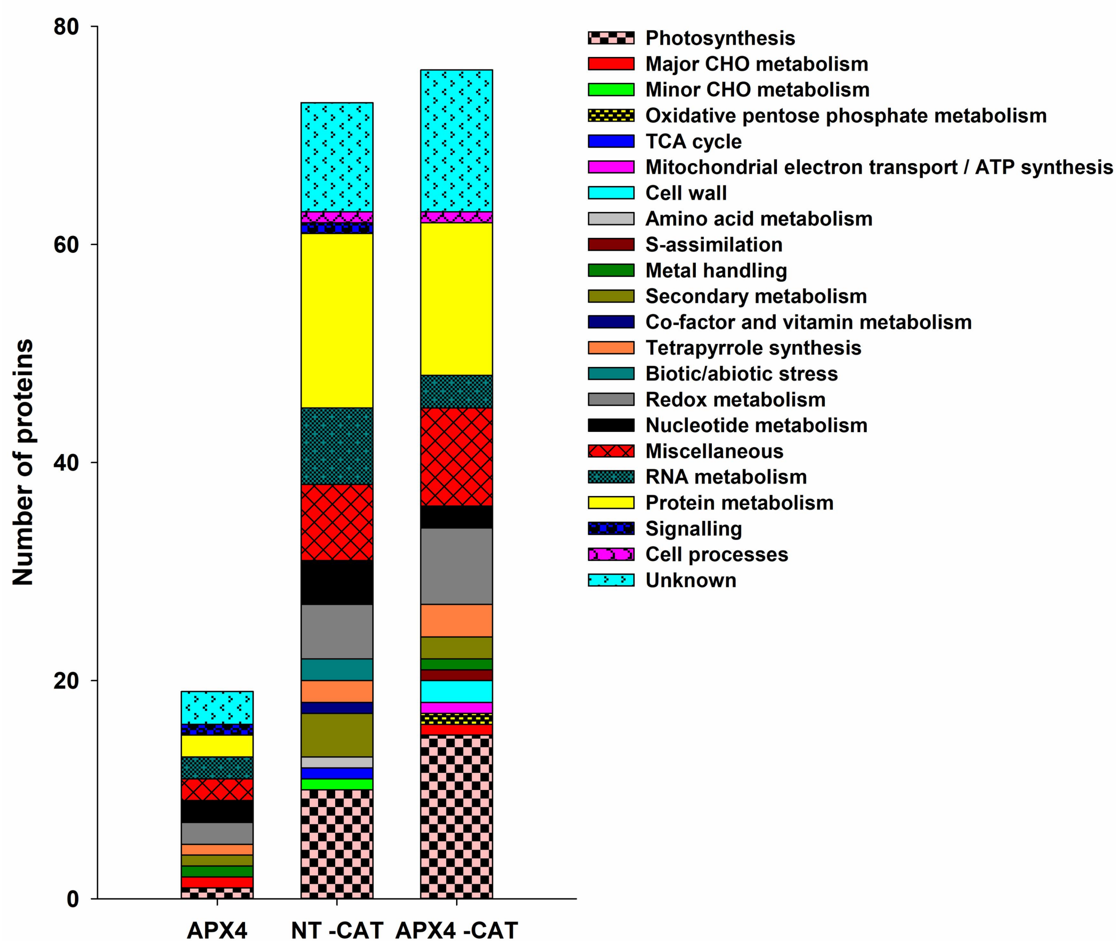


Figure 6. Functional classification of proteins with decreased abundance. The amount of each protein in leaves of RNAiOsAPX4 under normal growth condition and in NT and RNAiOsAPX4 plants exposed to 10 mM AT (-CAT) were all compared to the respective protein amount found in NT control plants. Each protein content was considered increased if the $\log_2$ was higher than 0.5. Proteins were classified by the software (MapMan) according to their involvement in each metabolic process.

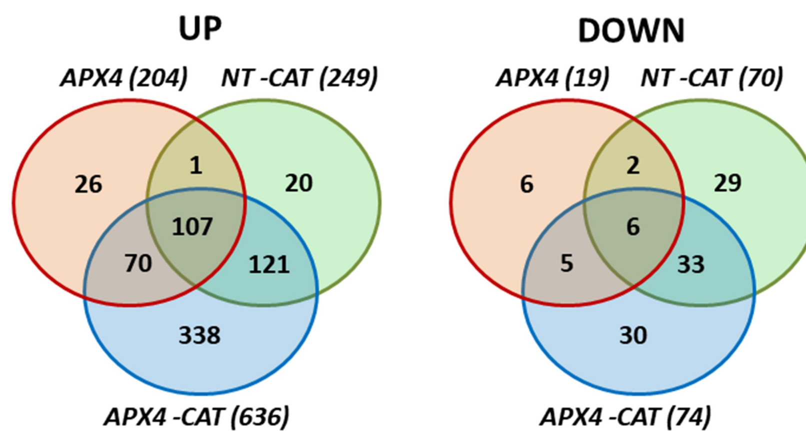


Figure 7. Venn's diagrams depicting proteins with different abundance in leaves of RNAiOsAPX4 under normal growth condition and in NT and RNAiOsAPX4 plants exposed to 10 mM AT (-CAT) were all compared to the respective protein amount of NT control plants. Each protein content was considered increased if the log_2 was higher than 0.5. The Venn diagram was constructed in the Pangloss Venn diagram generator (http://www.pangloss.com/seidel/Protocols/venn.cgi).

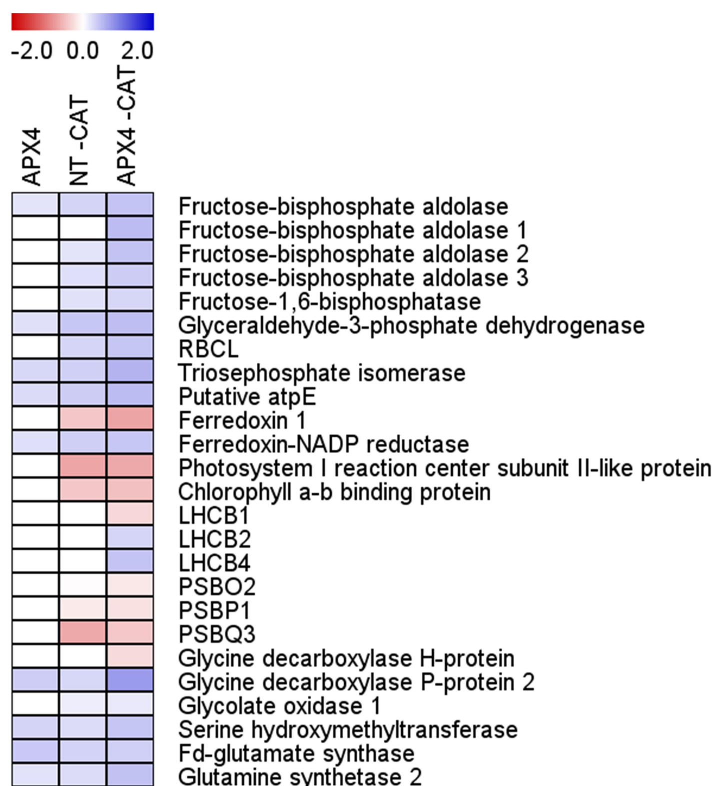


Figure 8. Heat map of differently expressed proteins related to photosynthetic metabolism in leaves of RNAiOsAPX4 under normal growth condition and in NT and RNAiOsAPX4 plants exposed to 10 mM AT (-CAT) were all compared to the respective protein amount of NT control plants. The fold change from proteomics was plotted into a heat map using MEV software framework.

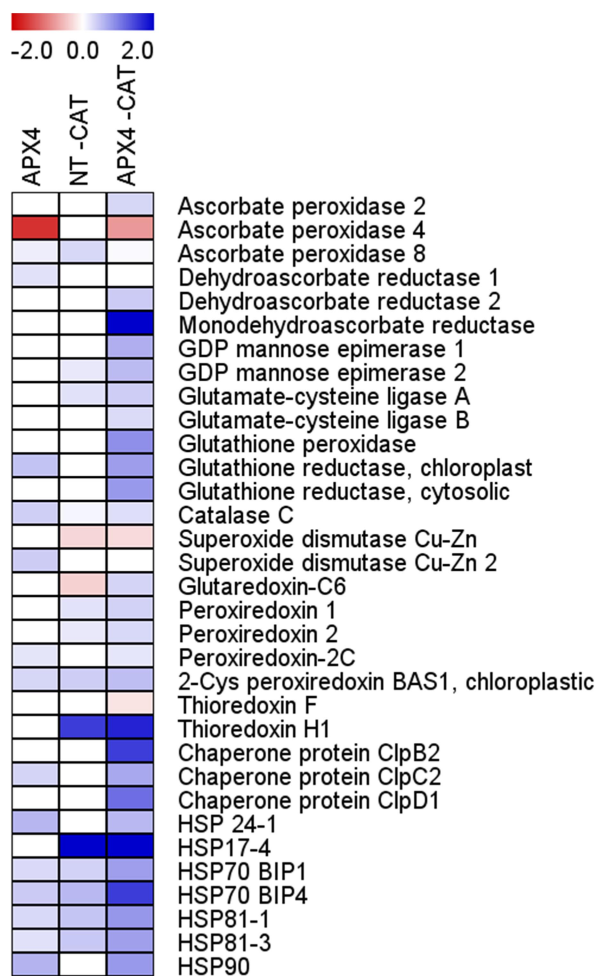


Figure 9. Heat map of differently expressed proteins related to stress metabolism in leaves of RNAiOsAPX4 under normal growth condition and in NT and RNAiOsAPX4 plants exposed to 10 mM AT (-CAT) were all compared to the respective protein amount of NT control plants. The fold change from proteomics was plotted into a heat map using MEV software framework.

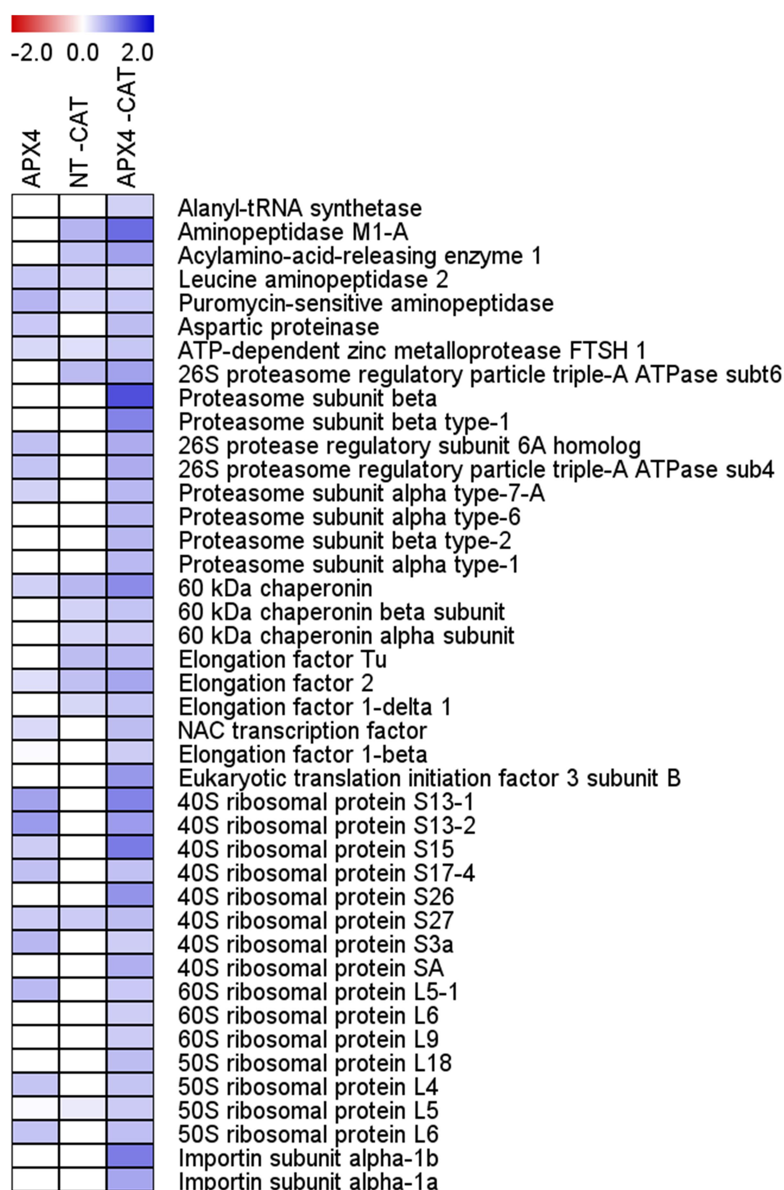


Figure 10. Heat map of differently expressed proteins related to protein metabolism in leaves of RNAiOsAPX4 under normal growth condition and in NT and RNAiOsAPX4 plants exposed to 10 mM AT (-CAT) were all compared to the respective protein amount of NT control plants. The fold change from proteomics was plotted into a heat map using MEV software framework.

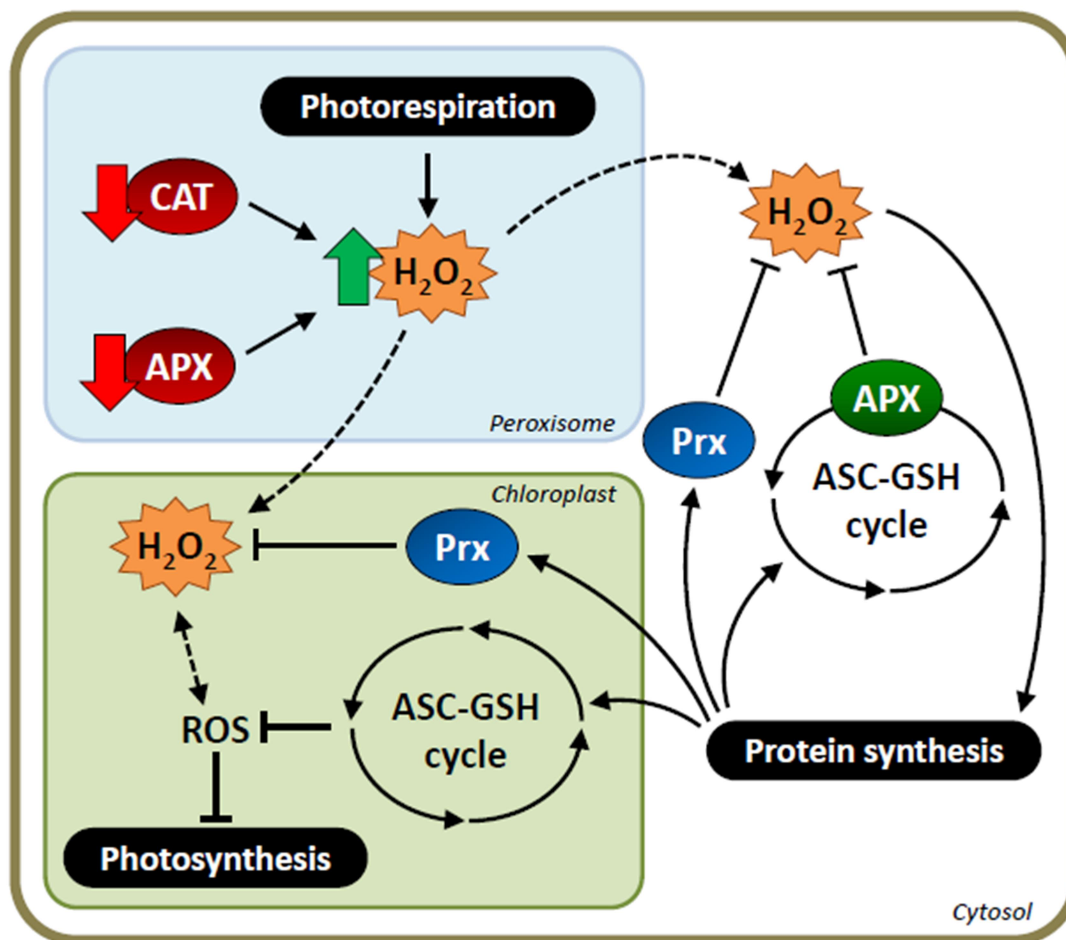
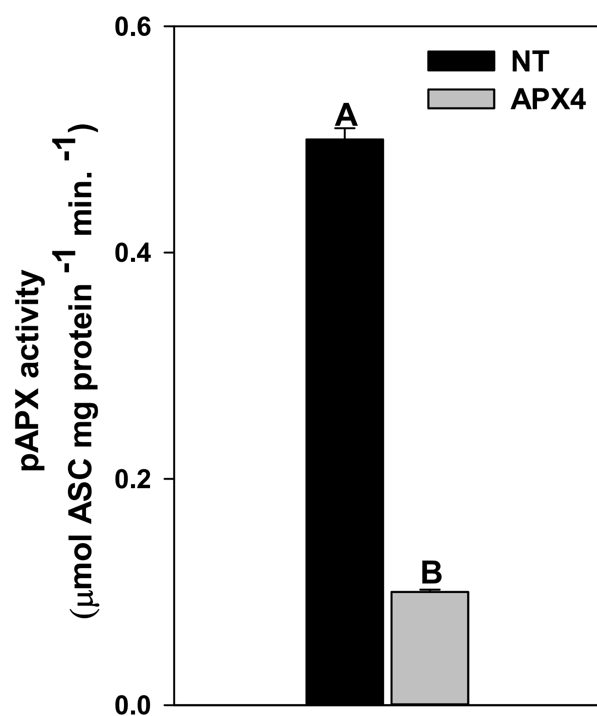
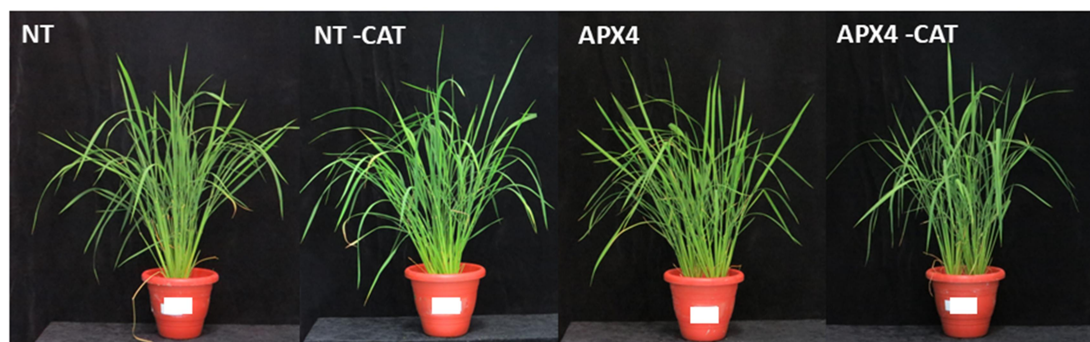


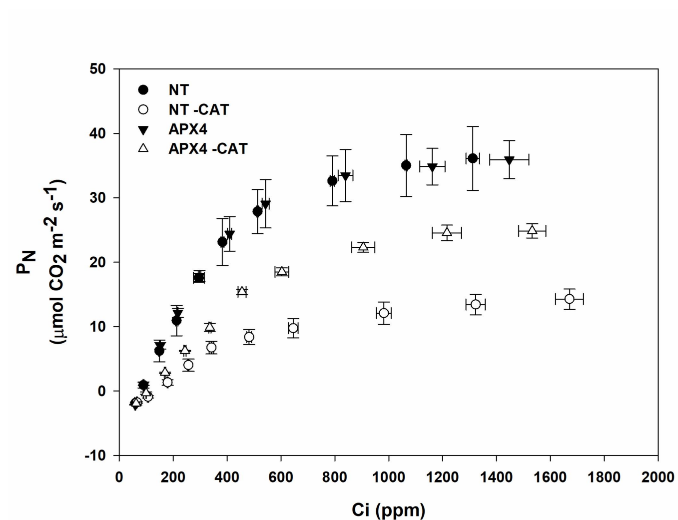
Figure 11. Schematic model summarizing the main mechanisms in RNAiOsAPX4 plants related to mitigation of photosynthesis impairment in presence of CAT inhibition. Previously to CAT inhibition, the pAPX deficiency in peroxisomal membrane could have stimulated an initial H_2O_2 flux towards cytosol, triggering signaling mechanisms. After CAT inhibition, both pAPX silenced and NT plants displayed a strong H_2O_2 burst inside peroxisomes. This ROS might have crossed membranes reaching cytosol and chloroplasts. In these cellular compartments (peroxisomes, cytosol and chloroplasts), H_2O_2 or other derivative redox signals could have triggered signaling for synthesis increase of several protective proteins involved with photosynthesis. It is postulated that the main important proteins for mitigation of the adverse effects induced by CAT inhibition are those belonging to cytosolic and chloroplast ASC/GSH cycle and other important antioxidant and protective proteins. These proteins might have acted as excess ROS scavengers, contributing to improve the photosynthetic capacity in RNAiOsAPX4 plants, in comparison to NT plants under CAT inhibition.



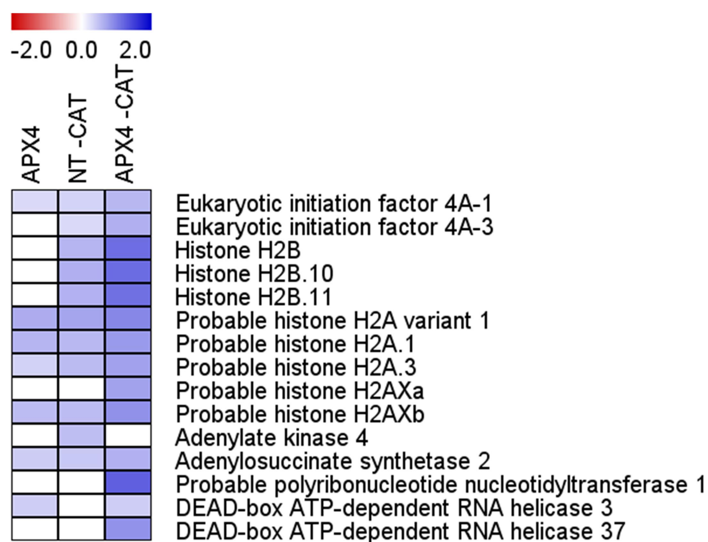
Supplementary figure 1. Peroxisomal ascorbate peroxidase activity in leaves of peroxisomal APX4 knockdown and NT rice plants leaves. The bars represent means of four replicates ±SD. Capital letters indicate significant differences between treatments (control and -CAT) within the same genotype and lower cases represent significant differences between genotypes (NT and APX4) within each treatment.



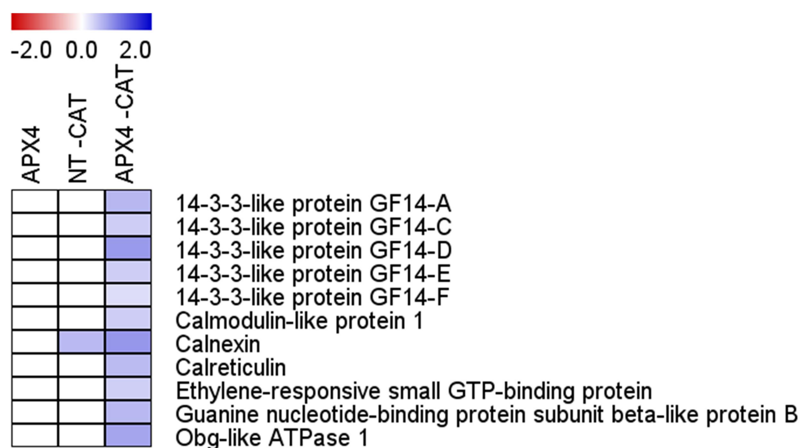
Supplementary figure 2. Morphological aspects of shoots from peroxisomal APX4 knockdown and NT rice plants under control or exposed to 10 mM AT (-CAT). Plants are the most representative from four biological replicates.



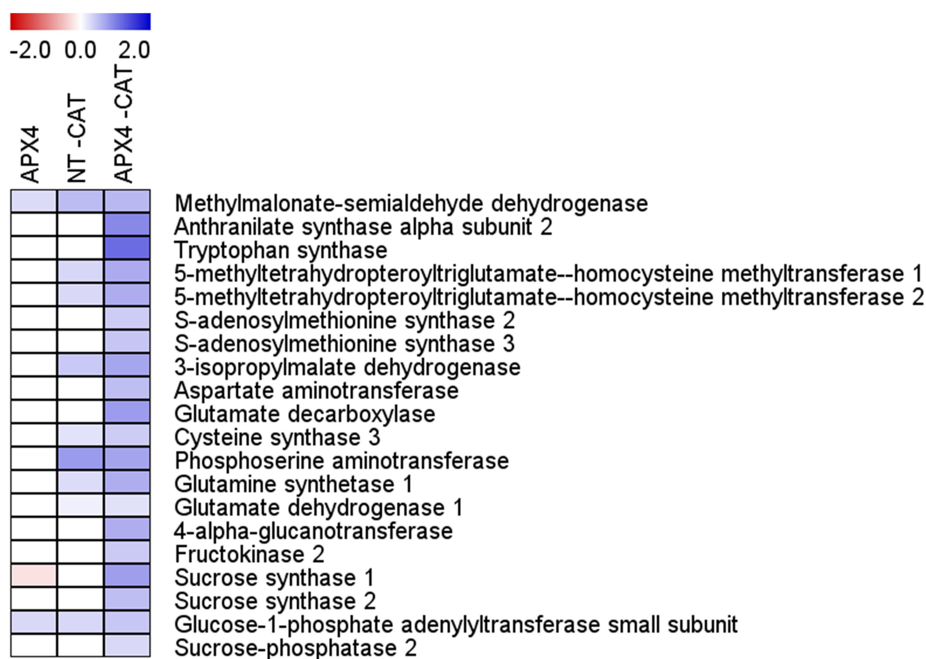
Supplementary figure 3. P_N – Ci curves in peroxisomal APX4 knockdown and NT rice plants under control condition and 10 mM AT (-CAT). The vertical bars indicate standard deviation (SD) of P_N and horizontal bars designate SD of Ci averages.



Supplementary figure 4. Heat map of differently expressed proteins related to nucleus metabolism from leaves of NT plants exposed to 10 mM AT (-CAT) and APX4 silenced rice exposed to distilled water (APX4-control) or AT (APX4-CAT), all compared to the respective protein amount found in NT control plants. The fold change from proteomics was plotted into a heat map using MEV software framework.



Supplementary figure 5. Heat map of differently expressed proteins related to signaling in leaves from leaves of NT plants exposed to 10 mM AT (-CAT) and APX4 silenced rice exposed to distilled water (APX4-control) or AT (APX4-CAT), all compared to the respective protein amount found in NT control plants. The fold change from proteomics was plotted into a heat map using MEV software framework.



Supplementary figure 6. Heat map of differently expressed proteins related to amino acids and sugar metabolism from leaves of NT plants exposed to 10 mM AT (-CAT) and APX4 silenced rice exposed to distilled water (APX4-control) or AT (APX4-CAT), all compared to the respective protein amount found in NT control plants. The fold change from proteomics was plotted into a heat map using MEV software framework.

3 CHAPTER III: IMPROVEMENT OF PHOTOSYNTHETIC PERFORMANCE UNDER HIGH LIGHT CONDITION ACHIEVED BY PEROXISOMAL APX4 KNOCKDOWN RICE PLANTS AS CONSEQUENCE OF COMBINED PROTECTIVE MECHANISMS

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Abstract – Photorespiration is the main source of H_2O_2 in the plant cell due to the glycolate oxidase reaction in peroxisomes. It is known that APX4 knocked-down rice plants are less sensitive to oxidative stress induced by catalase inhibition, probably because of a primer effect triggered by H_2O_2-related signaling. However, the contribution of the photorespiratory flux for the H_2O_2 balance is not understood. To better understand the photorespiratory involvement in the peroxisomal H_2O_2 signaling, we exposed APX4-silenced (APX4) rice plants to opposite photorespiratory conditions (high light and high CO_2) and combined gas exchange, biochemical and proteomics analysis. Our overall results show that the photorespiratory flux was increased under the high light treatment in APX4 plants. We found that APX4 plants have higher photosynthesis resilience under high light condition associated with catalase inhibition than non-transformed plants. The proteomic analyses showed that high light induced an accumulation of chloroplastic proteins from ASC/GSH cycle and Trx M5 in APX4 plants. We concluded that APX4 plants have a better photosynthesis performance under high light as a consequence of an increased photorespiratory flux associated to an efficient chloroplastic antioxidant system.

Keywords: H_2O_2 signaling, Ascorbate peroxidase, Photorespiration.

3.1 Introduction

Photorespiration is an inevitable cellular process that occurs when the enzyme ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco) of the Calvin–Benson cycle (CB cycle) fixes O_2 instead of CO_2 (Bowes et al., 1971). In C3 plants, every third to fourth molecule of ribulose 1,5-bisphosphate (RuBP) is oxygenated rather than carboxylated at air 0.04% CO_2 / 21% O_2 ratio (Tcherkez, 2013; Sharkey, 1988). As consequence of the Rubisco oxygenation, there is the production of a toxic molecule, 2-phosphoglycolate, instead of 3-phosphoglycerate. Thus, to recover the 3-phosphoglycerate, the photorespiratory pathway involves more than 20 different enzymes and transporters that are distributed over at least three compartments in plant cells: chloroplasts, peroxisomes, and mitochondria (Hagemann & Bauwe, 2016).

Many works have shown that the knockout of genes encoding photorespiratory enzymes result in the called ‘photorespiratory phenotype’ and such mutants cannot grow in the ambient air. However, this phenotype can be rescued in high CO_2 concentration air, where RuBP oxygenation becomes inhibited (Somerville, 2001; Bauwe et al., 2012). In addition, the photosynthetic carbon assimilation becomes impaired when carbon or nitrogen flow through the photorespiratory pathway is artificially restricted (Timm & Bauwe, 2013). The metabolic impairment caused by photorespiration disruption is, in most of the cases, a consequence of excessive accumulation of photorespiratory products as 2-phosphoglycolate, glyoxylate, glycolate, hydrogen peroxide (H_2O_2) and glycine (Lu et al., 2014; Xu et al., 2009; Mhamdi et al., 2012).

The H_2O_2 is an important player in the plant metabolism, especially under photorespiratory conditions. This molecule is the reactive oxygen species (ROS) with the highest half-life and can act both as a toxic molecule and as a signaling molecule, depending on its concentration (Foyer & Noctor, 2005; Lin et al., 2012; Del Río, 2015). This ROS is considered the main plant signaling molecule and can regulate processes such as hormonal control, growth, gene expression and modulation of signaling proteins (Foyer & Noctor, 2005). The long half-life associated to the small size of H_2O_2 molecule allows it to migrate into different cellular compartments, facilitating signaling functions, as retrograde signaling (Maruta et al., 2012; Noctor et al., 2014). On the other hand, the H_2O_2 excessive accumulation results in damage to biomolecules and inactivation of essential cell functions (Del Rio, 2015).

The main site of H_2O_2 production is the peroxisomes, which occurs through the photorespiratory enzyme glycolate oxidase (GO) (Del Río & López-Huertas, 2016). Under abiotic stress, photorespiration can represent 50% of the Rubisco activity, resulting in a high H_2O_2 production (Peterhansel & Maurino, 2011). Therefore, an efficient antioxidant system is necessary to control H_2O_2 levels. To scavenge the peroxisomal H_2O_2, plants have an efficient antioxidant apparatus, composed of enzymatic and non-enzymatic components (Nyathi & Baker, 2006). Catalase (CAT) is the most important enzyme in H_2O_2 scavenging (Mhamdi et al., 2012). Although the peroxisomal ascorbate peroxidase (pAPX) also can detoxify H_2O_2, its physiological role is still under discussion. Recent evidences indicate that this enzyme acts in the regulation of H_2O_2 signaling (Sousa et al., 2015).

A study with Arabidopsis plants accumulating H_2O_2 in chloroplasts and peroxisomes showed that H_2O_2 triggers gene expression differently in accordance to its

site of production, concluding thus that the H_2O_2 response is site-dependent (Sewelam et al., 2014). Many works have shown that plants doubly deficient in CAT and cytosolic APX are less sensitive to oxidative stress than plants with only lacking only CAT (Rizhsky et al., 2002, Vanderauwera et al., 2011; Bonifácio et al., 2016). This result is somehow unexpected, once the absence of two important antioxidant enzymes implies in scenery better than the absence of only one of both. However, now it is more clearly understood that the cytosolic APX deficiency results in a slight H_2O_2 accumulation that acts as a signal molecule inducing early antioxidant responses (Vanderauwera et al., 2011; Bonifácio et al., 2016). There are many works showing the effect of cytosolic H_2O_2 accumulation, however, the mechanisms triggered by the peroxisomal H_2O_2 signaling are still unclear.

The CAT knockout and pharmacological inhibition of CAT activity are useful ways to induce high peroxisomal H_2O_2 accumulation. CAT-knocked-out Arabidopsis plants display leaf senescence, leaf necrosis, stunted growth and programmed cellular death (PCP) (Vandenabeele et al., 2004; Vanderauwera et al., 2011; Smith, 1984). However, we have shown that APX4-knocked-down rice plants under pharmacological CAT inhibition have a higher photosynthetic capacity and lower oxidative damage than NT plants (Sousa et el., 2015). This improvement was attributed to a priming signaling by H_2O_2 resulted from the lack of pAPX.

Our previous results were enough to show the less sensitivity of APX4 plants to CAT activity inhibition, but mechanisms triggered for these plants under high photorespiration are not understood since this natural process is the main producer of peroxisomal H_2O_2. The question of this study is: if the H_2O_2 accumulation previously observed in APX4 plants is capable to trigger an efficient antioxidant response under

CAT inhibition (Sousa et al., 2015), would the photorespiration also be involved in this protective response or it is an exclusive consequence of the antioxidant protection? We concluded that under high photorespiration, APX4 plants had a higher photosynthetic resilience than NT plants due to a higher photorespiratory flux associated with antioxidant chloroplast enzymes.

3.2 Material and methods

Growth and treatment conditions

Knocked-down rice (*Oryza sativa* L. ssp. japonica cv. Nipponbare) plants deficient in peroxisomal ascorbate peroxidase 4 (APX4) were obtained as previously described (Sousa et al. 2015). Three lines of APX4-silenced plants were selected based on their transcript amounts: Lg, Lh and Lj, which expressed 10, 15 and 14%, respectively, of the transcript amount in the NT plants. To perform the current study, the Lg line was chosen because it presented the lowest level of APX4 expression. After germination, both non-transformed (NT) and APX4 plants were transferred to 2-L pots containing Hoagland-Arnon's nutritive solution (Hoagland & Arnon 1950) and grown in controlled growth chamber for 40 days.

For High light associated to CAT inhibition treatment (HL+AT), 40-day-old NT and APX4 plants were sprayed with 10 mM 3-amino-1,2,4-triazole (3-AT) dissolved in 10 mM N-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid (HEPES) buffer at pH 6.5 containing 1.5 mM $CaCl_2$ and 0.1% Triton X-100. Plants were sprayed once with 50

mL of 3-AT solution at 6:00 pm and plants were kept in the dark during 12 hours. Afterwards, the plants were exposed to high light treatment (1000 µmol photons m^{-2} s^{-1}) for 8 hours. After the 20 hours of treatment, the gas exchange determinations and leaf sampling for proteomic analysis were performed. The control plants were sprayed with a 3-AT-free solution and exposed to ambient light (400 µmol photons m^{-2} s^{-1}). For the low photorespiration treatment (CO_2), plants were grown in 3000 ppm CO_2.

Exchange measurements

The intercellular CO_2 partial pressure (C_I)-dependent photosynthesis (A) curve was measured using a portable infrared gas analyzer system, equipped with an LED source and a leaf chamber (IRGA LI-6400XT, LI-COR, Lincoln, NE, USA). For the A/C_I curve, the C_I was varied between 50 and 1,800 ppm CO_2 and the photosynthetically active radiation (PAR) was set as 1,000 µmol photons m^{-2} s^{-1}. The maximum Rubisco carboxylation rate (V_{cmax}) and maximum photosynthetic electron transport rate (J_{max}) were calculated from the A/C_I curve (Sharkey et al. 2007). Electron flux used for RuBP carboxylation (J_C) and RuBP oxygenation (J_O) and photorespiration rate (P_R) were estimated according to Valentini et al. (1995) and Bagard et al. (2008). A_{pot} was measured by the photosynthesis rate under 1,800 ppm CO_2.

Amino acids photorespiration-related

For determination of amino acid concentrations in leaves, free amino acids were collected in aqueous fraction, after extraction in methanol:chloroform:water (12:5:3, v/v/v) solution. The amino acids were derivatized with o-phthaldialdehyde (OPA) and separated for high-pressure liquid chromatography (HPLC) (Shimadzu, Tokyo, Japan) using a reverse-phase C18 column (5lm, 250 mm 9 4.6 mm). The mobile phase comprised two solutions. The first solution contained 50 mM sodium phosphate, 50 mM sodium acetate, 2% tetrahydrofuran, and 2% methanol (pH 7.25), while the second solution contained 65% methanol (in water, v/v). The flow rate of 8.0 mL $min.^{-1}$ and column temperature of 30 °C were utilized for elution. The amino acid peaks were detected using a SPD-20A detector (Shimadzu, Tokyo, Japan) at 240 nm and quantified from the standard curves.

Glycolate oxidase activity

The protein extraction was performed utilizing fresh leaf matter in the presence of 100 mM phosphate buffer, pH 7.5, containing 2 mM EDTA. The GO activity was assayed by the formation of the glyoxylate-phenylhydrazone complex and measured after reading at 324 nm (Baker & Tolbert 1966). GO activity was calculated using the molar extinction coefficient of the glyoxylate-phenylhydrazone complex (17 mM^{-1} cm^{-1}) and expressed as ηmol $glyoxylate^{-1}$ mg $protein^{-1}$ min^{-1}.

Protein extraction, digestion and desalt for proteomic analysis

Approximately 700 mg of leaf fresh matter from each plant were ground to a powder, frozen and dried by lyophilization. The extracted proteins were precipitated in 10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone, and resuspended in a lysis buffer containing 8 M urea, 2 M thiourea, 5% CHAPS and 2 mM tributylphosphine. The protein concentrations were determined using the Bradford method (Bradford, 1976) with bovine serum albumin as standard. The samples were purified with methanol and chloroform to remove detergent from samples and centrifuged at 20,000 × *g* for 10 min to achieve phase separation. The upper phase was discarded and methanol was added to the lower phase. The solutions were again centrifuged at 20,000 × *g* for 10 min and the resulting pellets were dried. Dried samples were reduced with 25 mM dithiothreitol and alkylated with 30 mM iodoacetamide. 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 the dark. Peptides were acidified with 20% formic acid (pH < 3) and desalted with a C18-pipette tip (Nikkyo Technos, Tokyo, Japan). The samples were analyzed by nano-liquid chromatography with *in tandem* mass spectrometry (LC-MS/MS).

Protein identification using nano-liquid chromatography mass spectrometry

The peptide samples were separated using an Ultimate 3000 nano-LC system (Dionex, Germering, Germany), and the peptide ions were detected using a LTQ Orbitrap Discovery MS nanospray (Thermo Fisher Scientific, San Jose, CA, USA) with data-dependent acquisition mode with Xcalibur software (version 2.1, Thermo Fisher Scientific). The peptide samples were loaded onto a C18 PepMap trap column (300 µm

I.D. × 5 mm, 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^{-1}. The eluted peptides were loaded and separated on a C18 capillary tip column (75 μm I.D. × 120 mm, NikkyoTechnos) 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 90 sec to prevent the repetitive selection of peptides (Zhang *et al.* 2009).

Data acquisition by mass spectrometry analysis

Identification of proteins was performed using Mascot search engines (version 2.4.1., Matrix Science, London, UK) with a rice protein database (50,253 sequences and 15,266,515 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_ 2013-4-24) and protein sequences predicted computationally (IRGSP-1.0_ predicted protein_2013-3-9). The Proteome Discoverer software (version 1.4, Thermo Fisher Scientific) was used to process the acquired raw data files. For the Mascot searches, the carbamido methylation 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 at 0.5 Da, and the peptide charge 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

The commercial label-free quantification package SIEVE was used for the differential analysis of relative abundances of peptides and proteins between samples. 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 bin codes (http://mapman.gabipd.org/) (Usadel *et al.* 2005).

Statistical Analyses

The experiments were carried out in a completely randomized design with four replicates, each one represented by an individual pot containing two plants. Data were analyzed by ANOVA and averages were compared by Tukey's test at the 0.05 confidence level. The standard deviation is plotted in all graphics. The proteomics analyses were performed as previously described ($p < 0.05$).

3.3 Results

Photosynthesis performance in APX4 plants under CAT inhibition and high light

To study the impact of photorespiration on the photosynthetic performance of APX4 plants, we have induced photorespiration by CAT inhibition associated with high light (1000 µmol photons $m^{-2}s^{-1}$). The absence of CAT activity did not induce photorespiration but exacerbates the photorespiratory H_2O_2 accumulation. In parallel, for low photorespiration treatment, another group of NT and APX4 plants were grown in non-photorespiratory condition (3000 ppm CO_2). High light treatment caused a strong decrease in the photosynthetic rate and Rubisco amount (data not shown),

consequently, a reduction of photorespiration. However, the photorespiration relative to photosynthesis rate was increased, confirming that this treatment induced a relative increase in the photorespiratory rate (P_R/P_N). The photorespiration was strongly induced in APX4 plants since the P_R/P_N ratio increased from 0.3 to 0.9; whilst in NT plants the increase was lower, from 0.3 to 0.5 (Figure 1A). Under low photorespiration, the Pr/P_N was lower than in control condition and was not different between the genotypes (Figure 1A). In the HL+AT treatment, the electron flow to oxygenation/electron flow to carboxylation ratio (J_O/J_C) was slightly increased in NT plants while in APX4 plants the increase was more apparent (Figure 1B). Similar to Pr/P_N, the CO_2 treatment induced a marked reduction of the J_O/J_C rate in both genotypes (Figure 1B).

The potential photosynthesis (A_{pot}) expressed the maximal photosynthetic capacity in non-limiting CO_2 concentration. The exposition time to high light under absence of CAT activity, HL+AT treatment, was enough to drastically reduce the A_{pot}, from 36.6 to 9.5 in NT and from 37.3 to 14.8 in APX4 (Figure 2A). Under low photorespiration, the A_{pot} was similar to the control condition in both NT and APX4. The electron transport rates (ETR) were similar under control and high CO_2 condition, while reduced 85% and 67% in HL+AT treatment, in NT and APX4, respectively (Figure 2B). A similar result was observed in maximum carboxylation velocity (V_{cmax}) since NT and APX4 plants showed a reduction of 94% and 80%, respectively, in comparison to the control treatment, although the low photorespiration condition induced a slight increase in V_{cmax} in NT plants (Figure 2C). Taken together, these findings demonstrate that CAT inhibition under high light induced strong photosynthesis impairment in both genotypes and that APX4 plants seem to have better maintenance of the photosynthetic apparatus.

Metabolite changes induced by photorespiration

The glycine/serine ratio expresses the efficiency of the glycine to serine conversion by glycine decarboxylase complex (GDC) in the photorespiratory cycle. The high photorespiration induced an increase at the Gly/Ser ratio in both genotypes. However, this increase was higher in NT plants, from 0.2 to 0.5, than in APX plants, from 0.2 to 0.4 (Figure 3A). Under low photorespiration, the Gly/Ser ratio was changed only in APX4 plants, which showed an increase in this rate (Figure 3A). Thus, the difference in the Gly/Ser ratio between NT and APX4 observed for the control condition was increased in HL+AT and inverted in CO_2 treatment. The glycolate oxidase (GO) activity was slightly reduced in APX4 under high light condition but did not change in NT plants, both compared to their respective control conditions (Figure 3B). As expected, the low photorespiration induced a reduction in GO activity and abolished the difference between NT and APX4 plants.

To figure out the profile of photorespiration-related amino acids in APX4 plants under high and low photorespiration, we analyzed the contents of glutamine (Gln), glutamate (Glu), asparagine (Asn) and aspartate (Asp). The glutamine (Gln) content was slightly higher in APX4 than in NT plants under control condition. However, the high photorespiration induced an increase of 3-fold in NT and 2-fold in APX4, therefore, a significant higher Gln accumulation in NT than in APX4 plants (Figure 4A). Under reduced photorespiration, the Gln concentration was not altered in NT plants and was slightly reduced in APX4 plants (Figure 4A). The glutamate content was almost the

same between NT and APX under the three conditions, except in HL+AT, in which NT had a slightly higher Glu concentration (Figure 4B).

Asparagine is believed to be involved in the photorespiratory conversion of glyoxylate to glycine. Thus, a higher Asn consumption is expected under conditions with high photorespiratory rates. Indeed, the Asn content in both genotypes was lower in HL+AT treatment but, unexpectedly, this trend was also observed in CO_2 treatment, both in comparison to the ambient condition. Possibly, the lower Asn content found in low photorespiration is a consequence of a reduced photorespiratory rate, once the concentration of other photorespiration-related amino acids was also lower than in ambient condition (Figure 4). Under control condition, the Asn content is already lower in APX4, when compared with NT. Maybe, this is consequence of a possible higher photorespiratory rate in APX4 plants under growth conditions. The aspartate content was somewhat similar in both genotypes in all conditions tested, except in low photorespiration, where the Asp content reduced 40% in NT and 74% in APX4 plants, compared to control (Figure 4D).

Proteomic changes triggered by high and low photorespiration

To better understand the effects of photorespiration on the cellular metabolism of APX4 plants, a proteomic analysis was performed. All the treatments were normalized to NT plants under control conditions. High light associated to CAT inhibition induced a large rise in the number of proteins with increased amount in APX4 plants (365), while only 69 proteins had their amount increased in NT plants at the same

condition (Figure 5). Among these proteins, only 20 and 190 were accumulated in NT and APX4 leaves, respectively, under high photorespiration (Figure 5A). Even with the reduction of photorespiration, the protein profile between NT and APX4 plants was different. Among the 327 and 193 proteins increased in NT and APX4 plants, respectively, only 71 had increased abundance exclusively in both genotypes under low photorespiration (Figure 5A). The HL+AT treatment induced a decrease in the amount of a large number of proteins (Figure 5B). Under high light, 206 and 396 proteins had their abundance decreased in NT and APX4, respectively, and the amount of 45 of them were decreased exclusively in both genotypes in the same treatment (Figure 5B). These results indicate that APX4 plants had a higher number of proteins exclusively with increased and decreased amounts under induction of photorespiration. These proteins are possibly involved with the better photosynthetic resilience observed for those plants under oxidative stress.

The categorization graphic classifies proteins according to their metabolic function and shows the number of proteins with increased or decreased amounts (Figure 6). The most expressive class among the proteins with increased amount in NT leaves under HL+AT was that related to biotic/abiotic stress, expressing 46% of all proteins with increased abundance. Interestingly, among these proteins, 84% were also accumulated in APX4 leaves in HL+AT treatment. In accordance with the better photosynthetic performance in APX4 plants in HL+AT treatment, the number of proteins related to the photosynthesis metabolism with increased abundance was markedly higher in APX4 plants than NT plants. The proteins from the protein metabolism category were highlighted in APX4 plants under high photorespiration, with 65 proteins with increased amount. When compared with the HL+AT treatment, the CO_2 treatment induced a higher number of proteins with increased amount in NT and a

lower number in APX4 leaves. The protein metabolism was the category with the highest number of proteins that had increased amount in both genotypes under low photorespiration, with 113 in NT and 49 in APX4 (Figure 6).

The abundances of a large number of proteins were also decreased in APX4 under high light associated to CAT inhibition (Figure 7). Among these proteins, the most representative class with decreased amount in APX4 plants was the protein metabolism. These findings demonstrate that the proteins from the protein metabolism category might be involved in the synthesis of proteins for protection against high peroxisomal H_2O_2 accumulation in APX4 plants under high photorespiration. Probably, there was a higher production of antioxidant proteins which may be involved in the more efficient antioxidant protection showed in APX4 plants (Figure 7).

Accumulation of photosynthesis- and stress-related proteins in APX4 plants under high photorespiration

The heat map of photosynthesis-related proteins shows the main proteins differently expressed (Figure 8). HL+AT treatment did not induce accumulation of photosynthesis proteins in NT plants. This result corroborates the marked reduction in photosynthesis parameters in this treatment. On the other hand, the APX4 plants under the same condition showed an increase in the amount of many proteins from both Calvin-Benson (CBB) cycle and photochemical reactions (Figure 8). The main proteins with increased abundance in this treatment were fructose biphosphate aldolase, Rubisco activase, and glyceraldehyde-3-phosphate dehydrogenase, from Calvin-Benson cycle,

and ferredoxin 1, ferredoxin-NADP reductase 1 and 2, LHCB2 and LHCB4, from photochemical. Probably, the induction of photosynthesis proteins in APX4 plants contributed to the maintenance of photosynthesis under high light associated to CAT inhibition. Unexpectedly, the amount of proteins related to photorespiration was not altered under high light condition, in both genotypes. In low photorespiration, the abundance of serinehydroxymethyl transferase and glycolate oxidase 1, 2 and 5 was decreased in APX4 plants (Figure 8).

Among the stress-related proteins, the amount of proteins involved with the ascorbate and glutathione metabolism (probable L-ascorbate peroxidase 7 and 8, dehydroascorbate reductase 2, monodehydroascorbate reductase 3 and 4, and glutathione reductase) increased only in APX4 plants HL+AT treatment (Figure 9). The abundance of the chloroplastic thioredoxin M5 was also markedly increased in APX4 plants under high light combined to CAT inhibition. This enzyme is involved in the regulation of chloroplast enzymes and hydrogen peroxide scavenging system in chloroplasts. The amounts of chaperones and heat shock proteins (HSP) were increased in high photorespiration in both genotypes. In high CO_2, almost none protein was altered for both genotypes, except CAT B and few HSPs, which were accumulated (Figure 9).

Taken together, the above results suggest that, under high photorespiration and CAT-inhibited conditions, the proteins from ascorbate and glutathione metabolisms and Trx M5 in APX4 plants may be involved with the control of the H_2O_2 produced more efficiently than in NT plants. This more efficient antioxidant system in APX4 plants results in a lower ROS accumulation and, consequently, a photosynthetic machinery less damaged than in NT plants.

3.4 Discussion

Previously, we showed that APX4-silenced rice plants have an improved photosynthetic capacity under CAT inhibition (Sousa et al., 2015). However, it is not understood how APX4 plants cope with the production of peroxisomal H_2O_2 intensified by high photorespiration. In light of the importance of this process for cellular metabolism, we tested whether photorespiration would change differently the photosynthetic performance of APX4-silencied rice plants. The treatment utilized to induce photorespiration was not specific since high light does not trigger only high Rubisco oxygenation. High light conditions increase the photorespiration rate as a consequence of a high electron pressure in thylakoid membrane but also triggers other effects (Li et al., 2009; Kangasjärvi et al., 2012). However, this treatment is widely applied to induce high photorespiratory rates. In addition to high light, the CAT activity was inhibited, mimicking a higher photorespiration and inducing accumulation of photorespiratory H_2O_2. The combination of the high light treatment with CAT inhibition induced a strong oxidative stress for peroxisomal H_2O_2 accumulation that has implied in a severe reduction of the photosynthetic rate. Nevertheless, this treatment was suitable to induce an increase in the relative photorespiratory rate, since the Pr/P_N ratio was increased in both genotypes under high light and CAT inhibition.

Interestingly, the higher net photosynthesis in APX4 under high light and CAT inhibition was associated with an also higher relative photorespiratory rate, in comparison to NT plants. Possibly, the better photosynthetic performance from APX4-silencing plants is associated with an increased photorespiratory flux. This conclusion

can be supported by the results of P_R/P_N ratio, J_O/J_C ratio, Gly/Ser ratio, and Asn content. Additionally, these results allowed us to confirm that the relative photorespiratory rate was the key process to explain the differences in the following results between the treatments applied. A high photorespiratory flux metabolizes more efficiently the photorespiratory intermediates, that are, the most of them, harmful for photosynthesis through inhibition of key photosynthetic enzymes. The glyoxylate has been reported as a toxic photorespiratory intermediate. Its accumulation causes an intense impairment in the photosynthetic CO_2 assimilation (Lu at al., 2014) as well as decrease in Rubisco activation and reduced RuBP regeneration (Chastain & Ogren, 1989).

It is already known that APX4-silenced plants accumulate less glyoxylate than non-transformated plants under CAT-inhibited activity. (Sousa et al., 2015). Probably, under high photorespiration, the glyoxylate accumulation is still lower in APX4 plants than in NT plants, as observed under CAT inhibition. During photorespiration, glyoxylate can be alternatively detoxified by serine:glyoxylate aminotransferase, using asparagine as an amino donor (Lea et al., 2007; Zhang et al., 2013). As asparagine content was lower in APX4 than NT plants under normal conditions and even lower under high photorespiration, it is possible that the asparagine pool is metabolizing glyoxylate in APX4 plants, and thus contributing to the better photosynthetic performance in APX4 plants of HL+AT treatment.

The lower Gly/Ser ratio in APX4 plants compared to NT plants under control and HL+AT conditions indicates a high glycine decarboxylase complex (GDC) activity. Many works have been reported that the overexpression of photorespiratory enzymes implies in improved photosynthesis. Arabidopsis plants overexpressing H-protein from

the GDC complex showed increased net photosynthesis and growth (Timm et al., 2012). A similar result was found with Arabidopsis plants overexpressing dihydrolipoamide dehydrogenase (L-protein subunit) of the GDC complex, as well as the mitochondrial serinehydroxymethyl aminotransferase SHMT (Timm et al. 2015; Wu et al., 2015). The high photorespiratory flux results in a more efficient consumption of photorespiratory intermediates. Therefore, the low Gly/Ser ratio in APX4 plants might express a high GDC complex activity, contributing to a better photosynthesis under CAT inhibition associated to high light. Under high CO_2 concentration, the differences between NT and APX plants, concerning photosynthesis parameters and photorespiration indicators, disappeared, except the Gly/ser ratio that was kept higher in APX4 plants than NT plants. This result is expected, once in high CO_2 concentration the photorespiration and, consequently, peroxisomal H_2O_2, are abolished.

The slight reduction of the GO activity under high photorespiration might be a protective mechanism to reduce the glyoxylate and H_2O_2 production in APX4 plants. A reduction of the peroxisomal H_2O_2 accumulation is a manner to attenuate the oxidative damage caused by H_2O_2 in APX4 plants under HL+AT, once in these plants CAT is inhibited and the amounts of APX4 proteins are much lower than in NT. Obviously, the reduction of GO activity might not be the crucial point for the photosynthesis resilience in APX4 plants under high photorespiration but certainly it contributed for a lower H_2O_2 production. In addition, it is already known that a reduction of GO activity above 60% causes photosynthetic impairment (Lu et al., 2014) Thus, a strong suppression of GO activity would stop the photorespiratory flux, causing the accumulation of toxic photorespiratory intermediates.

The higher accumulation of some antioxidants proteins in APX4 compared to NT plants under high photorespiration might collaborate for a better photosynthetic efficiency, since a higher ROS scavenger system implies in lower photosynthesis impairment. The chloroplastic thioredoxins M is involved in the activation of photosynthesis-related proteins such as malate dehydrogenase NADP-dependent (NADP-MDH), glucose-6-phosphate dehydrogenase (G6PDH) and the regeneration of the activity of enzymes related to the antioxidant mechanisms like 2-Cys-Prxs (Collin et al., 2003; Vieira Dos Santos et al., 2007). In a study with rice plants, the knockdown of the Trx M expression by RNA interference induced abnormal chloroplast development and reduced growth and seed production (Chi et al., 2008). In agreement with these reports, APX4 plants from HL+AT treatment had a 7-fold increase in the abundance of Trx M5, compared to NT plants under control condition.

Concomitant to this increased amount of Trx M5, the amount of proteins of the Calvin-Benson cycle were increased and photosynthesis was also higher in APX4 than in NT plants, both under high light combined to CAT inhibition. The Trx M5 might have regulated the proteins from Calvin cycle, inducing an accumulation of these proteins under high photorespiration. Among the numerous chloroplastic thioredoxins, the M-type Trx proteins have been suggested to be involved in leaf development, chloroplast morphology, cyclic electron flow, and tetrapyrrole synthesis (Chi et al., 2008; Benitez-Alfonso et al., 2009; Luo et al., 2012; Courteille et al., 2012). Indeed, our proteomic analysis showed that the amounts of some proteins of the tetrapyrrole synthesis were increased in APX4 under high photorespiration (data not shown). The accumulation of Cys-Prxs in APX4 in HL+AT treatment indicates a probable antioxidant protection, since Cys-Prxs are abundant chloroplast proteins that are oxidized by reactions with hydrogen peroxide (Dietz, 2003). Despite the oxidation state

of Trx M5 and Cys-Prx have not been quantified, the accumulation of these proteins lead to the comprehension that they are acting in the oxidative protection of APX4 plants under photorespiration.

Another important antioxidant system that was clearly induced in APX4-silenced plants is the ascorbate-glutathione (ASC-GSH) cycle. Interestingly, most of the proteins of the ASC-GSH cycle that were accumulated are addressed to the chloroplasts. In accordance, the two chloroplastic APX isoforms, APX7 and APX8, were also accumulated at high photorespiration treatment. The importance of the ASC-GSH cycle in H_2O_2 signaling for antioxidant response to stress, mainly by the glutathione metabolism, has been constantly highlighted (Han et al., 2013; Rahantaniaina et al., 2017). A recent work with loss-of-function mutants for the three Arabidopsis dehydroascorbate reductase (DHAR) in combination with a CAT-deficiency background has concluded that DHARs operate redundantly to ensure glutathione oxidation in response to intracellular H_2O_2. The results obtained in this work also provide evidences that reinforce the ASC-GSH cycle as a major player in scavenging intracellular H_2O_2 (Rahantaniaina et al., 2017).

It is important to highlight the single difference between NT and APX4 plants is the APX4 knockdown and, as a result, the elimination of APX activity in peroxisomes. Our previous results with APX4-silenced plants have been suggested that the peroxisomal APX displays a role in the regulation of the H_2O_2 signaling. It is assumed that the absence of peroxisomal APX induces a slight H_2O_2 accumulation and this molecule act as a signal to induce an early antioxidant response efficient to cope with oxidative stress (Sousa et al., 2015). Other works with absence of different APX isoforms have shown the possible role of APX in triggering antioxidant protection

possibly by H_2O_2 signaling (Vanderauwera et al., 2011; Bonifácio et al., 2016). Despite the outcome is somewhat the same when different APX isoforms have loss of function, it is known that the H_2O_2 signal is compartment-dependent and thus, possibly, different isoforms trigger different H_2O_2 signal (Sewelam et al., 2014).

Despite the peroxisomes are the main site for H_2O_2 accumulation under high photorespiration, it is clear that the antioxidant mechanisms triggered in APX4 plants were predominantly localized in chloroplasts. Possibly the H_2O_2 generated in peroxisomes migrates to chloroplasts or induces antioxidant protection in chloroplasts by signaling. This result reinforces the importance of H_2O_2 as a signal molecule at the antioxidant response. In conclusion, the photosynthesis impairment caused by high photorespiratory H_2O_2 accumulation was attenuated in APX4-silenced rice plants. This attenuation is a consequence of different mechanisms, probably triggered by a previous H_2O_2 signaling, capable to induce a high photorespiratory flux and an efficient antioxidant protection in chloroplasts, protecting the photosynthetic apparatus against oxidative damage.

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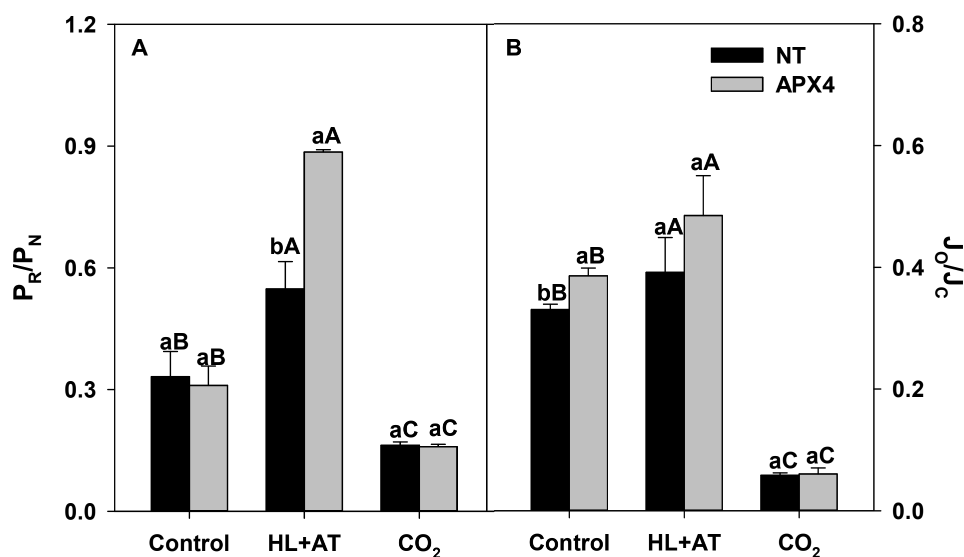

Figure 1. Photorespiration (Pr) / net photosynthesis (P_N) ratio (A) and electron flow devoted to oxygenation (Jo) / electron flow devoted to carboxylation (J_C) ratio (B) of NT and APX4 plants under growth conditions (Control), exposed to 10 mM 3-AT + high light (1000 µmol photons m^{-2} s^{-1}) (High Pr), or exposed to 3% CO_2 (Low Pr). Capital letters indicate significant differences between treatments (control, High Pr and Low Pr) within the same genotype and lower cases represent significant differences between genotypes (NT and APX4) within each treatment by analysis of variance (ANOVA) followed by the Tukey's test ($p < 0.05$).

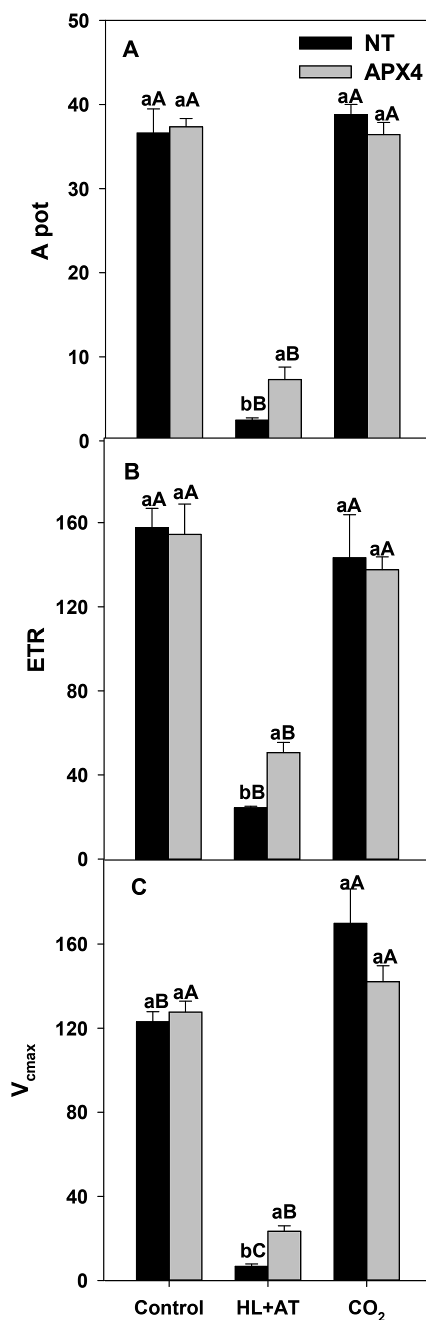

Figure 2. Potential photosynthesis (A_{pot}) (A), electron transport flux (ETR) (B), and maximum carboxylation velocity (V_{cmax}) (C) of NT and APX4 plants under growth conditions (Control), exposed to 10 mM 3-AT + high light (1000 µmol photons m^{-2} s^{-1}) (High Pr), or exposed to 3% CO_2 (Low Pr). Capital letters indicate significant differences between treatments (Control, High Pr and Low Pr) within the same genotype and lower cases represent significant differences between genotypes (NT and APX4) within each treatment by analysis of variance (ANOVA) followed by the Tukey's test ($p < 0.05$).

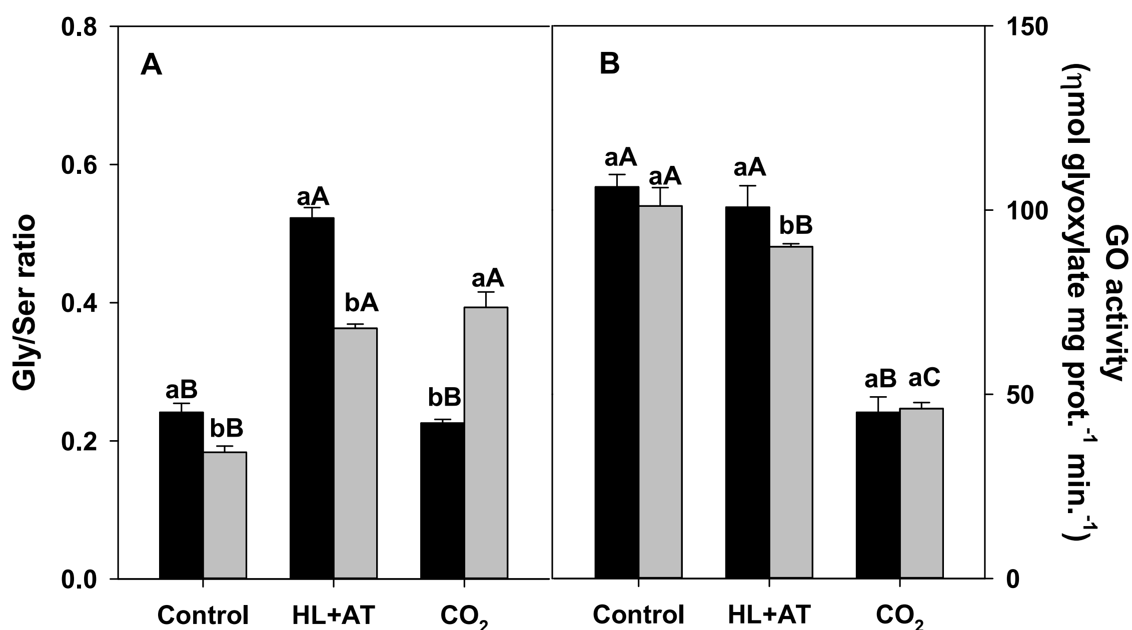

Figure 3. Changes in glycine (Gly)/serine (Ser) ratio (A) and glycolate oxidase activity (B) leaves of NT and APX4 plants under growth conditions (Control), exposed to 10 mM 3-AT + high light (1000 µmol photons m^{-2} s^{-1}) (High Pr), or exposed to 3% CO_2 (Low Pr). Capital letters indicate significant differences between treatments (Control, High Pr and Low Pr) within the same genotype and lower cases represent significant differences between genotypes (NT and APX4) within each treatment by analysis of variance (ANOVA) followed by the Tukey's test ($p < 0.05$).

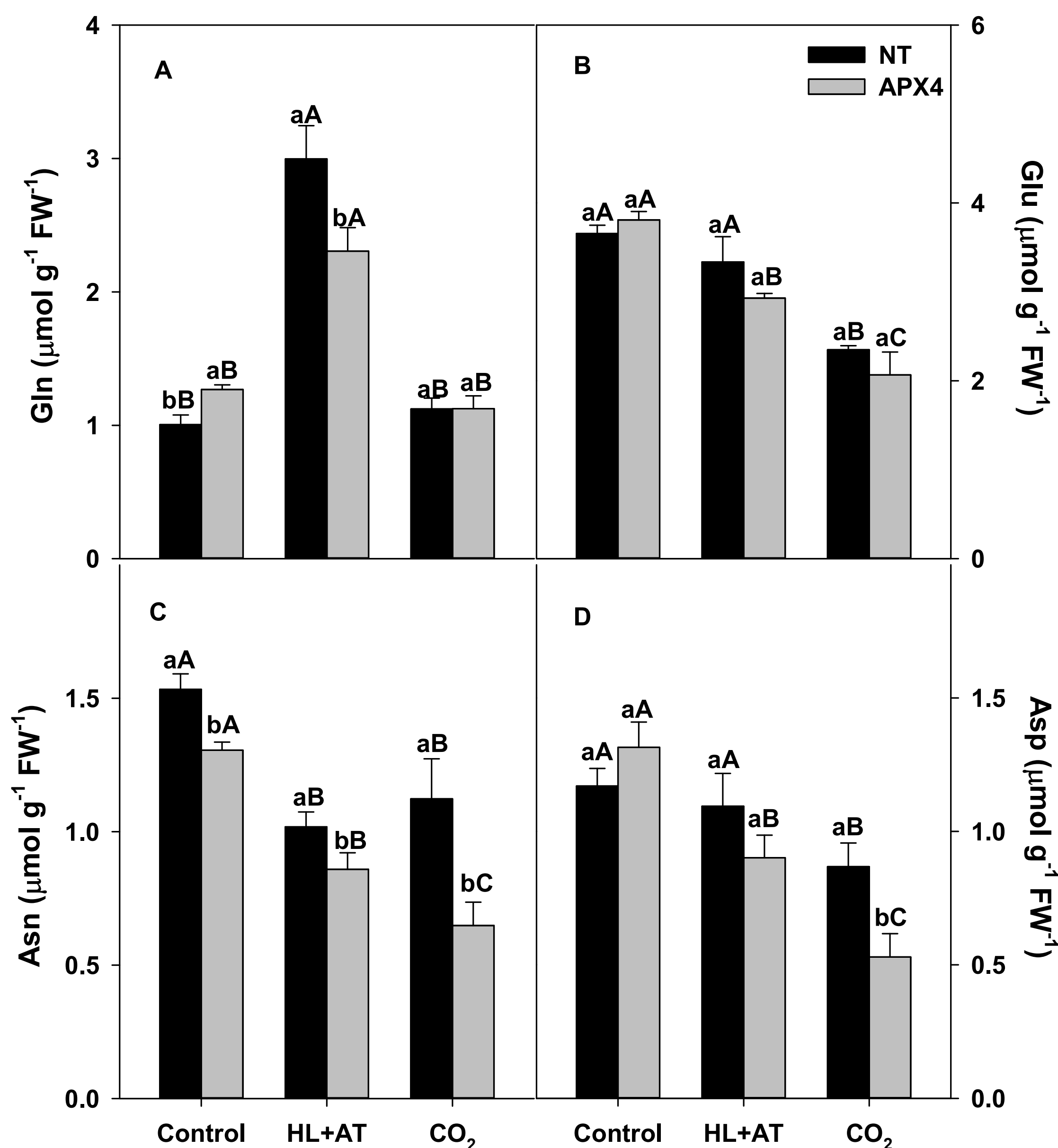

Figure 4. Glutamine (Gln) (A), glutamate (Glu) (B), asparagine (Asn) (C) and Aspartate (Asp) (D) contents in leaves of NT and APX4 plants under growth conditions (Control), exposed to 10 mM 3-AT + high light (1000 μmol photons m^{-2} s^{-1}) (High Pr), or exposed to 3% CO_2 (Low Pr). Capital letters indicate significant differences between treatments (Control, High Pr and Low Pr) within the same genotype and lower cases represent significant differences between genotypes (NT and APX4) within each treatment by analysis of variance (ANOVA) followed by the Tukey's test ($p < 0.05$).

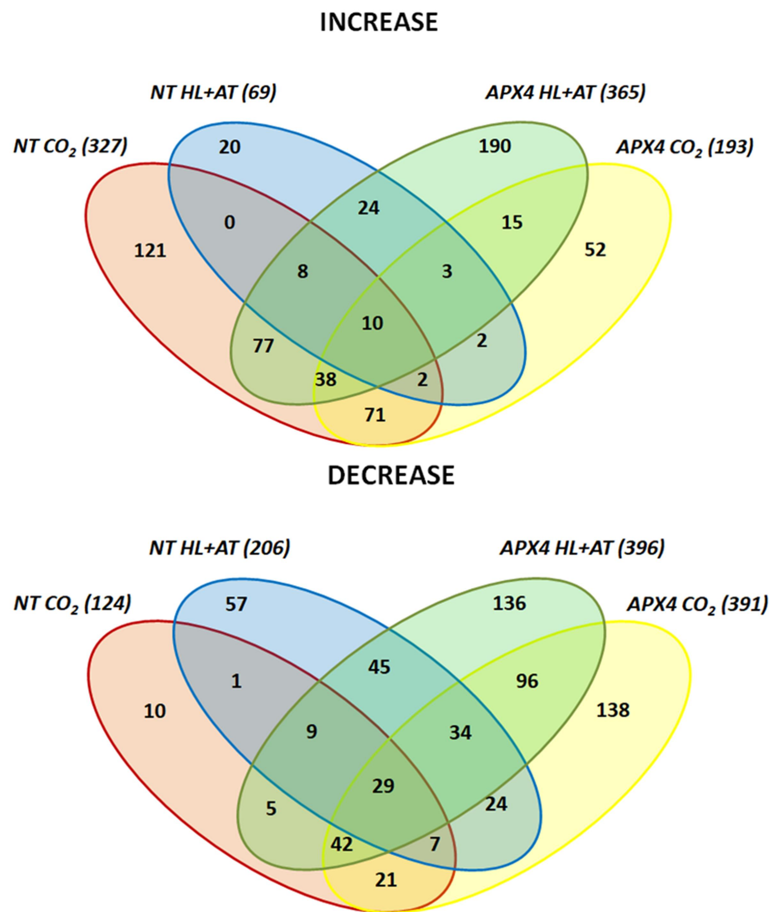

Figure 5. Venn diagram showing proteins with different abundances from leaves of NT and APX4 plants exposed to 10 mM AT + high light (1000 µmol photons m^{-2} s^{-1}) (High Pr) or 3% CO_2 (Low Pr), all compared to their respective protein amounts found under control conditions.

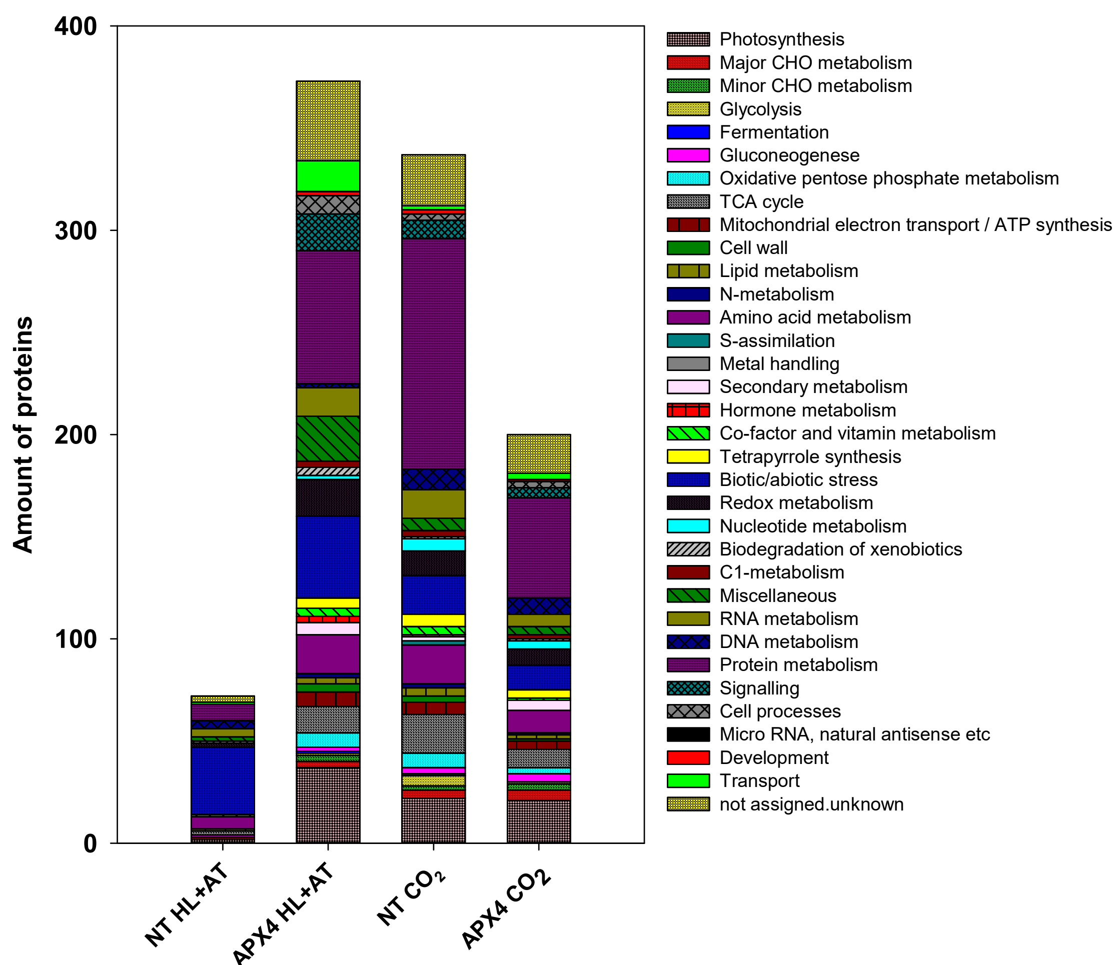

Figure 6. Functional classification of proteins with increased amount in leaves of NT and APX4 plants exposed to 10 mM AT + high light (1000 µmol photons m^{-2} s^{-1}) (High Pr) or 3% CO_2 (Low Pr). The values are relative to their respective protein amounts found under control conditions. Each protein content was considered decreased if the log_2 was lower than 0.5. The proteins were classified by the software (MapMan), according to their involvement in each metabolic process.

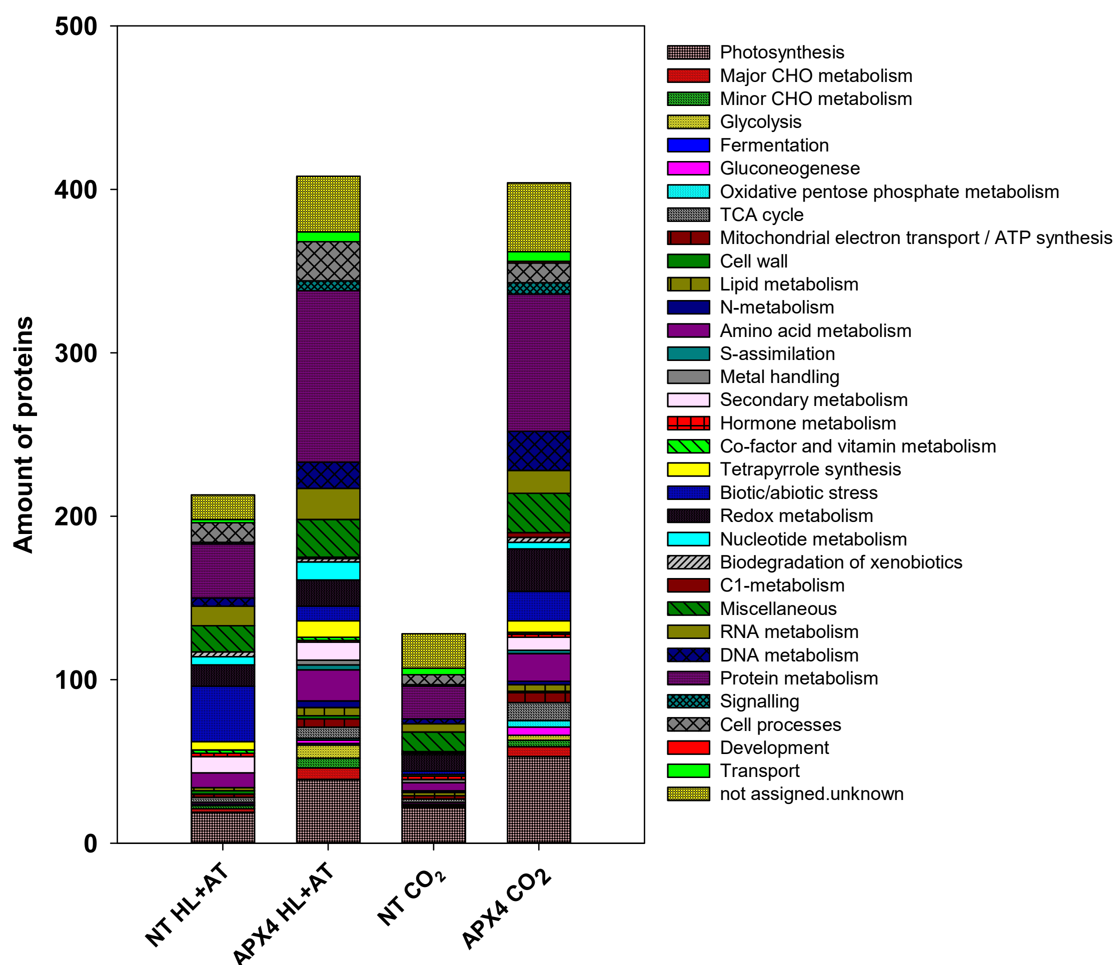

Figure 7. Functional classification of proteins with decreased amount in leaves of NT and APX4 plants exposed to 10 mM AT + high light (1000 µmol photons m^{-2} s^{-1}) (High Pr) or 3% CO_2 (Low Pr). The values are relative to their respective protein amounts found under control conditions. Each protein content was considered decreased if the log_2 was lower than 0.5. The proteins were classified by the software (MapMan), according to their involvement in each metabolic process.

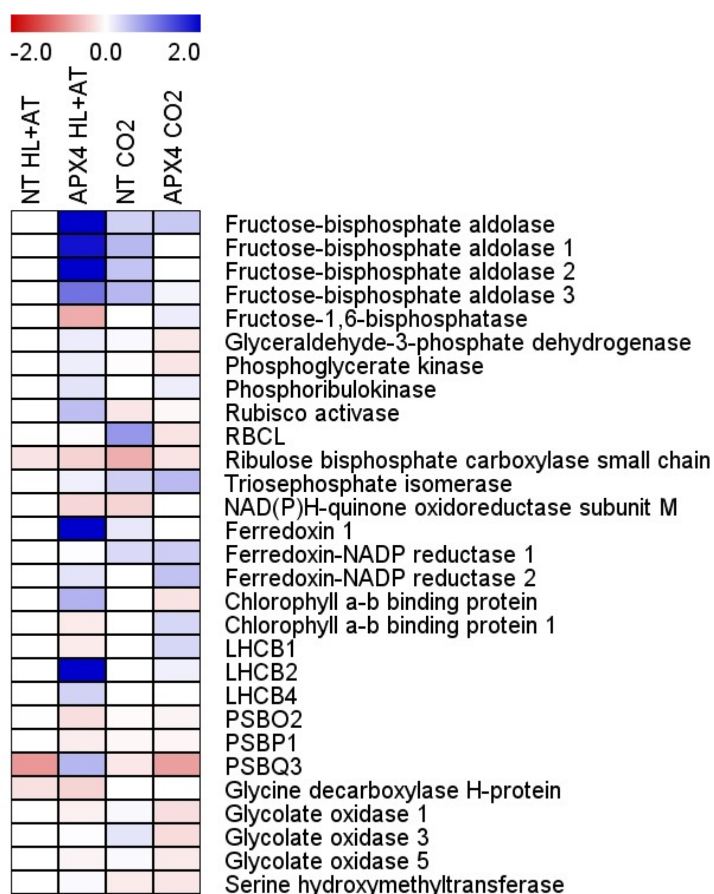

Figure 8. Heat map of differentially expressed proteins related to photosynthesis from leaves of NT and APX4 plants exposed to 10 mM AT + high light (1000 µmol photons m^{-2} s^{-1}) (High Pr) or 3% CO_2 (Low Pr). The values are relative to their respective protein amounts found under control conditions. The fold change from proteomics was plotted into a heat map using MEV software framework.

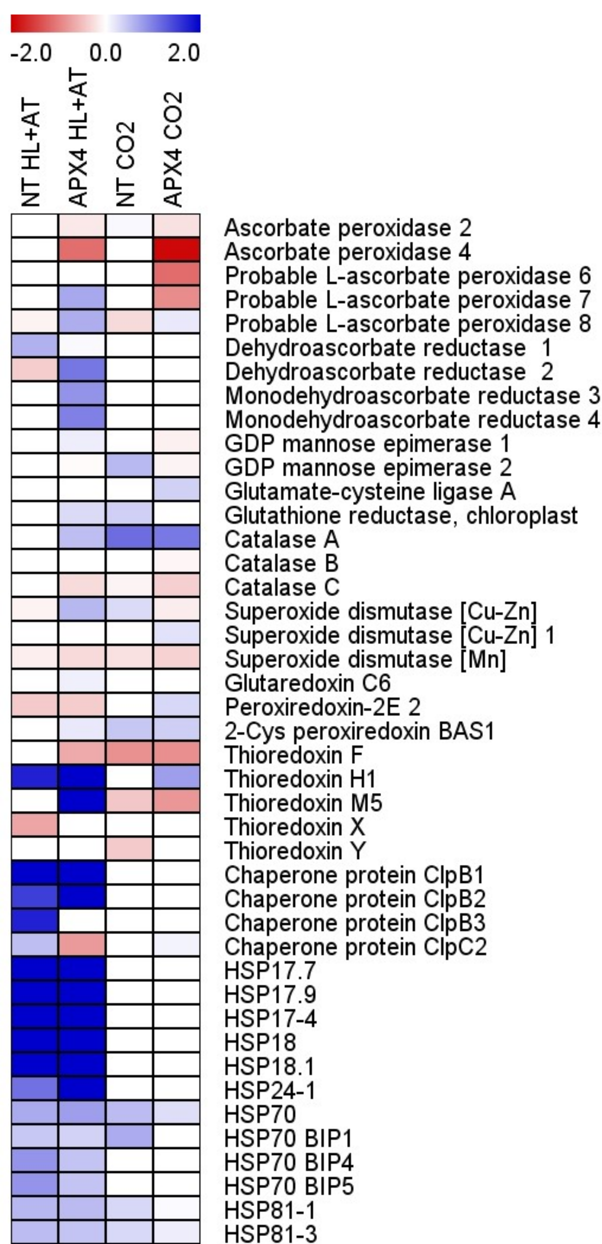

Figure 9. Heat map of differentially expressed proteins related to stress metabolism from leaves of NT and APX4 plants exposed to 10 mM AT + high light (1000 µmol photons m^{-2} s^{-1}) (High Pr) or 3% CO_2 (Low Pr). The values are relative to their respective protein amounts found under control conditions. The fold change from proteomics was plotted into a heat map using MEV software framework.

4. CONCLUSION

This study provided results enough to support the following conclusions:

1. APX4 deficiency triggers several proteomic changes in rice plants with inhibited CAT activity.
2. Peroxisomal H_2O_2 accumulation induced by APX4 knockdown might act as a signaling molecule which triggers a better photosynthetic performance upon CAT inhibition.
3. Enhanced photorespiratory flow contributes to photosynthesis resilience in APX4-knocked-down rice plants under high photorespiration associated to CAT inhibition.
4. APX-deficient rice plants have a specific metabolic rearrangement, possibly triggered by H_2O_2 and/or GSH oxidation which induce less oxidative damage caused by high peroxisomal H_2O_2 concentration.
5. The chloroplastic antioxidant system contributes for better photosynthesis resilience in APX4-knockd-down rice plants upon high H_2O_2 accumulation in peroxisomes.

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